



Increased acetylation of H3K14 in the genomic regions that encode trained immunity enzymes in lysophosphatidylcholine-activated human aortic endothelial cells – Novel qualification markers for chronic disease risk factors and conditional DAMPs

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ABSTRACT

To test our hypothesis that proatherogenic lysophosphatidylcholine (LPC) upregulates trained immunity pathways (TIPs) in human aortic endothelial cells (HAECs), we conducted an intensive analyses on our RNA-Seq data and histone 3 lysine 14 acetylation (H3K14ac)-CHIP-Seq data, both performed on HAEC treated with LPC. Our analysis revealed that: 1) LPC induces upregulation of three TIPs including glycolysis enzymes (GE), mevalonate enzymes (ME), and acetyl-CoA generating enzymes (ACE); 2) LPC induces upregulation of 29% of 31 histone acetyltransferases, three of which acetylate H3K14; 3) LPC induces H3K14 acetylation (H3K14ac) in the genomic DNA that encodes LPC-induced TIP genes (79%) in comparison to that of in LPC-induced effector genes (43%) including ICAM-1; 4) TIP pathways are significantly different from that of EC activation effectors including adhesion molecule ICAM-1; 5) reactive oxygen species generating enzyme NOX2 deficiency decreases, but antioxidant transcription factor Nrf2 deficiency increases, the expressions of a few TIP genes and EC activation effector genes; and 6) LPC induced TIP genes (81%) favor *inter*-chromosomal long-range interactions (CLRI, *trans*-chromatin interaction) while LPC induced effector genes (65%) favor *intra*-chromosomal CLRI (cis-chromatin interaction). Our findings demonstrated that proatherogenic lipids upregulate TIPs in HAECs, which are a new category of qualification markers for chronic disease risk factors and conditional DAMPs and potential mechanisms for acute inflammation transition to chronic ones. These novel insights may lead to identifications of new cardiovascular risk factors in upregulating TIPs in cardiovascular cells and novel therapeutic targets for the treatment of metabolic cardiovascular diseases, inflammation, and cancers. (total words: 245).

1. Introduction

Atherosclerotic diseases such as ischemic heart disease, stroke, and peripheral artery disease are estimated to cause 8.9 million deaths per year, making it the single most significant cause of death worldwide [1]. Activations of human aortic endothelial cells (HAEC) play

significant roles at the early stage in recruiting inflammatory cells [2–7] into arteries and amplify atherogenic inflammation as we reported [7–11]. Based on the functional comparisons of activated endothelial cells to the prototypic innate immune cells - macrophages on several aspects such as danger associated molecular pattern (DAMPs) recognition, phagocytosis, migration, antigen processing and presentation

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capacity as well as other characteristics, we proposed a novel concept that activated endothelial cells are innate immune cells [12]. Our recent work on studying the role of caspase-1/inflammasome in recognizing atherogenic DAMPs in aortic endothelial cells [13–18] consolidated this new concept. Based on our new RNA sequencing (RNA-Seq) data analyses in human aortic endothelial cells stimulated with proatherogenic lipids lysophosphatidylcholine (LPC) and lysophosphatidylinositol (LPI), we proposed another new concept that upregulation of additional DAMP receptors and T cell co-stimulation receptors and major histocompatibility complex (MHC) molecules in addition to upregulation of cytokines/chemokines and adhesion molecules are the features for prolonged endothelial cell activation, and innate immune *trans*-differentiation of endothelial cells [19]. These new concepts improved our understanding on the roles of endothelial cell activation in not only the early phase [13] but also throughout the later phase of atherogenic process. However, a significant issue remains poorly characterized whether endothelial cells are capable of developing an immune memory that facilitate prolonged endothelial cell activation due to constant exposure to cardiovascular risk factors.

It is a well-documented concept that adaptive immune system can develop antigen-specific memory T cells [20] and B cells [21] that have previously encountered and responded to their cognate antigen. Recently, it was discovered that innate immune cells are also capable of developing an immune memory when exposed to certain inflammatory stimuli, and this type of memory was termed as innate immune memory. Unlike adaptive immune memory, innate immune memory is non-specific. Innate immune cells such as monocytes, macrophages, dendritic cells, and natural killer (NK) cells and some non-immune cells [22] were shown to develop trained immunity by undergoing functional reprogramming when exposed to inflammatory stimuli, that elicit changed responses to subsequent inflammatory challenges. This long-term reprogramming depends on the rewiring of cell metabolism and epigenetic processes; and they stay at the basis of induction of both innate immune memory (also termed trained immunity) and innate immune tolerance [23]. It was identified that three metabolic pathways (trained immunity pathways - TIP) including glycolysis pathway, mevalonate pathway and acetyl coenzyme A (acetyl-CoA) generation are responsible for initiating innate immune memory formation. These metabolic changes lead to activation alter the epigenetics of the innate immune cell [24] that serve as the sustained memory links between rewiring of cell metabolism and transcriptomic changes.

As described above, we presented the novel concept that endothelial cells are conditional innate immune cells, where they undergo innate-immune transition due to chronic exposure to cardiovascular risk factors. However, whether endothelial cells as conditional innate immune cells can develop trained immunity after repeatedly encountering the cardiovascular risk factors had not been investigated so far. Whether the trained immunity is designated only for professional innate immune cells and whether trained immunity can be used as key marker for qualification of chronic inflammatory disease risk factors among all the stimuli, by which qualified chronic inflammatory insults can alter the functionality and the heighten responsiveness of the innate immune system in the long term [23] had not been studied as well.

To investigate whether endothelial cells can develop trained immunity, we treated human aortic endothelial cells (HAEC) with proatherogenic lysophospholipid named lysophosphatidylcholine (LPC) and conducted RNAseq analysis [19]. Previously, we have reported that the lysophospholipid family can act as conditional DAMP, where they regulate physiological functions at a normal plasma concentration, but induce inflammation at a high plasma concentration [25]. We tested whether expression of enzymes involved in TIP is changed with LPC treatment in HAEC. Previously, we have shown that acetylation of histone 3 lysine 14 (H3K14) residue plays an important role in mediating LPC mediated endothelial cell activation. Modifications of the histones including histone 3 lysine 4 methylation 1 (H3K4me1), H3K4me3 and H3K27 acetylation (H3K27ac) in monocytes and

macrophages [26] were reported to regulate development of trained immunity. Therefore, we investigated whether LPC treatment promote acetylation of H3K14 in the genomic regions of inflammatory genes such as TNF-alpha, IL-6, IL-1beta, etc. and in genomic regions that encode for TIP enzymes (anti-acetylated H3K14 antibody – CHIP-seq data) [7]. Further, we analyzed whether the signaling pathways altered by LPC when eliciting trained immunity and endothelial activation (effector function) are similar or distinct to each other.

Our analysis revealed that conditional DAMP such as LPC has the potential to induce the expression of TIP enzymes, and also to modulate epigenetic changes by promoting acetylation of histones. Furthermore, our analysis revealed that LPC treatment induced H3K14 acetylation in the genomic regions of inflammatory markers associated with trained immunity. Also, the signaling mechanisms involved in effector function and the trained immunity function mediated by LPC are distinct from each other. Interestingly, we observed that the genes that regulate trained immunity and effector function in endothelial cells have differential interaction patterns with distant genomic regions (CLRI – chromatin long range interactions). Additionally, we observed that acetylation level of H3K14 in the genomic regions are quite distinct between the genes that regulate trained immunity and effector function in response to LPC treatment in HAEC. Therefore, we hypothesize that conditional DAMP such as LPC can promote trained immunity and effector function, which are two exclusive mechanisms in endothelial cells by promoting differential epigenetic modifications and differential CLRI patterns in the genes that regulate the two mechanisms.

Our findings demonstrated that conditional DAMPs, proatherogenic lipids, upregulate TIP in HAECs, which are new category of markers for prolonged EC activation and qualification of chronic disease risk factors. These novel insights may lead to identifications of new cardiovascular risk factors out of many compound stimuli in diets and environments, that are capable in upregulating TIP and potentially amplifying secondary and chronic, prolonged as we recently termed [19], innate immune responses in vascular cells, for novel therapeutics of metabolic cardiovascular diseases, inflammations, and cancers.

2. Materials and methods

2.1. Modulation of trained immunity pathway enzymes in HAEC

Raw RNA-Seq data was obtained from our previous study [19,27]. Raw reads generated by the Illumina HiSeq control software were assessed using FastQC [28]. Samples with poor sequence quality were discarded. Sequence reads were mapped to the hg38 genome using STAR [29]. A list of mRNAs common to both control and LPC-stimulated HAECs was generated using the VLOOKUP function in Microsoft Excel. Using previously obtained mRNA expression data [19,27], 95% confidence interval (mean \pm 2*SD) of mRNA fold changes were calculated using the ten housekeeping genes [30]: C1orf43, CHMP2A, GAPDH, EMC7, GPI, PSMB2, PSMB4, RAB7A, SNRPD3, and VPS29.

2.2. Genome-wide analysis of H3K14ac binding positions

H3K14ac CHIP seq data was obtained from our lab previous study [7]. Genomic DNA regions of interest were isolated by performing IP using specific antibody against H3K14ac (Active Motif, cat. 39599). H3K14Ac enriched regions were identified using the spatial clustering for identification of CHIP-enriched regions (SICER algorithm, threshold FDR 1E-10, gap size 600 bp). Using a cutoff of FDR 1E-20, significant changed-regions between the control sample and the LPC-treated sample were further identified. Signal maps and peak locations were used as input data to acquire detailed information on sample comparison, peak metrics, peak locations and gene annotations.

2.3. Analysis of long-range interactions

A complete list of long-range chromatin interaction sites in the human genome was obtained from the 4DGenome database (<https://4dgenome.research.chop.edu/>) as a tabulated text file [31]. The grep command line utility was used to filter for entries detected using 4C methodology. The resulting filtered data was imported into Microsoft Excel and raw interaction distances calculated as the differences between gene start coordinates. An AWK script was used to determine whether the target gene was downstream or upstream of its partner in each interaction pair and to add this information to the data file. The signs of distance values were then updated, with downstream entries designated as positive and upstream values designated as negative, using a Python script. These updated distance values were separated into each group and used in pairwise two-sample Kolmogorov-Smirnov tests (described further below under “Statistical analysis”). Distance distributions for all upregulated and all downregulated mRNAs were compared by groups, respectively.

2.4. Pathway analysis

QIAGEN Ingenuity Pathway Analysis (IPA) software, which constructs predicted upstream and downstream causal networks for input datasets from a curated research literature base, was used to elucidate potential downstream pathways for TIP enzymes [32]. Lists of TIP enzyme mRNAs were input into IPA and ran through the expression target filter to generate a list of potential mRNA targets. The list of mRNA target genes was then run through an IPA core analysis. All canonical downstream pathways returned in the resulting output were exported into a Microsoft Excel spreadsheet. The top ten pathways were extracted for further qualitative consideration.

2.5. Graphical figure generation

For Venn diagrams, lists of genes were input into an online Venn diagram generator (<http://bioinfo.gp.cnb.csic.es/tools/venny/>, VENNY 2.1 by Juan Carlos Oliveros, BioinfoGP, CNB-CSIC) [33]. This tool was used to produce both diagrams and lists of overlapped genes between groups. Explanatory and conceptual graphics were produced using Microsoft Paint.

2.6. Statistical analysis

Descriptive summary statistics were reported by group. Data were checked for normality assumption and, if found to be not normally distributed, subsequently transformed using various functions such as the log10 and cubic-root to find the optimal transformation for the underlying chromatin long-range interaction distance distribution data. Chromatin long-range interaction distance density functions were then estimated and plotted by group under the optimal transformation using the non-parametric kernel density approach with a normal weight function [34–36]. Pairwise comparisons of median chromatin long-range interaction distance among the groups were performed with multiple comparison adjustments using the Dwass, Steel, and Critchlow-Fligner method based on the Wilcoxon test for downstream and upstream data separately [36–39]. Pairwise comparisons between groups for chromatin long-range interaction distance distribution, median distance location shift, and distance distribution scale were implemented using the Kolmogorov-Smirnov two-sample test, Hodges-Lehmann estimation method and Fligner-Policello test, and Ansari-Bradley test, respectively, again for downstream and upstream data separately [36–40]. SAS version 9.4 was used to perform these analyses and generate density function plots for the chromatin long-range interaction distance data.

For rest of the data, single-factor ANOVA and non-parametric Kruskal-Wallis tests were conducted for all multi-group analyses using

Table 1

LPC does not significantly modulate the expressions of house-keeping mRNAs in human aortic endothelial cells (HAECs), suggesting that the RNA-seq experiments in HAECs treated with LPC was well performed; and the data were in high quality.

Gene ID	House-keeping gene	Fold Change (LPC)
25912	C1orf43	0.97
27243	CHMP2A	1
2597	GAPDH	1
56851	EMC7	0.98
2821	GPI	1
5690	PSMB2	0.98
5692	PSMB4	1.02
7879	RAB7A	0.98
6634	SNRPD3	1
51699	VPS29	1.04
Mean ± 2SD		1.01 ± 0.1

The expression changes of mRNAs are identified by using a confidence interval as justified by a statistical analysis of expression variation ranges of house-keeping genes as Mean ± 2SD. RNA-seq data was deposited as E-MTAB-6604 at ArrayExpress.

the Real Statistics Resource Pack add-in for Microsoft Excel (downloaded from <http://www.real-statistics.com/>) [41].

3. Results

3.1. LPC induces upregulation of three trained immunity pathways, 14 out of 71 glycolytic enzymes (17%), 6 out of 7 mevalonate pathway enzymes (86%), 4 out of 24 acetyl-CoA enzymes (17%) in HAECs

As reported, memory T cells are generated from antigen-specific T cell activation [42]. Thus, we hypothesize that, similar to that of in adaptive immune memory T cells, modulation of trained immunity (innate immune memory) pathways can be found in human aortic endothelial cells activated by conditional DAMPs such as proatherogenic LPC. To examine this hypothesis, we analyzed the RNA-Seq data from LPC-activated HAEC as we reported [19] (our RNA-Seq data were deposited as E-MTAB-6604 at the ArrayExpress). Of note, we confirmed the high quality of our RNA-Seq data by re-examining the expression levels of ten house-keeping genes [30]. As shown in Table 1, the confidence intervals of the expression changes in HAEC before (control) and after stimulation with LPC fell in to a narrow range (mean ± 2 x standard deviation = 1.01 ± 0.1). It has been reported that increased activity of the three metabolic pathways including glycolysis pathway, mevalonate pathway and acetyl-CoA synthesis contribute to the establishment of trained immunity [23] (Fig. 1A). We collected and made a list of 102 enzymes that regulate these three pathways. 71 of the listed 102 enzymes regulate glycolysis as per the glycolytic database (https://rgd.mcw.edu/rgdweb/pathway/pathwayRecord.html?acc_id=PW:0000640&species=Human#annot) (S. Table 1A), 24 enzymes involved acetyl-CoA generation [43,44] (S. Table 1B and Fig. 1B) and 7 enzymes regulate the mevalonate pathway (S. Table 1C and Fig. 1B) [45]. As shown in Table 2, LPC induced 12 out of 71 glycolysis enzymes (17%) (Table 2A, Fig. 2A), 6 out of 7 mevalonate pathway enzymes (86%) (Table 2B, Fig. 2A), and 4 out of 24 acetyl-CoA generating enzymes (17%) (Table 2C, Fig. 2A–B). The fact that endothelial cells act as conditional innate immune cells [12,19] and in the presence of proatherogenic stimulus such as LPC, endothelial cells increased the expression of some of the components in the metabolic pathways suggests that endothelial cells may have the potential to develop innate immune memory. Previously, it was shown that some conditional DAMP such as LPC act via G-protein coupled receptors (GPCR) [25], suggesting that GPCR may play a role in eliciting innate immune memory via cellular signaling mechanisms that are yet to be elucidated.

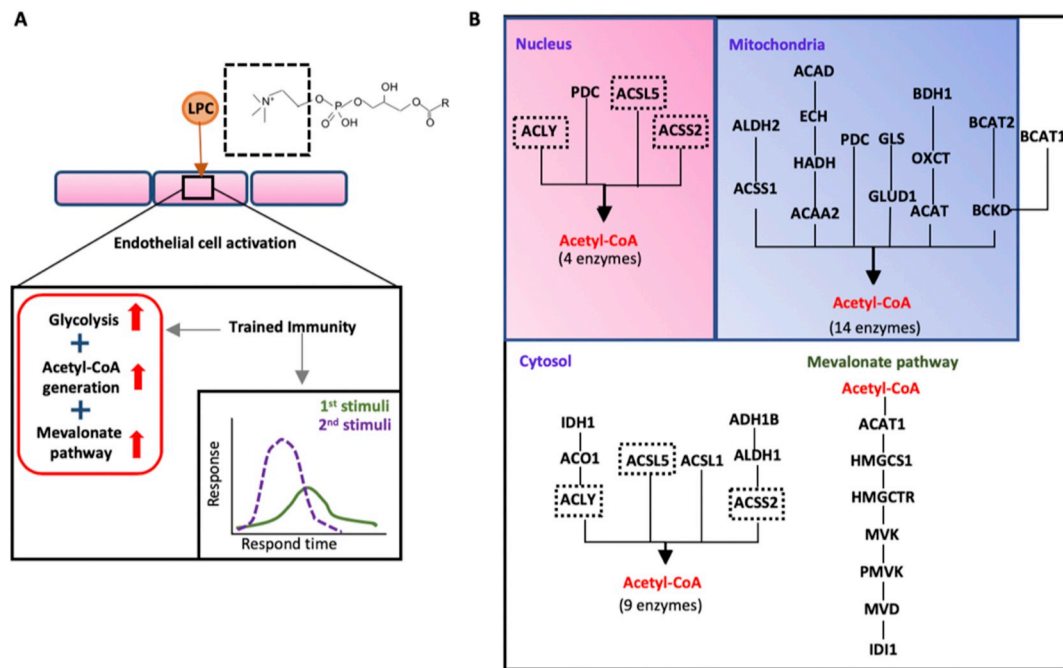


Fig. 1. LPC induces upregulation of three trained immunity pathways. **A.** Human aortic endothelial cells (HAECs), treated with lso-phosphatidylcholine (LPC), upregulate the three metabolic pathway enzymes, which contribute to trained immunity (innate immune memory). Activated aortic endothelial cells may take shorter time to be activated again when encounter risk factors for the second time. **B.** 24 acetyl-CoA generating enzymes and 7 mevalonate enzymes are localized in mitochondria, cytosol and nucleus; and three acetyl-CoA generating enzymes (in box) are localized in two subcellular locations.

3.2. LPC upregulates 6 out of 31 histone acetyltransferases, three of them are reported to acetylate histone 3 lysine 14 (H3K14ac); and acetylation level of H3K14 was increased in the genomic regions of LPC-induced trained immunity enzymes

Trained immunity is orchestrated by epigenetic reprogramming

such as histone methylation and acetylation (Table 3A). These changes are broadly defined as sustained changes in gene expression and cell physiology for a prolonged period of time that do not involve permanent genetic changes such as mutations and recombination, which are essential for adaptive immunity [26]. Our data revealed that LPC can induce the expression of several enzymes that regulate acetyl-coa

Table 2A

The 12 out of 71 mRNAs[#] (17%) of glycolysis enzymes are regulated in LPC-induced HAEC activation, including 12 increased mRNAs (17%) and 2 decreased mRNAs (3%).

ID	Gene Symbol*	Object name	Fold change (>1.11 or <0.91)
218	ALDH3A1	aldehyde dehydrogenase 3 family member A1	1.34
226	ALDOA	aldolase, fructose-bisphosphate A	1.15
2027	ENO3	enolase 3	1.65
5106	PCK2	phosphoenolpyruvate carboxykinase 2, mitochondrial	1.25
5160	PDHA1	pyruvate dehydrogenase E1 alpha 1 subunit	1.14
5210	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	1.53
5211	PFKL	phosphofructokinase, liver type	1.12
441531	PGAM4	phosphoglycerate mutase family member 4	1.36
5230	PGK1	phosphoglycerate kinase 1	1.13
5236	PGM1	phosphoglucomutase 1	1.21
5315	PKM	pyruvate kinase M1/2	1.31
5208	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	1.39
130589	GALM	galactose mutarotase	0.83
2645	GCK	glucokinase	0.58

*Red fonts indicate that gene expressions were increased in RNAseq; and green fonts indicate that gene expressions were decreased.

Table 2B

The 6 out of 7 mRNAs (86%) of mevalonate pathway enzymes are regulated in LPC-induced HAEC activation; and all 6 of the enzymes are upregulated.

ID	Gene Symbol	Entrez Gene Name	Fold change
38	ACAT1	acetyl-CoA acetyltransferase 1	1.26
4598	MVK	mevalonate kinase	1.94
4597	MVD	mevalonate diphosphate decarboxylase	1.83
3422	IDI1	isopentenyl-diphosphate delta isomerase 1	1.31
3157	HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	1.58
3156	HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	1.26

synthesis, which is known to induce histone acetylation by donating acetyl groups. In order to identify the molecular mechanisms underlying our observation that LPC increases the expression of enzymes involved in metabolic pathways that propagate innate immune memory, we hypothesized that LPC not only increases acetyl-CoA generation by upregulating acetyl-CoA generating enzymes but also induce histone modifications by promoting the expression of histone acetyltransferases that can mediate the transfer of acetyl group, thus eliciting innate immune memory function.

Previously, we reported that cardiovascular, and other disease risk factors can modulate the expression of as many as 31 histone acetyltransferases [46]. As shown in Table 3B, LPC modulated the expression of nine histone acetyltransferases where expression of six enzymes were upregulated in HAECs. Our extensive literature search showed that histone acetyltransferases modulated by LPC can acetylate as many as 25 different lysine residues that reside in the four types of histones (Fig. 3). According to our analysis, LPC upregulated three histone acetyltransferases that have the potential to acetylate lysine 14 in histone 3 protein (H3K14ac), which is the unique lysine among the 10 lysine residues that can undergo acetylation in histone 3 protein (Fig. 3, Table 3C). We have experimentally verified that LPC can indeed induce H3K14 acetylation in LPC treated HAECs with mass spectrometry, and had published our findings [7].

H3K14ac is recognized as a histone modification that promote gene expression [7,47]. Therefore, we hypothesized that one of the mechanisms that LPC may utilize to upregulate the gene expression of metabolic enzymes that elicit trained immunity is by increasing the acetylation of H3K14 in their promoter regions. Out of 12 enzymes in glycolysis pathway that were induced by LPC, 8 genes (66.7%) were shown to have H3K14ac in their genomic region compared to the control (genes highlighted in Table 4A). Of note, LPC does not induce acetylation of H3K14 in the genomic regions of house-keeping genes, and according to our RNA-seq data, the expression of these genes were

not modulated by LPC.

Similarly, out of six enzymes in the mevalonate pathway that were upregulated by LPC treatment, 4 enzymes showed an increase in acetylation in H3K14 residue with LPC treatment compared to the control (genes highlighted in Table 4B). Interestingly, we observed an increased acetylation of H3K14 residues in three acetyl-CoA generating enzyme genes that were upregulated by LPC treatment. These results indicate that LPC may potentially induce epigenetic modifications by acetylation of the histones via increasing the expression of enzymes that are responsible for acetyl-CoA synthesis (donors – Tables 2C–4C) and enzymes that regulate the acetylation process in histones (acetyl transferases – Table 3B).

3.3. Acetylation level of histone 3 lysine 14 residue in the genomic regions of enzymes that mediate trained immunity (79%) is higher than that of in the genomic regions of the genes that regulate endothelial cell activation including ICAM-1 (effectors) (43%)

We and others reported that the signaling mechanisms that exert T cell memory function can be separated from mechanisms that propagate T effector cell functions [48] potentially via differential epigenetic and gene regulation [49,50]. Previously, we reported that LPC induces HAEC activation by upregulating a list of genes that regulate EC activation including intercellular adhesion molecule-1 (ICAM-1). This list includes genes that regulate the expression of EC adhesion molecules, cytokines/chemokines, conditional DAMP receptors and mediators of innate immunity [19]. Unlike in T cells, whether the epigenetic changes that regulate trained immune memory are different from epigenetic changes that regulate activation of EC had not been investigated before.

From herein, we refer the genes that mediate EC activation as “EC effector genes”.

We hypothesized that LPC treatment in HAECs has the tendency to promote acetylation of H3K14 residue in the genomic regions of the

Table 2C

LPC upregulate the expressions of 4 of 24 Acetyl-CoA generating enzyme mRNAs (17%) in HAECs, suggesting that LPC increases the availability of cellular acetyl donor Acetyl-CoA (substrate for histone acetyltransferases) for potential histone acetylation.

Gene ID	Gene Symbol	Objective name	Subcellular Location	Fold Change
587	BCAT2	branched chain amino acid transaminase 2	Mitochondria	1.16
3417	IDH1	isocitrate dehydrogenase (NADP(+)) 1, cytosolic	Cytosol	1.24
51703	ACSL5	acyl-CoA synthetase long chain family member 5	Cytosol/Nucleus	1.16
47	ACLY	ATP citrate lyase	Cytosol/nucleus	1.21
3033	HADH	hydroxyacyl-CoA dehydrogenase	Mitochondria	0.87
2180	ACSL1	acyl-CoA synthetase long chain family member 1	Cytosol	0.88

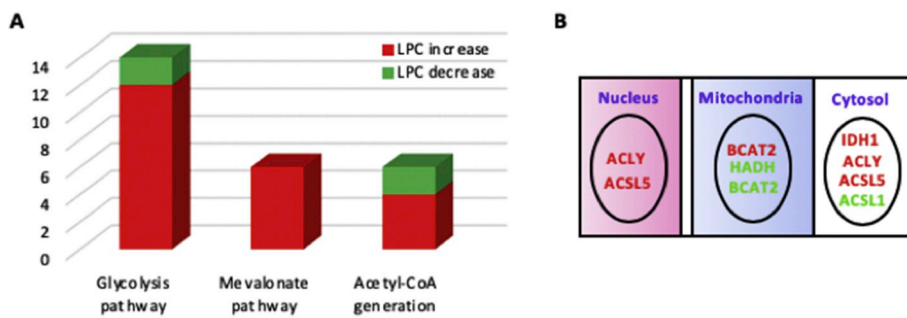


Fig. 2. LPC regulates trained immunity by more upregulating metabolic enzymes. **A.** LPC regulates trained immunity by more upregulating than downregulating metabolic enzymes, suggesting that LPC has the potent in establishing innate immune memory in human aortic endothelial cells. **B.** The analysis showed that the subcellular locations for LPC regulated acetyl-CoA generating enzymes.

genes that regulated trained immunity rather than inducing similar epigenetic modifications in the effector genes (Fig. 4A). As shown in Fig. 4B, we observed that LPC treatment induced the acetylation of H3K14 in 19 trained immunity genes, among which 15 genes were upregulated according to our RNAseq data. Therefore, almost 79% of the trained immunity genes that were upregulated by LPC treatment had acetylated H3K14 in the genomic regions. In contrast, only 43% (22 genes out of a total of 51 of effector genes that were upregulated by LPC including ICAM-1 [7,19]) had acetylated H3K14 in the genomic regions (Fig. 4B).

Previously, it was shown that the stimuli that are capable of eliciting an innate immune response in immune cells, induce epigenetic modifications in the inflammatory genes such as TNF- α [51] and IL-6 [52]. These epigenetic changes lasts even when the stimuli that triggered the modifications removed from the experimental system. Presence of such epigenetic modifications in inflammatory genes produce an enhanced response when the cells are exposed to secondary inflammatory stimuli. Therefore, we hypothesized that LPC may trigger acetylation of H3K14 in the genomic DNA that encodes for inflammatory genes such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), IL-1 β , CD131 (cytokine receptor common subunit β), monocyte chemoattractant protein-1 (MCP-1), matrix metalloproteinase-2 (MMP2) and MMP9 (readouts). The results in Table 4D shows that 4 out of 7 trained immunity read-out genes have acetylated H3K14 residues in their genomic regions in LPC-treated HAEC although LPC treatment did not increase their mRNA

transcripts. Therefore, these results suggest that increased H3K14Ac of these trained immunity read out genes may have these genes in a ready-state to induce the inflammation when exposed to the second inflammatory stimuli.

Of note, as we reported previously, we observed the presence of H3K14Ac epigenetic modification in the genomic areas of genes that were downregulated by LPC treatment in EC. Our results are well correlated with the reports which identified H3K14ac as an epigenetic marker for active regulatory regions [47], which significantly induced changes in gene expression (both upregulation and downregulation) in a diet induced obese model [53]. Taken together, these results suggest that EC activation mediated by conditional DAMP such as LPC may use differential cellular programs to acetylate lysine residues on histone 3 in the genomic regions of the effector genes from that of trained immunity enzyme genes.

3.4. The metabolic pathways that are affected by trained immunity genes and effector genes in EC are completely independent

To determine the upstream and downstream signaling pathways affected by LPC-modulated trained immunity enzymes and LPC-modulated effector genes, we used Ingenuity pathway analysis (IPA). For our analysis, we categorized our genes of interest in to four groups (Table 5A): 1) LPC-upregulated trained immunity genes; 2) LPC-downregulated trained immunity genes; 3) LPC-upregulated effector

Table 3A

Trained immunity has been reported to be regulated by histone 3 methylations and acetylation; and different modifications of histones have different transcriptional regulation functions.

Modification	Histone modification	Transcription regulation	Cell type	PMID	Reference
Methylation	H3K4me1	Activation	Macrophage	27102489	Science (New York, N.Y.), Vol.352(6284)
			Natural killer cells		
	H3K4me2	Activation	Macrophage	27102489, 28694812	Science (New York, N.Y.), Vol.352(6284); Front Immunol. 2017 Jun 26;8:734
			Natural killer cells		
	H3K4me3	Activation	Macrophage	27102489, 28694812	Science (New York, N.Y.), Vol.352(6284); Front Immunol. 2017 Jun 26;8:734
			Natural killer cells		
Acetylation	H3K9me2	Repression	Macrophage	26556024	Cell Cycle. 2016;15(1):3-4.
			Monocyte	28694812	Front Immunol. 2017 Jun 26;8:734
	H3K27me3	Repression	Monocyte	28694812	Front Immunol. 2017 Jun 26;8:734
	H3K4Ac	Activation	Macrophage	27102489	Science (New York, N.Y.), Vol.352(6284)
			Natural killer cells		
	H3K27Ac	Activation	Monocyte	28694812	Front Immunol. 2017 Jun 26;8:734

Table 3B

LPC modulate the expressions of 9 out of 31 histone acetyltransferases* (29%) and upregulate six out of nine modulated histone acetyltransferases (67.7%) in HAECs, suggesting that upregulated histone acetyltransferases may mediate LPC-induced histone acetylation in HAEC activation.

Gene ID	Histone acetyltransferase	Fold Change
2648	KAT2A: K(lysine) acetyltransferase 2A	1.22
10524	KAT5: K(lysine) acetyltransferase 5	1.15
84148	KAT8: K(lysine) acetyltransferase 8	1.21
26151	NAT9: N-acetyltransferase 9 (GCN5-related, putative)	1.12
57106	NAT14: N-acetyltransferase 14 (GCN5-related, putative)	1.13
8260	NAA10: N(alpha)-acetyltransferase 10, NatA catalytic subunit	1.18
79903	NAA60: N(alpha)-acetyltransferase 60, NatF catalytic subunit	0.83
1387	CREBBP: CREB binding protein	0.8
2033	EP300: E1A binding protein p300	0.8

genes; and 4) LPC-downregulated effector genes.

According to our IPA analysis, none of the top ten metabolic pathways in the four groups we analyzed were shared (Table 5A). We recently reported that 63–70% of the T cell effector signaling pathways and T cell memory signaling pathways regulated by 14 co-stimulation receptors, and four dual function receptors are shared [48]. The significant overlap between effector signaling pathways and memory signaling pathways of T-cells is shown in Table 5B. Unlike in T-cells, all the metabolic signaling pathways that are affected by LPC modulated trained immunity enzymes and LPC modulated effector genes in EC did not overlap (Table 5B). What we inferred from these data is that ECs utilize distinct signaling mechanisms to elicit trained immunity to that of EC activation in response to conditional DAMP such as LPC. Partly,

this may be due to differential H3K14Ac binding levels seen between the genomic regions of the genes that induce trained immunity function and EC activation (Fig. 4B).

3.5. Inter-chromosomal long-range interactions (CLRI, trans chromatin interaction) (from different chromosomes) favor LPC induction (81%) over reduction (7.7%) of trained immunity genes. In contrast, intra-chromosomal CLRIs (cis-chromatin interaction) favor both LPC mediated induction (65%) and LPC reduction (61.2%) of EC activation effector genes

Rapid development of technologies such as chromosome conformation capture sequencing (3C-Seq) [54], chromosome conformation capture-on-chip (4C-Seq) [55,56], and chromosome conformation

Table 3C

Three out of 4 upregulated histone acetyltransferases are functional in mediating histone 3 lysine 14 acetylation (H3K14ac), while 2 decreased histone acetyltransferases are not, suggesting that LPC may induce H3K14ac presumably through the function of three LPC-upregulated histone acetyltransferases. Of note, among six LPC-upregulated histone acetyltransferases, the histone acetylation sites of only four enzymes have been documented in the PubMed.

Histone acetyltransferase Alias	Substrates	PMID
KAT2A	GCN5, GCN5L2, HGCN5	H3K9, H3K14, H3K18, H3K23, H3K27, H3K36, 26637399, H3K56, H4K16 23437046
*KAT9	ELP3	H3K14, H4K8 12466527, 15902492
KAT8	MOF, MYST1, PP7073	H4K16 19698979
*KAT5	HTATIP, TIP60	H2AK4; H2AK5; H2AK7; H2AK11; H2AK13; 17694074, H3K14; H4K5; H4K8; H4K12; H4K16 19698979, 24316985
CREBBP	CBP; KAT3A	H2AK15; H3K27; H3K56; H3K122; H4K20 26603525, 24704920
EP300	p300; KAT3B	H2AK5; H2AK15; H2AK36; H2BK43; H2BK46; 19698979, H3K56; H3K79; H3K115; H3K122; H4K5; 22464331, H4K20; H4K44 26625199, 24704920

http://weram.biocuckoo.org/BrowseGeneral.php?enzyme=HAT&fam=p300_CBP&spe=Homosapiens.

* Three enzymes upregulated by LPC have the potential to acetylate H3K14.

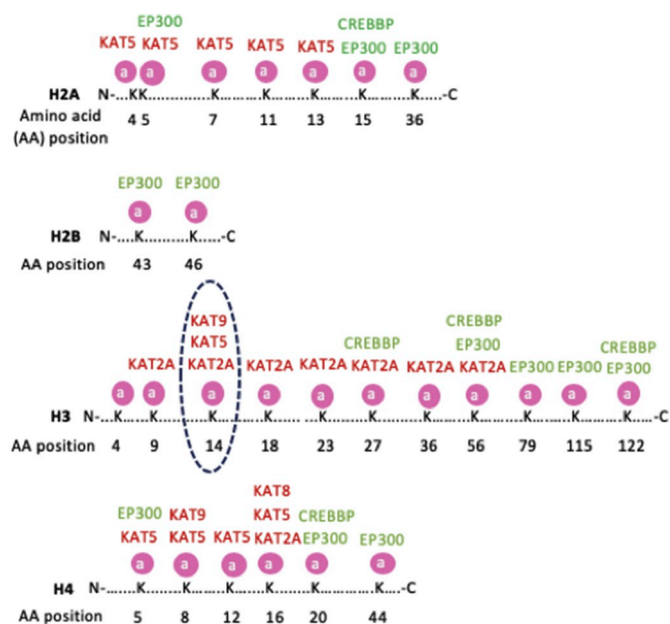


Fig. 3. LPC-upregulated three histone acetyltransferases all have potential to acetylate histone 3 lysine 14, which is the unique lysine among 10 acetyltable lysine residues in histone 3. LPC-modulated histone acetyltransferases can acetylate potential 25 lysine residues in four histones.

capture carbon copy sequencing (5C-Seq) [57] allow capturing chromosome conformations. 3C-Seq, 4C-Seq and 5C-Seq techniques are utilized to determine the relative frequency of direct physical contact between a pair of linearly separated chromatin segments, genome-wide interactions involving a single anchor genomic region (encoding the genes of interest), and interactions involving multiple genomic regions,

respectively. Differences in chromatin long-range interaction (CLRI) patterns between genes have previously been hypothesized to influence alternative splicing [58], the transcription of inflammatory genes such as cytokine [59], cytokine receptor [60] and cardiovascular disease-causative genes [61].

A group of proteins that are referred to as insulators bind to distinct regions of the genome and mediate intra- and inter-chromosomal interactions [62]. Previously, it was demonstrated that changes in the expression of insulators and its binding patterns alter the chromosomal interactions, thus alter the gene expression patterns [62]. We hypothesized that the distinct cellular signaling mechanisms affected by trained immunity genes and effector genes due to LPC treatment in EC (Table 5B) may be due to differential CLRI patterns in the genomic regions where these genes are located.

In order to support this hypothesis, we first examined whether LPC treatment alter the expression of eight characterized chromosome architectural insulators including RAD21, CTCF, ZNF143, ATF2, PML, ELK4, FOXM1, and POU2F2 [63]. As shown in Table 6A, two chromosome conformation regulators such as ELK4 and FOXM1 were downregulated by LPC treatment in HAEC, suggesting that conditional DAMP such as LPC may have the potential to alter chromosomal interactions that may alter the gene expression patterns. To find more evidence to support our hypothesis, we obtained CLRI data for all the trained immunity genes and effector genes modulated by LPC treatment in HAEC. The CLRI data for our analysis were collected from well-accepted 4DGenome database that has a huge collection of 4,433,071 CLRI that were experimentally-derived [31].

In our analysis, we designated the distance between the gene of interest and the upstream genomic regions that the gene interact with as positive, while the distance between the gene and the downstream genomic regions that the gene interacts as negative. We calculated the distances between the gene of interest (trained immunity genes and effector genes) and the upstream and downstream genomic regions which the gene interacts with. The two-sample Kolmogorov-Smirnov

Table 4A

The 10 out of 14 LPC-regulated glycolysis enzymes (71%) are mediated by histone 3 lysine 14 acetylation, as judged by increased histone 3 lysine 14 (acetylated) binding in the genomic regions encoding enzymes in comparison to that non-acetylated histone 3 lysine 14 binding in the same genomic regions encoding the enzymes.

ID	Gene Symbol*	Object name	H3K14ac Ratio (LPC/control)
218	ALDH3A1	aldehyde dehydrogenase 3 family member A1	1.539
226	ALDOA	aldolase, fructose-bisphosphate A	1.140
2027	ENO3	enolase 3	1.339
5106	PCK2	phosphoenolpyruvate carboxykinase 2, mitochondrial	1.993
5210	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	1.690
5211	PFKL	phosphofructokinase, liver type	2.173
5236	PGM1	phosphoglucomutase 1	1.367
5315	PKM	pyruvate kinase M1/2	1.187
5208	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	0.628
5160	PDHA1	pyruvate dehydrogenase E1 alpha 1 subunit	--
441531	PGAM4	phosphoglycerate mutase family member 4	--
5230	PGK1	phosphoglycerate kinase 1	--
130589	GALM	galactose mutarotase	1.034
2645	GCK	glucokinase	1.840

#LPC treated H3K14 Ac binding peak versus H3K14 Ac binding peak of vehicle control. *Genes in red are LPC-upregulated in HAECs and genes in green are LPC-downregulated. Unchanged expressions of house-keeping genes in RNA-seq are not modulated in LPC H3K14 acetylated bindings.

Table 4B

The 4 out of 6 LPC-upregulated mevalonate pathway enzymes (71%) are mediated by histone 3 lysine 14 acetylation.

ID	Gene Symbol	Gene Name	Ratio (LPC/control)
38	ACAT1	acetyl-CoA acetyltransferase 1	1.220
4598	MVK	mevalonate kinase	1.985
4597	MVD	mevalonate diphosphate decarboxylase	1.551
3422	IDI1	isopentenyl-diphosphate delta isomerase 1	1.813
3157	HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	0.830
3156	HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	--

Table 4C

The 5 out of 6 LPC upregulated acetyl-CoA generating enzymes (83%) are mediated by histone 3 lysine 14 acetylation.

Gene ID	Gene Symbol	Gene name	Ratio (LPC/control)
587	BCAT2	branched chain amino acid transaminase 2	1.604
51703	ACSL5	acyl-CoA synthetase long chain family member 5	1.154
47	ACLY	ATP citrate lyase	1.281
3417	IDH1	isocitrate dehydrogenase (NADP(+)) 1, cytosolic	0.628
3033	HADH	hydroxyacyl-CoA dehydrogenase	1.735
2180	ACSL1	acyl-CoA synthetase long chain family member 1	1.675

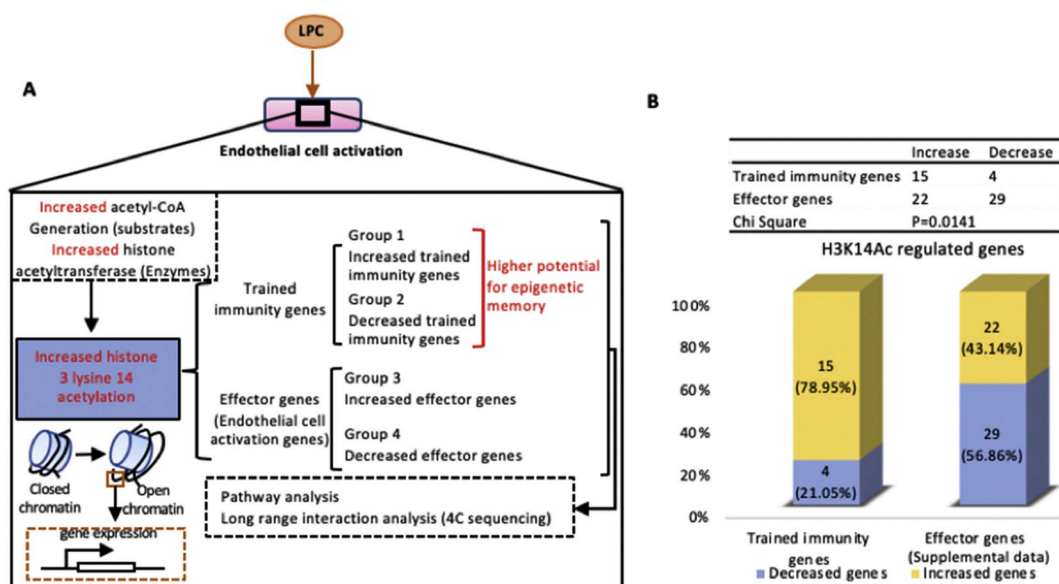


Fig. 4. Acetylation level of histone 3 lysine 14 residue in the genomic regions of enzymes that mediated trained immunity is higher than that of endothelial cell activation. **A.** LPC treatment regulates the expression of trained immunity genes and effector genes via H3K14Ac or not; and the 4 groups are compared with pathway analysis and 4c sequencing dataset for long range interaction. **B.** H3K14Ac regulates 19 trained immunity genes and 51 effector gene; 15 out of 19 genes are increased (78.95%) and 22 out of 51 genes are increased (43.14%).

test confirmed that the CLRI distance distribution between the trained immunity genes and effector genes are significantly different to each other ($p < 0.001$).

For our analysis, we categorized the genes of our interest in to following groups: 1) LPC – downregulated effector genes, 2) LPC-upregulated effector genes, 3) LPC-upregulated trained immunity genes, and 4) LPC-downregulated trained immunity genes.

According to our analyses, effector genes and trained immunity

genes do interact with distant genomic regions (Table 6B). All the four groups we analyzed interact with distinct genomic regions to each other. Interestingly, we found significant differences between the CLRI pattern of the trained immunity genes and effector genes that were upregulated by LPC treatment in EC. 18.8% of the distant genomic regions that upregulated trained immunity genes interact with were located on the same chromosome (*cis*-chromatin interactions) while 81.2% of the distant genomic regions that these genes interact with

Table 4D

4 out of 7 trained immunity response genes are increased in H3K4ac bindings while the expression levels are not significantly changed in LPC induced RNA seq data.

Trained					
immunity readouts	Gene name	Ratio	Control peak	LPC peak	PMID
TNF- α	tumor necrosis factor alpha	1.314	30	45	29328908, 27733422
Il-1 β	Interleukin 1 beta	1.183	12	18	30380404, 29328908, 27733422
CD131	cytokine receptor common subunit beta	1.966	8	16	29367213
MMP2	Matrix metalloproteinase 2	1.088	48	43	29367213
IL-6	Interleukin 6	0.76	28	18	27733422
MCP1	monocyte chemoattractant protein-1	--	--	--	29367213
MMP9	Matrix metalloproteinase 9	--	--	--	29367213

Table 5A

LPC activated aortic endothelial cells (ECs) via ① increasing or ② decreasing EC activation effector genes; and ③④ trained immunity genes. The top 10 metabolic pathways for the four groups are not overlapped.

	Effector	Memory
① LPC increased endothelial cell activation effector genes		
Hepatic Fibrosis/Hepatic Stellate Cell Activation	✓	
TREM1 Signaling	✓	
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	✓	
Atherosclerosis Signaling	✓	
Granulocyte Adhesion and Diapedesis	✓	
Lanosterol Biosynthesis	✓	
Role of Cytokines in Mediating Communication between Immune Cells	✓	
VDR/RXR Activation	✓	
Neuroinflammation Signaling Pathway	✓	
p38 MAPK Signaling	✓	
② LPC decreased endothelial cell activation effector genes		
ATM Signaling	✓	
Adenine and Adenosine Salvage III	✓	
Purine Ribonucleosides Degradation to Ribose-1-phosphate	✓	
Adenosine Nucleotides Degradation II	✓	
Purine Nucleotides Degradation II (Aerobic)	✓	
GADD45 Signaling	✓	
DNA damage-induced 14-3-3 σ Signaling	✓	
Estrogen-mediated S-phase Entry	✓	
Sonic Hedgehog Signaling	✓	
April Mediated Signaling	✓	
③ LPC increased trained immunity genes		
Mevalonate Pathway I		✓
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)		✓
Glycolysis I		✓
Superpathway of Cholesterol Biosynthesis		✓
Gluconeogenesis I		✓
AMPK Signaling		✓
Sirtuin Signaling Pathway		✓
Ketogenesis		✓
Isoleucine Degradation I		✓
Acetyl-CoA Biosynthesis III (from Citrate)		✓
④ LPC decreased trained immunity genes		
Fatty Acid β -oxidation I		✓
Type II Diabetes Mellitus Signaling		✓
Acetate Conversion to Acetyl-CoA		✓
Trehalose Degradation II (Trehalase)		✓
Galactose Degradation I (Leloir Pathway)		✓
Sucrose Degradation V (Mammalian)		✓
GDP-glucose Biosynthesis		✓
Glucose and Glucose-1-phosphate Degradation		✓
UDP-N-acetyl-D-galactosamine Biosynthesis II		✓
Fatty Acid Activation		✓

were located on a different chromosome (*trans*-chromatin interactions). In contrast, upregulated effector genes had 65% *cis*-chromatin interactions and 35% *trans*-chromatin interactions (Table 6C). Overall, this data suggest that *trans*-chromatin interactions may play a significant role in upregulating trained immunity genes while its contribution to upregulate effector genes is comparatively low in EC treated with LPC (Fig. 5A). Interestingly, the upstream CLRI regions of LPC-downregulated effector genes span in a narrow range when compared to that of LPC-upregulated effector genes (Fig. 5A). Of note, very few upstream CLRIs found in LPC-increased trained immunity genes group in the database prevented us from obtaining the statistical analysis data. Moreover, most of the genomic regions that trained immunity genes interact with are located at a larger distance in the chromosome to that of effector genes of HAEC (Fig. 5B).

Overall, our results suggest that *trans*-chromatin interactions may play an essential role in upregulating the genes we included in upregulated trained immunity group in responses to LPC in HAEC. In contrast, *cis*-chromatin interactions seem to be favorable for both LPC-upregulated effector genes (65%) and LPC-downregulated gene groups (61.2%). Since the 4DGenome database contains the experimental data derived from human non-aortic endothelial cells [31], future work will be needed to consolidate our findings in aortic endothelial cells by utilizing circular chromosome conformation capture sequencing (4C-Seq) technique. Of note, our findings are well correlated with previous report, suggesting that *trans*-chromatin interactions have multiple functions, not only regulating gene expressions such as olfactory receptors, interferon responsive genes, facilitating the formation of chromatin domains such as centromere clusters more globally in the establishment of X-inactivation [62], but also promoting trained immunity pathway gene upregulation demonstrated here.

4. Discussion

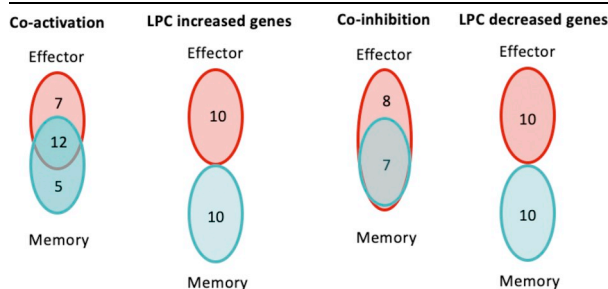
Previously, we have introduced the concept that endothelial cells can act as conditional innate immune cells [12]. We recognized endothelial cells as conditional innate immune cells because of their capability to express MHC (major histocompatibility complex) I and II molecules, pattern recognition receptors, adhesion molecules and produce pro-inflammatory cytokines, chemokines in response to chronic stresses. Expression of MHC class I and II may facilitate the endothelial cells to act as antigen presenting cells and propagate adaptive immune responses [48]. Therefore, endothelial cells may function similar to professional innate immune cells when exposed to different stresses for a prolonged time.

The adaptive immune memory had been studied extensively before [48]. Recently, the ability of innate immune cells to develop memory in

Table 5B

The top metabolic signaling pathways in regulating trained immunity genes and endothelial activation genes are not overlapped. In contrast, co-stimulation and co-inhibition receptor pathways in regulating T cell effector function and memory functions are partially overlapped.

		Numbers			Shared Percentage	
		Effector	Memory	Shared by Effect and memory	Effect	Memory
T cell (Adaptive immune cell)	Coactivation	19	17	12	63.16%	70.59%
	Coinhibition	15	7	7	46.67%	100.00%
Endothelial cell (Innate immune cell)	LPC increased genes	10	10	0	0.00%	0.00%
	LPC decreased genes	10	10	0	0.00%	0.00%

**Table 6A**

LPC modulates the expressions of chromosomal architectural insulator insulators, such as ELK4 and FOXM1, suggesting that LPC may modulate chromatin long-range interaction.

Gene ID	Gene Symbol	Fold Change		Control peak	LPC peak
		in RNA seq	(H3K14Ac CHIP-seq)		
2005	ELK4	0.86	2.83	6	17
2305	FOXM1	0.79	0.94	17	16

8 architectural protein genes (RAD21, CTCF, ZNF143, ATF2, PML, ELK4, FOXM1, POU2F2) are identified in the literature. 2 out of 8 insulators are downregulated in RNA-seq data of LPC treated human aortic endothelial cells.

response to adverse stimuli was also demonstrated. This phenomena is referred to as innate immune memory or “trained immunity” [26]. This is a primitive form of adaptation in host defense, resulting from chromatin structure rearrangement, which provides an increased but non-specific response to a reinfection. In contrast to innate immune memory, adaptive immune memory is more advanced, with increased magnitude of response mediated through epigenetic changes, as well as specificity mediated by gene recombination [72]. Although trained immunity has been studied extensively in innate immune cells such as monocytes, macrophages, natural killer cells, ability of non-immune cells such as hematopoietic cells, mesenchymal stem cells, and epithelial cells to elicit trained immunity had been reported [22].

Our previous reports demonstrated that epigenetic mechanisms such as histone 3 lysine 14 acetylation [7], miR155 [64], and potentially miR221 [65] play important roles in promoting human aortic

Table 6B

LPC modulates chromatin long-range interaction around trained immunity genes and endothelial cell activation effector genes.

Obs	Type	Stream	Count
1	LPC decreased effector genes	DownStream	23
2	LPC decreased effector genes	UpStream	15
3	LPC decreased trained immunity genes	DownStream	2
4	LPC decreased trained immunity genes	UpStream	10
5	LPC increased effector genes	DownStream	9
6	LPC increased effector genes	UpStream	17
7	LPC increased trained immunity genes	DownStream	3

endothelial cell activation, metabolic cardiovascular disease [46] and CD4+Foxp3+ regulatory T cells [50]. Although Immunological memory is an important evolutionary trait that improves host survival upon reinfection [66], a comprehensive analyses of trained immunity pathways (TIP) and trained immunity enzyme expression related to cardiovascular diseases remain in its infancy [67]. Despite the fact that atherosclerosis-relevant human aortic endothelial cells can act as conditional innate immune cells when exposed stresses, their ability to formulate innate immune memory in response to these stresses had not been reported. To fill in this important knowledge gap, we profiled the expression of 102 metabolic enzymes that mediate the formation of trained immunity in human aortic endothelial cells (HAECs) stimulated with LPC, which we have previously proposed as a “conditional danger associated molecular pattern (conditional DAMP)” [19,68]. The metabolomic analysis we conducted on the aortas of 3 week high fat diet fed Apolipoprotein E deficient mice showed a significant increase in the LPC species including palmitoyl-LPC (16:0), stearoyl-LPC (18:0), and oleoyl-LPC (18:1) [10]. In our *in-vitro* experimental systems, we have used LPC at a concentration of 10 μ M. Our previous study demonstrated that LPC at a concentration of 10 μ M in HAEC induced mitochondrial ROS production, which played an essential role in promoting H3K14Ac in ICAM-1 promoter, leading to EC activation [10].

We made the following findings: *first*, LPC induces upregulation of three trained immunity pathways, 17% of 71 glycolysis enzymes, 86% of seven mevalonate enzymes, 17% of 24 acetyl-CoA enzymes, in HAECs; *second*, LPC upregulate six out of 31 histone acetyltransferases, three reported to acetylate histone 3 lysine 14 (H3K14ac); *third*, Histone 3 lysine 14 residue is acetylated in genomic regions of enzymes that

Table 6C

The chromatin long range interactions for LPC increased trained immunity genes are different from that of LPC-increased endothelial cell activation effector genes. The increased trained immunity genes have 18.8% chromatin long range interactions on the same chromosome (*cis*-chromatin interaction) and 81.2% chromatin long range interactions on different chromosomes (*trans*-chromatin interaction). In contrast, LPC increased EC activation effector genes have 65% *cis*-chromatin interactions and 35% *trans*-chromatin interactions.

Long range interaction	Cis-chromatin interaction	Percentage (%)	Trans-chromatin interaction	Percentage (%)	Total (%)
Increased Trained immunity	3	18.75	13	81.25	100
Decreased Trained immunity	12	92.31	1	7.69	100
Increased effector	26	65	14	35	100
Decreased effector	38	61.29	24	38.71	100

Increased Trained immunity	Decreased Trained immunity	P < 0.0001
Increased effector P = 0.0027	Decreased effector P = 0.0491	P = 0.6605

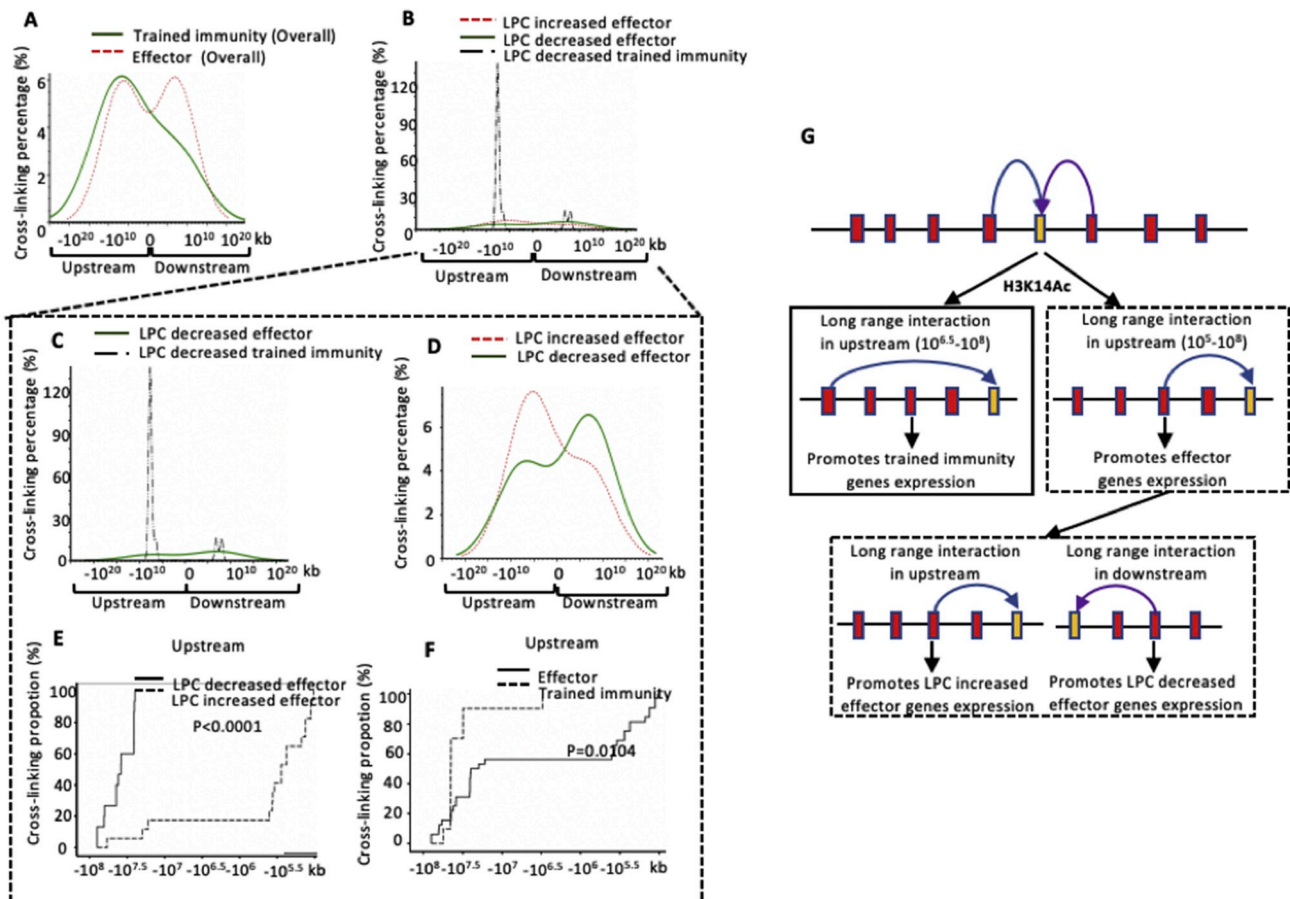
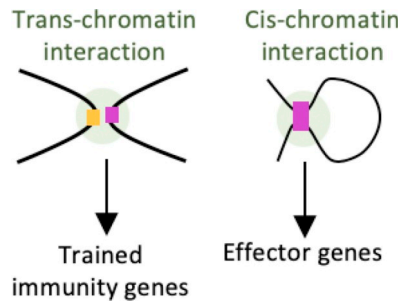


Fig. 5. Inter-chromosomal long-range interactions favor LPC induction of trained immunity genes. A. The long-range interaction in upstream promotes trained immunity in LPC treated HAECs. The long-range interaction in upstream is significant different between LPC increased effector genes and decreased effector gene; and between effectors and trained immunity genes. B. Working model of long-range regulation of genes.

Table 7A

Nicotinamide adenine dinucleotide phosphate oxidase 2 (NADPH oxidase 2, NOX2) deficiency or inhibition downregulates some trained immunity enzymes and endothelial activation effectors although the cell types used in the microarray analyses were not matched. These results suggest that reactive oxygen species (ROS) may promote both trained immunity pathways and endothelial activation pathways.

GEO dataset ID*	Species	Genotype	Cell type	Gene symbol	Fold change	Gene group	Percentage [#]
GSE100671	<i>Homo sapiens</i>	NOX2 - / -	Peripheral blood leukocyte	PFKL	1.21	Glycolytic enzyme	Upregulation
				ENO3	0.80	Glycolytic enzyme	5/22 (22.7%)
				PCK2	1.83	Glycolytic enzyme	Downregulation 6/22 (27.3%)
				PKM	0.68	Glycolytic enzyme	
				ALDOA	0.83	Glycolytic enzyme	
				PGAM4	0.76	Glycolytic enzyme	
				PGM1	1.27	Glycolytic enzyme	
				PGK1	0.82	Glycolytic enzyme	
				ACATI	1.32	Mevalonate enzyme	
				ACSL5	0.85	Acetyl-CoA generating enzyme	
				ACLY	1.19	Acetyl-CoA generating enzyme	
				SLC2A6	0.89	Effectors (Endothelial cell activation genes)	Upregulation
				S100a4	0.45	Effectors (Endothelial cell activation genes)	5/26 (19.2%)
				IL32	3.10	Effectors (Endothelial cell activation genes)	Downregulation 5/26 (19.2%)
				ZNF185	1.64	Effectors (Endothelial cell activation genes)	
				ccdc24	1.63	Effectors (Endothelial cell activation genes)	
				TMEM158	1.61	Effectors (Endothelial cell activation genes)	
rotdi	0.71	Effectors (Endothelial cell activation genes)					
tm7sf2	0.61	Effectors (Endothelial cell activation genes)					
sult1a1	0.85	Effectors (Endothelial cell activation genes)					
IL1RL1	1.72	Effectors (Endothelial cell activation genes)					
GSE36446	<i>Rattus norvegicus</i>	NOX2 inhibitor	Kidney Cortex	PFKFB2	0.88	Glycolytic enzyme	Upregulation
				PFKL	1.30	Glycolytic enzyme	2/22 (9.1%)
				PGM1	0.84	Glycolytic enzyme	Downregulation 3/22 (13.6%)
				HMGCS1	1.16	Mevalonate enzyme	
				MVD	0.78	Mevalonate enzyme	
				ST6GALNAC1	0.91	Effectors (Endothelial cell activation genes)	Upregulation
				IGF2	0.73	Effectors (Endothelial cell activation genes)	0/26 (0%)
				FADS2	0.80	Effectors (Endothelial cell activation genes)	Downregulation 3/26 (11.5%)
				PFKL	0.59	Glycolytic enzyme	Upregulation
				PKM	0.85	Glycolytic enzyme	1/22 (4.5%)
PCK2	0.79	Glycolytic enzyme	Downregulation 4/22 (18.2%)				
PFKFB2	0.91	Glycolytic enzyme					
MVD	1.49	Mevalonate enzyme					
GSE36446	<i>Rattus norvegicus</i>	NOX2 inhibitor	Kidney medulla	tm7sf2	0.76	Effectors (Endothelial cell activation genes)	Upregulation
				IL1RL1	1.25	Effectors (Endothelial cell activation genes)	1/26 (3.8%)
							Downregulation 1/26 (3.8%)

* GSE is the dataset ID in NCBI-GEO datasets (<https://www.ncbi.nlm.nih.gov/gds>).

The number of upregulated or downregulated genes is divided by the total number of increased trained immunity enzymes [22] or increased endothelial cell activation gene [26].

mediate trained immunity (79%) in comparison to genes that regulate endothelial cell activation (effectors) (43%); *fourth*, the pathway differences between trained immunity and EC activation effector pathways are bigger than the difference between T cell effector and T memory pathways; and *fifth*, *trans*-chromatin interactions favor LPC induction (81%) over reduction (7.7%) of trained immunity genes. In contrast, *cis*-chromatin interactions favor both LPC induction (65%) and LPC reduction (61.2%) of EC activation effector genes.

Our data revealed that conditional DAMP such as LPC can activate distinct cellular signaling pathways during development of innate immune memory and during endothelial cell activation (effector function). Our data is not sufficient to describe whether these signaling pathways lie upstream or downstream of LPC mediated trained immunity function or effector function in EC. However, the fact that endothelial cells may have the potential to separate innate immune memory function from effector function in response to stimuli is indeed novel and interesting. Based on the top pathways identified in the Ingenuity Pathway Analysis, the dominant features of trained immunity pathways in aortic ECs are metabolic generation of acetyl-CoA for histone epigenetic reprogramming while the dominant features of EC activation effector pathways are for recruiting inflammatory cells. One of the potential mechanisms that endothelial cells may utilize to separate the trained immunity function from effector function is differential epigenetic modulation in the genes responsible for exerting these two functions. We observed that there is a differential level of acetylation at the lysine 14 residues of H3 histones bound in the genomic regions of the genes that regulate trained immunity function versus that regulate EC effector function. In our study, we analyzed only one type of epigenetic modifications. We acknowledge that, as we reported previously [46], other epigenetic modifications such as histone methylation, histone SUMOylation (a post-translational modification with small ubiquitin-like modifiers), histone phosphorylation, histone ubiquitination, as well as histone acetylation on other lysine residues in histones need to be studied in future to fully elucidate how these epigenetic changes may impact trained immunity and effector functions in endothelial cells.

Furthermore, we observed that there is a significant difference between the genes that regulate the trained immunity function and effector function in their interacting patterns with distant chromosomal regions. Our data revealed that the trained immunity genes that were upregulated by the LPC treatment in endothelial cells preferentially

have *trans*-chromatin interactions, while effector genes have *cis*-chromatin interactions. Furthermore, distant chromatin interaction regions of the trained immunity genes seem to be located at a larger distance when compared to that of effector genes. Also, distant chromatin regions that the downregulated trained immunity genes interact with seem to span in a narrow region when compared to that of downregulated effector genes. However, as we mentioned in the results section, well controlled experiments are required in the future to fully discern the differential distant genomic interaction patterns of trained immunity genes and effector genes in endothelial cells.

One of the interesting findings of our study is that the pathway differences between trained immunity and EC activation effector pathways are larger than the differences between T cell effector and memory pathways. The extent of epigenetic changes and long range chromosomal interaction patterns that contribute to this difference we observed need to be investigated in future. However, epigenetic changes are indeed crucial for formation of adaptive immunity. Failure to generate and maintain epigenetic changes in adaptive immune cells results in poor adaptive immune memory [69]. Moreover, DNA methylation, histone modifications and chromatin structure are involved in controlling T-cell responses [70]. In addition, interchromosomal interactions have proven to play an important role at the early stage of T-cell activation and differentiation [71,72]. How *trans*-, and *cis*-chromosomal interactions can affect the formation of adaptive immune memory and its effector functions need to be investigated further in the future.

Of note, recent reports suggest that accumulation of fumarate from the tricarboxylic acid (TCA) cycle and mevalonate from the cholesterol synthesis pathway induce TIP; and that statins inhibit TIP but 6-fluoro-mevalonate (6-FM) and IL-1 β boost TIP [73]. Recent work had indicated that IL-1 family of cytokines, especially IL-1 β play a role in regulating trained immunity [74]. Exposure of macrophages from mice to IL-1 β resulted in an increase in glucose uptake compared to untreated control cells [75]. Previously, it was shown that the metabolic shift to aerobic glycolysis induced by the activation of Akt/mammalian target of rapamycin (mTOR)/hypoxia-inducible factor-1 α (HIF-1 α) pathway promotes trained immunity in human monocytes [76]. Recent studies also revealed associations between IL-1, HIF-1 α and cellular metabolism, which could potentially lead to development of trained immunity [77].

In addition, it has been well documented that reactive oxygen species (ROS) play a key role in regulating pathophysiological signaling

Table 7B

Antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) deficiency upregulates some trained immunity enzymes and endothelial activation genes although the cell types used in the microarray analyses were not matched. These results suggest that antioxidant Nrf2 inhibits both trained immunity pathways and endothelial activation pathways.

GEO dataset	Species	Genotype	Cell type	Gene symbol	Fold change	Gene group	Percentage
GSE867	<i>Mus musculus</i>	Nrf2 -/-	liver	PFKFB2	2.74	Glycolytic enzyme	Upregulation
				HMGCS1	3.02	Mevalonate enzymes	3/22 (13.6%)
				ID1	3.37	Mevalonate enzymes	Downregulation 0/22 (0%)
				IGF2	2.06	Effectors (Endothelial cell activation genes)	Upregulation
				LSS	2.83	Effectors (Endothelial cell activation genes)	2/26 (7.7%)
							Downregulation 0/26 (0%)
GSE7810	<i>Mus musculus</i>	Nrf2 -/-	Lung type II cell	PFKL	1.45	Glycolytic enzyme	Upregulation
				HMGCR	0.75	Mevalonate enzymes	4/22 (18.2%)
				ACLY	1.99	Acetyl-CoA generating enzyme	Downregulation 1/22 (4.5%)
				ACSL5	1.40	Acetyl-CoA generating enzyme	
				IDH1	1.37	Acetyl-CoA generating enzyme	
				S100a4	0.63	Effectors (Endothelial cell activation genes)	Upregulation
				MMAB	0.75	Effectors (Endothelial cell activation genes)	3/26 (11.5%)
				IGFBP5	5.91	Effectors (Endothelial cell activation genes)	Downregulation 4/26 (15.4%)
				SLC2A6	2.74	Effectors (Endothelial cell activation genes)	
				ICAM1	0.60	Effectors (Endothelial cell activation genes)	
				Qpct1	1.40	Effectors (Endothelial cell activation genes)	
				CALD1	0.48	Effectors (Endothelial cell activation genes)	

in endothelial cell activation [78] and cardiovascular diseases [79]. We also reported that mitochondrial ROS play a significant role in EC activation [7,11]. Thus, we also determined whether ROS generation pathway and antioxidant pathway could regulate trained immunity enzyme expression and EC activation effector expression. By mining the microarray datasets in the NIH-NCBI-GeoDataset database (<https://www.ncbi.nlm.nih.gov/gds/>), we found that the deficiency or inhibition of nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) [79] decreases (Table 7A), but the deficiency of antioxidant transcription factor Nuclear factor erythroid 2-related factor 2 (Nrf2) [79] increases (Table 7B), the expression of a few trained immunity enzymes and EC activation effectors, although the cell types used in the microarray analyses were not endothelial cells. These preliminary database-mining analyses suggest that ROS may have the potential to increase the activity of both trained immunity pathways and EC activation effector pathways (Fig. 6), which are correlated with a recent report that mammalian target of rapamycin (mTOR)-dependent ROS formation promotes trained immunity in human monocytes [80]. We also acknowledge that the future studies are required using human aortic ECs will be warranted to examine this issue carefully and further determine whether any ROS pathways, reactive nitrogen species (RNS) and antioxidant/anti-RNS pathways [81] could modulate the activity of

trained immunity pathways and use distinct cellular signaling mechanisms to regulate trained immunity pathways and EC activation effector pathways reported here.

LPC is a pro-inflammatory lipid that promote development of endothelial cell activation and atherosclerosis [82]. Our analysis suggests that LPC may elicit trained immunity function in addition to activation in endothelial cells. Regardless of significant progress in the understanding of metabolic cardiovascular diseases, how the risk factors such as hyperlipidemia, hyperglycemia, hyperhomocysteinemia, cigarette smoke, hypertension and metabolic syndrome trigger vascular inflammation and atherosclerosis remain poorly defined [83]. Based on our analysis, it can be hypothesized that chronic exposure of endothelial cells to these risk factors may trigger formation of innate immune memory.

On the other hand, nutrition is also identified as a modifiable factor in impacting immune function and has been studied for several decades. The research in this field has developed into a distinguished study subject called nutritional immunology [84]. A dietary inflammation index has also been proposed previously [85]. In addition to contribution of dietary components in developing hyperglycemia, hyperlipidemia and subsequent impact on manifesting cardiovascular diseases and other sterile inflammatory diseases is well recognized. We propose that conditional DAMP-upregulated trained immunity pathway

Table 7C
A new working model.

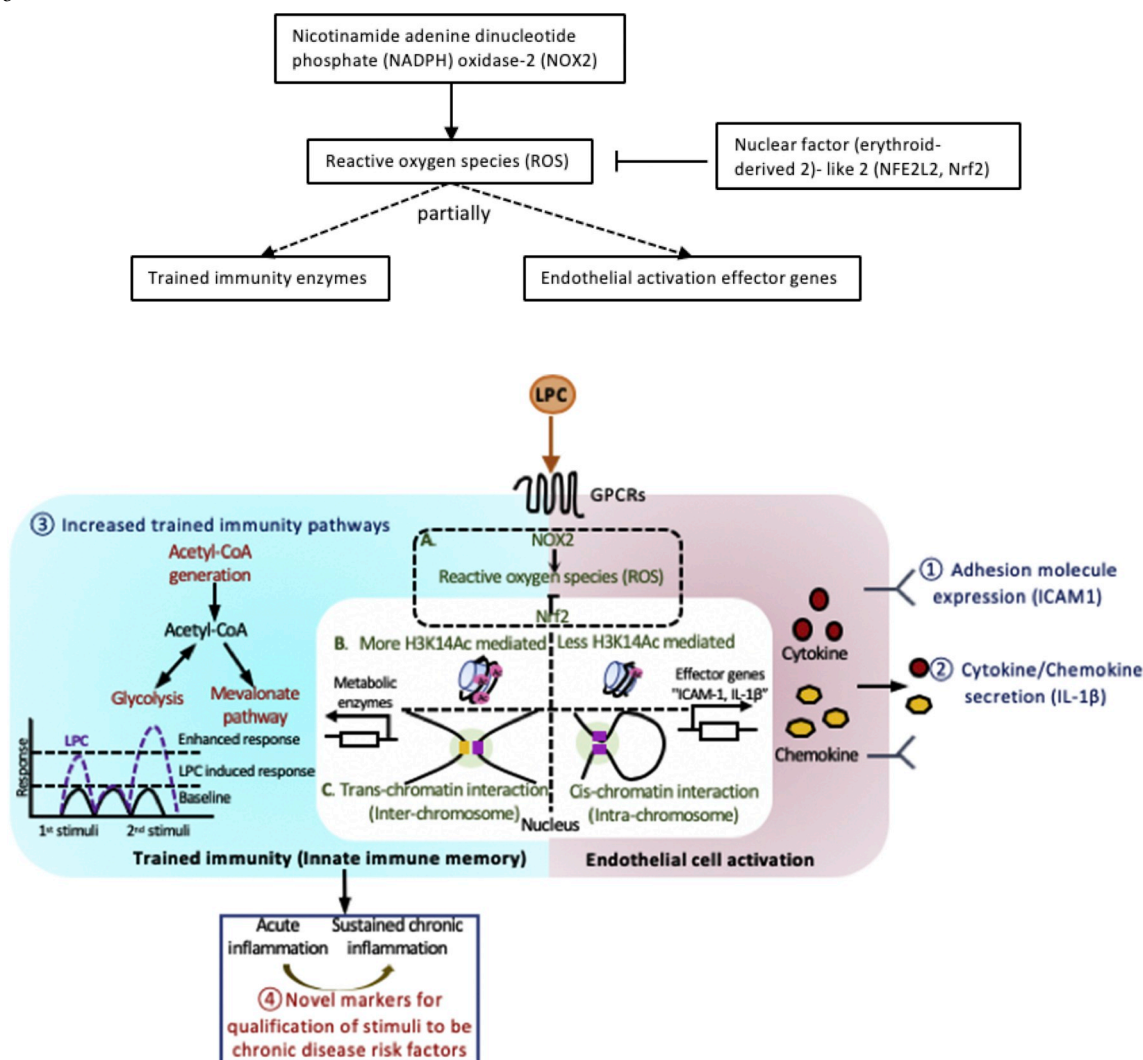


Fig. 6. A novel working model: LPC leads to different trained immunities which are the novel makers for quantification of chronic disease risk factor; and LPC leads to trained immunity through ROS production, H3K14 acetylation and long-range interaction.

enzymes and the acetylation status H3K14 can be used as novel qualification markers of chronic inflammation risk factors out of all the stimuli we exposed to foods (all the 26,625 compounds identified in the foods (<http://foodb.ca/compounds>) and environmental compounds (for example, more than 7000 compounds in cigarette smoke, <https://www.lung.org/stop-smoking/smoking-facts/whats-in-a-cigarette.html>).

We acknowledge that the expression of some of the genes of the TIP and histone acetyl transferases regulated by LPC in aortic EC appear to be modest. Previously, *Laurent* et al. had presented the evidence that small changes in levels of RNAs can provide biologically meaningful insights, suggesting modest changes in gene expression should not be ignored [86]. In their experimental system, approximately half of all biologically meaningful transcripts that change during the course of treatment was less than 2 folds. Therefore, an argument was made that small changes of the transcripts that represent a significant fraction of the transcriptional changes in a tissue indeed can be biologically meaningful. Likewise, it is highly likely that the modest changes we see in the gene expression in our study may translate in to meaningful biological effects.

Finally, we have summarized our findings in the working model shown in Fig. 6. Briefly, we suggest that conditional DAMP such as LPC may trigger innate immune memory and EC activation via distinct mechanisms. One of these mechanisms is induction of differential acetylation level at H3K14 in the genomic regions of trained immunity genes versus that of effector genes. Therefore, we suggest that conditional DAMP-upregulated trained immunity pathway enzymes and the acetylation status H3K14 can be used as novel qualification markers, which can be used to determine whether a particular stress can be a cardiovascular disease risk factor. Another mechanism, that endothelial cells may use to separate trained immunity function from effector function, is by utilizing differential interaction patterns between the distant genomic regions and the genes that induce memory and effector functions. These novel insights may lead to identifications of new cardiovascular risk factors in upregulating TIP in vascular cells and novel therapeutic targets of metabolic cardiovascular diseases, inflammations, and cancers.

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Availability of data and materials

None.

Authors' contributions

YL and YS carried out the data gathering, data analysis and prepared tables and figures. CDIV, GKN, YS, FS, CJ, RZ, DY, XL, WYY, JY, XHJ, ETC and HW aided with analysis of the data. XFY supervised the experimental design, data analysis, and manuscript writing. All authors read and approved the final manuscript.

Conflicts of interest

The authors have no competing interests to disclose.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2019.101221>.

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