**Short communications**

# DNA microarray analyses of genes expressed differentially in 3T3-L1 adipocytes co-cultured with murine macrophage cell line RAW 264.7 in the presence of the toll-like receptor 4 ligand bacterial endotoxin

**Short running title: Genes in adipocyte-macrophage interactions**

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**ABSTRACT**

Recent studies have suggested macrophages were integrated to adipose tissues to interact with adipocytes, thereby exacerbating inflammatory responses. Furthermore, both adipocytes and macrophages appear to express TLR-4, and free fatty acids may stimulate cells via TLR-4. Herein, we analyzed genes differentially expressed in adipocytes when co-cultured with macrophages in the presence of a ligand for TLR-4, bacterial lipopolysaccharide. RAW264.7, a murine macrophage cell line and differentiated 3T3-L1 adipocytes were co-cultured using a transwell system. Genes differentially expressed in adipocytes were analyzed by the DNA microarray method following 4, 8, 12 and 24h stimulation with 1 ng/ml of *E. coli* LPS. Randomly selected genes with high expressions were confirmed by quantitative methods at both the gene and the protein level. Co-culture of macrophages and adipocytes with a low LPS concentration (1 ng/ml) markedly up-regulated gene expressions associated with inflammation and/or angiogenesis, such as those of IL-6, MCP-1, RANTES and CXCL1/KC, in adipocytes. Furthermore, several genes associated with insulin resistance were differentially expressed. Up-regulations of genes encoding MCP-1, RANTES and CXC/KC were confirmed by quantitative methods. These results suggest that ligands for TLR-4 stimulate both adipocytes and macrophages to up-regulate the expressions of many genes associated with inflammation and/or angiogenesis.

**Key words**: adipocyte-macrophage interaction; TLR-4; endotoxin; gene expression; microarray analyses

## INTRODUCTION

It has been suggested that obesity is strongly associated with chronic inflammation, which, at least in part, explains why obese subjects are susceptible to both type 2 diabetes and atherosclerosis [1, 2]. Obese subjects characteristically have increased levels of circulating tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), and hence of c-reactive protein (CRP) [3]. Adipose has been postulated to be a primary tissue producing TNF-α and IL-6 in obese subjects, although the exact cell types responsible for the production of each cytokine remain unknown. Recent studies have suggested macrophages to be integrated into adipose tissues, interacting with adipocytes, and thereby exacerbating inflammatory responses, insulin resistance, and vascular complications [4, 5]. Co-culturing adipocytes with macrophages reportedly up-regulates some inflammatory gene expressions [6]. Furthermore, it has been reported that not only macrophages but also adipocytes express toll like receptor-4 (TLR-4), originally demonstrated to be a cell surface receptor for the bacterial endotoxin lipopolysaccharide (LPS) [7]. In addition, a recent report indicated that free fatty acid (FFA), byproducts of lipolysis and/or saturated fatty acids bind to TLR-4 and actually transduce signals into target cells [8].

We previously reported that co-culturing adipocytes and macrophages in the presence of endotoxin resulted in markedly enhanced (more than 100 fold increase) production of IL-6 as

compared to culturing each cell type alone, and suggested that co-existence of adipocytes and macrophages in the presence of TLR-4 ligands further exacerbates obesity-associated inflammatory conditions [9]. These data also suggest that gene expressions other than that of IL-6 may also be greatly influenced, in adipocytes co-cultured with macrophages, by the presence of TLR-4 ligand. Therefore, in this study, we analyzed gene expression profiles in adipocytes co-cultured with macrophages in the presence of endotoxin and attempted to detect genes with differential expressions under such conditions in order to further understand dynamic changes in gene expressions in adipocytes. These changes might be associated with the pathophysiology of diabetes and its vascular complications.

## METHODS

### *Cells and cell culture*

Mouse 3T3-L1 preadipocytes and the mouse macrophage cell line RAW264.7 were used. Pre-adipocytes were maintained in a Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, and were differentiated into mature adipocytes with 4.5 mM glucose, 1 mM insulin, 1 mM dexamethasone, and 0.5 mM 3-isobtyl-1-methylxanthine as described previously [9]. Mature adipocytes and RAW264.7 were co-cultured in a transwell system (Corning Inc., Acton,

MA) with a 0.4-μm porous membrane to separate the upper and lower chambers. Then, 1 x $10^5$ differentiated 3T3-L1 cells were cultured in the lower chamber, while 5 x $10^4$ RAW cells were cultured in the upper chamber [9].

*DNA microarray analyses*

The mouse focus array, based on a system containing 5693 gene probes (Affymetrix, Santa Clara, CA), was used to compare transcriptional profiles among 3T3-L1 adipocytes co-cultured with RAW cells in the presence or absence of 1 ng/ml of *E. coli* LPS (SIGMA, St. Louis, MO). Comparisons were made at 4, 8, 12 and 24h following LPS stimulation to assess the dynamic changes in gene expressions in adipocytes. This array contains a broad range of genes derived from publicly available, well-annotated mRNA sequences. Total RNA was isolated from 3T3-L1 adipocytes using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Preparations were quantified and their purity was confirmed by standard spectrophotometric methods. The results were expressed as the ratio of fluorescent intensity of the genes expressed in LPS-stimulated cells against that in unstimulated cells.

*Real-time PCR analyses*

To confirm the results obtained by DNA microarray analyses, we chose several highly expressed

cytokines by array analyses and confirmed their expressions by real-time PCR. The primers used are presented as supplementary information. Fold changes in mRNA were provided as the mRNA copy number of mouse 3T3-L1 cells stimulated with 1 ng/ml of LPS divided by the value of non-treated cells. Both were normalized with GAPDH as a housekeeping gene.

*Cytokine assay*

The cells were stimulated with or without *E. coli* LPS for 24h, and culture supernatants were collected. IL-6, MCP-1, RANTES and KC concentrations in the culture supernatants were measured using a commercial immunoassay (mouse IL-6, MCP-1, RANTES, and KC ELISA kits; R & D Systems, Inc., Minneapolis, MN).

*Statistical analyses*

Statistical analyses for determining significant differences in the production of each cytokine, according to culture conditions, were performed using the student t-test.

## RESULTS

*Expression profiles of genes expressed in adipocytes co-cultured with macrophages in the presence*

*of bacterial endotoxin in comparison to cells without LPS stimulation.*

Before performing microarray analyses, we first checked IL-6 protein production in supernatants from co-cultures to confirm the validity of samples (data not shown). Following confirmation of IL-6 production, we proceeded to perform microarray analyses. The full results of DNA microarray analyses are presented as supplementary information in excel format. Notably, the expressions of many genes associated with inflammation and/or angiogenesis were found to be up-regulated including that of IL-6, as expected. Besides IL-6, genes encoding MCP-1, RANTES, and CXCL1/KC were found to be highly expressed (Figure 1A). Expressions of many genes with unknown functions were also observed (see supplementary information). To confirm expressions of these genes to actually be up-regulated in LPS-stimulated adipocytes co-cultured with RAW cells, we performed real-time PCR analyses using the same samples as in the array analyses. The results are shown in Figure 1B. Gene expression profiles analyzed in two different experiments showed very similar patterns. Interestingly, there appeared to be two peaks at 4h and 12h in these cytokine gene expressions.

*3T3-L1 adipocytes produce higher amounts of MCP-1, RANTES, and CXCL1/KC proteins when co-cultuted with RAW cells in the presence of bacterial endotoxin.*

We next examined the protein expressions of these molecules using quantitative methods. The

results are shown in Figure 2. As expected, protein expressions of all these molecules (MCP-1, RANTES, and CXCL1/KC) were markedly up-regulated in adipocyte-macrophage co-cultures stimulated with endotoxin as compared to co-cultures without endotoxin stimulation, or with each cell type alone ($p<0.05$). Notably, the major source of all these molecules appeared to be 3T3-L1 adipocytes, as adipocytes alone produced detectable amounts of these molecules when stimulated with endotoxin, while RAW macrophages produced trace amounts of these proteins regardless of the presence or absence of endotoxin (Figure 2A-C).

## DISCUSSION

The present study demonstrated the LPS ligand for TLR-4 to dramatically change gene expression profiles in 3T3-L1 adipocytes in the presence of macrophages. Following DNA microarray analyses, we also confirmed high expressions of inflammation and/or angiogenesis associated molecules such as MCP-1, RANTES, and CXCL1/KC at both the gene and the protein level. It has been reported that macrophages infiltrating mature adipose tissues are primarily M1 macrophages with a pro-inflammatory phenotype which express inducible nitric oxide synthase (iNOS), TNF-α, and IL-6 [10]. These macrophages are postulated to interact with mature adipocytes, resulting in further exacerbation of the inflammatory cascade. We suggested in our previous study that TNF-α secreted

from RAW macrophages plays important roles in markedly up-regulating IL-6 translation in 3T3-L1 adipocytes, as the neutralizing TNF-α action with antibodies partially abolished the enhanced IL-6 production [9]. Therefore, we speculate that, at least in this situation, these RAW macrophages have the M1 phenotype. In fact, it has already been demonstrated that NF-kappaB is activated, and that cyclooxigenase-II, TNF-α, iNOS, and IL-12 are expressed in response to LPS in RAW macrophages. These are all typical features of M1 macrophages. We also confirmed the role of TNF-α in markedly up-regulating RANTES and CXC/KC production in our current study by using neutralizing antibody (data not shown). Interestingly, all IL-6, MCP-1, RANTES, and CXCL1/KC gene expressions appeared to show two peaks in our chronological study. We speculate that the first expression peak may be attributable to a direct effect of LPS, while the second may be due to the effects of TNF-α, considering the results of the neutralization assay described above. Il-6 and MCP-1 are cytokines well known to be associated with insulin resistance [11, 12]. Therefore, we speculate that TLR-4 ligands such as FFA and endotoxin further up-regulate inflammatory changes in adipose tissue, influencing insulin resistance and vascular disorders. In fact, besides IL-6, MCP-1, and RANTES, we found high expressions of several genes associated with inflammation such as serum amyloid A, TLR-2, plasminogen activator inhibitor-1, and vascular cell adhesion molecule-1 (supplementary information), all of which have also been reported to be highly expressed in adipose tissues of obese subjects [13-16]. In addition, the expression of suppressors of cytokine signaling-3 (SOCS-3) was

markedly enhanced (supplementary information), i.e. these inflammatory changes are highly suggestive of the insulin resistant state seen in obese subjects[17]. Cluster analyses indicated marked up-regulation of NF-kB-associated gene expression.

In contrast, GLUT-4 expression was down-regulated in endotoxin-stimulated adipocytes in the presence of RAW macrophages as compared with un-stimulated cells (supplementary information). In addition, we found that the expression of peroxisome proliferators-activated receptor (PPAR)-γ coactivator 1α (PGC-1α) was also suppressed, suggesting lipid and glucose metabolism to be greatly impaired under these conditions [18]. This may partially explain why subjects with morbid obesity are frequently resistant to physical exercise. Therefore, further studies looking at the up-stream and down-stream effector molecules will be necessary to understand the role of inflammatory signals in these impairments.

It is well known that mature adipose tissues are highly vascularized. The expressions of several genes associated with angiogenesis such as MCP-1 and CXCL1/KC were markedly enhanced [19, 20]. These gene products may promote neo-vascularization in mature adipose tissues, resulting in further recruitment of macrophages into these tissues, thereby accelerating the maturation of inflammatory adipose tissues. Our hypothesis on the role of these molecules in accelerating inflammatory changes in adipose tissues is presented schematically in Figure 2D.

Taken in total, these changes account well for our current knowledge of the molecular

mechanisms of obesity-associated insulin resistance. Analyses of the roles of many other genes, with as yet unknown functions, whose expressions were dramatically changed are currently in progress and will be reported elsewhere. We believe that our current analyses will contribute to further understanding of the molecular mechanisms associated with inflammation-induced insulin resistance in adipose tissues.

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**References**


**1.** Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA* 2001; **286**:327-334.

**2.** Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. *N Engl J Med* 1997; **336**:973-979.

**3.** Yudkin JS, Kumari M, Humphries SE, Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis* 2000; **148**:209-214.

4. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, Tartaglia LA, Chen H. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003; **112**:1821-1830.

5. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesiy is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; **112**:1796-1808.

**6.** Suganami T, Nishida J, Ogawa Y. A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol* 2005; **25**:2062-2068.

**7.** Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE. The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. *J Biol Chem* 2000; **275**:24255-24263.

8. Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 2006; **116**:3015-3025.

**9.** Yamashita A, Soga Y, Iwamoto Y, Yoshizawa S, Iwata H, Kokeguchi S, Takashiba S, Nishimura F. Macrophage-adipocyte interaction: Marked IL-6 production by co-cultures stimulated with LPS. *Obesity* 2007; **15**:2549-2552.

10. Lumenig CN, Bodzin JL, Saltiel AR. Obesity induces a phenotype switch in adipose tissue macrophage polarization. *J Clin Invest* 2007; **117**:175-184.
11. Rotter V, Nagaev I, Smith U. Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like Il-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *J Biol Chem* 2003; **278**:45777-45784.
12. Kamei N, Tobe K, Suzuki R, Ohsugi M, Watanabe T, Kubota N, Ohtsuka-Kowatani N, Kumagai K, Sakamoto K, Kobayashi M, Yamaguchi T, Ueki K, Oishi Y, Nishimura S, Manabe I, Hashimoto H, Ohnishi Y, Ogata H, Tokuyama K, Tsunoda M, Ide T, Murakami K, Nagai R, Kadowaki T. Overexpression of monocyte chemoattractant protein-1 in adipose tissue causes macrophage recruitment and insulin resistance. *J Biol Chem* 2006; **281**:26602-26614.
13. Cigolini M, Tonoli M, Borgato L, Frigotto L, Manzato F, Zeminian S, Cardinale C, Camin M, Chiaramonte E, De Sandre G, Lunardi C. Expression of plasminogen activator inhibitor-1 in human adipose tissue: a role for TNF-alpha? *Atherosclerosis* 1999; **143**:81-90.
14. Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE, Kwon BS, Erickson KL, Yu R. Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int J Obese* 2006; **30**:1347-1355.
15. Yang RZ, Lee MJ, Hu H, Pollin TI, Ryan AS, Nicklas BJ, Snitker S, Horenstein RB, Hull K, Goldberg NH, Goldberg AP, Shuldiner AR, Fried SK, Gong DW. Acute-phase serum amyloid A: an inflammatory adipokine and potential link between obesity and its metabolic complications. PLoS Med 2006; **3**:e287.
16. Huber J, Kiefer FZ, Zeyda M, Ludvik B, Silberhumber GR, Prager G, Zlabinger GJ, Stulnig TM. CC chemokine and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity. J Clin Endocrinol Metab 2008; [Epub ahead of print].
17. Ueki K, Kondo T, Tseng YH, Kahn CR. Central role of suppressor of cytokine signaling

proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. *Proc Natl Acad Sci USA* 2004; **101**:10422-10427.

18. Russell AP. PGC-1 alpha and exercise: important partners in combating insulin resistance. *Curr Diabetes Rev* 2005; **1**:175-181.

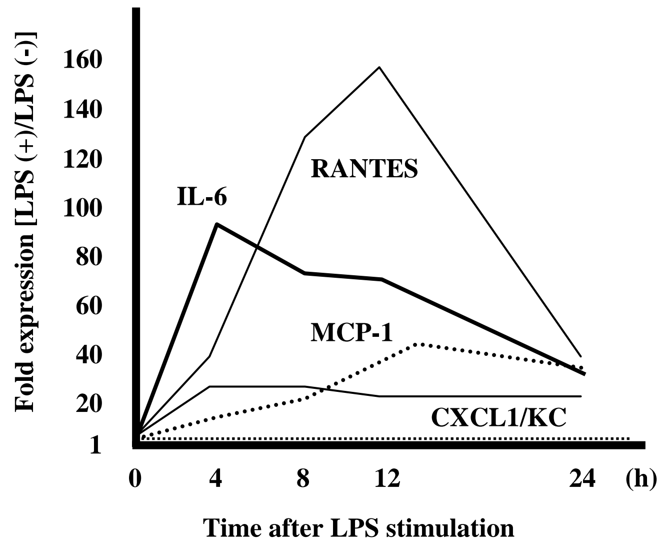
19. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 2000; **96**:34-40.

20. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 2006; **354**:610-621.

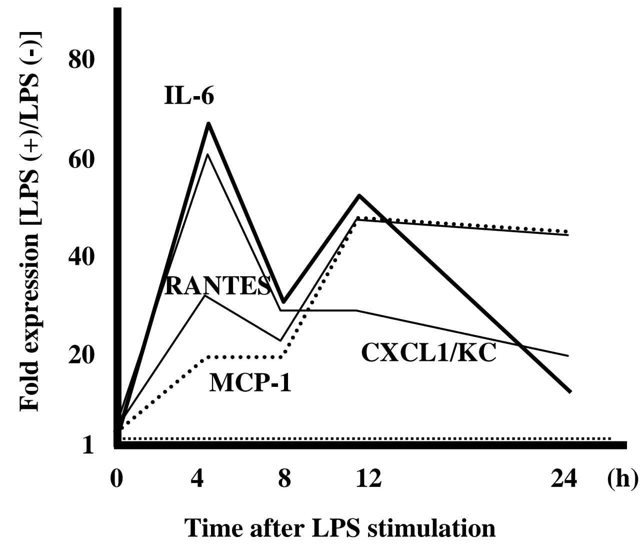
## Figure Legends

**Figure 1. Relative expressions of IL-6, RANTES, MCP-1, and CXCL1/KC mRNA in LPS-stimulated 3T3-L1 adipocytes co-cultured with RAW macrophages against those in un-stimulated adipocytes co-cultured with macrophages.**

Total RNA was isolated from adipocytes under each culture condition following 4, 8, 12 and 12h of LPS stimulation, and subjected to microarray analyses (A) and real-time PCR analyses (B) as described in "METHODS". Data are expressed as the fold expressions of genes in LPS-stimulated cells against those in un-stimulated cells.



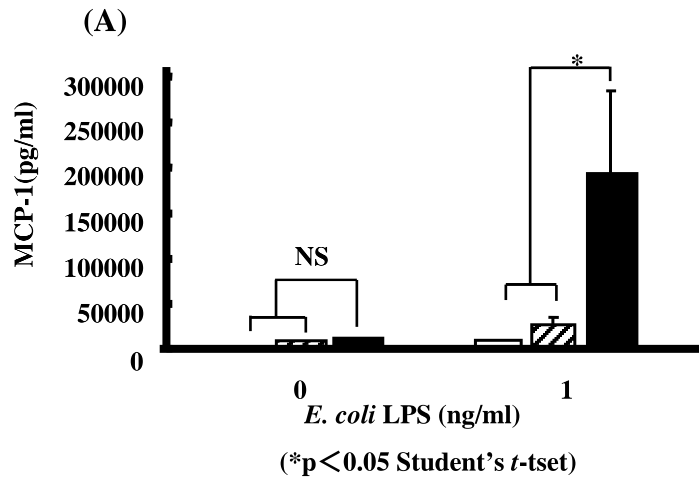

A) DNA microarray analyses

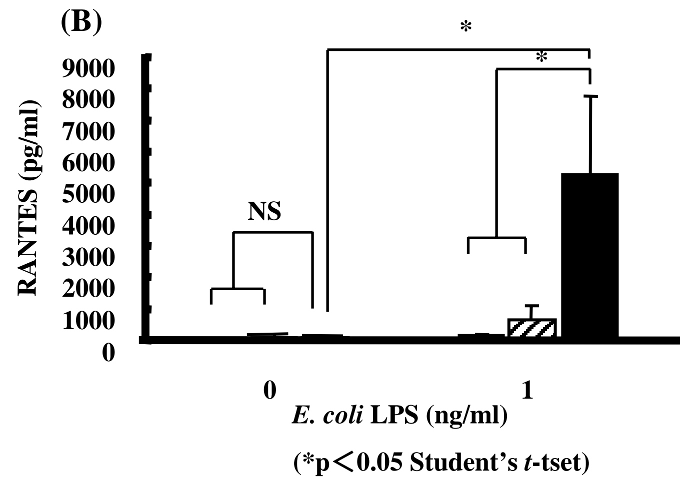



B) Real time PCR analyses

**Figure 2. MCP-1 (A), RANTES (B), and CXCL1/KC (C) productions from macrophage alone culture (open box), adipocyte alone culture (shadow box) and adipocyte-macrophage co-cultures (closed box) stimulated with LPS, and a schematic illustration of the roles of these molecules in enhanced inflammatory changes in adipose tissues.**

(A-C) Baseline productions of all these cytokines were very low in each culture. When the cells were stimulated with LPS, all of these cytokines were mainly produced by adipocytes. However, production was markedly up-regulated when co-cultured cells were stimulated with LPS. $*p < 0.05$, student t-test. (D) The TLR-4 ligand stimulates macrophages to produce trace amounts of TNF-α. TNF-α, in co-operation with other molecules, stimulates adipocytes to produce MCP-1, IL-6, RANTES, and CXC/L1. MCP-1 acts to recruit more macrophages into the adipose tissue. MCP-1 and IL-6 cause insulin resistance, as well as up-regulating CRP synthesis in the liver, while RANTES acts to recruit T cells into adipose tissue. On the other hand, CXC/L1 may enhance angiogenesis, which is a typical feature of adipose tissues. All of these molecules act to promote inflammatory changes in adipose tissues and may cause many other glucose and lipid metabolism impairments.


(A)
MCP-1(pg/ml)
300000
250000
200000
150000
100000
50000
0
NS
*
0
1
E. coli LPS (ng/ml)
(*p＜0.05 Student's t-tset)


(B)
RANTES (pg/ml)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
NS
*
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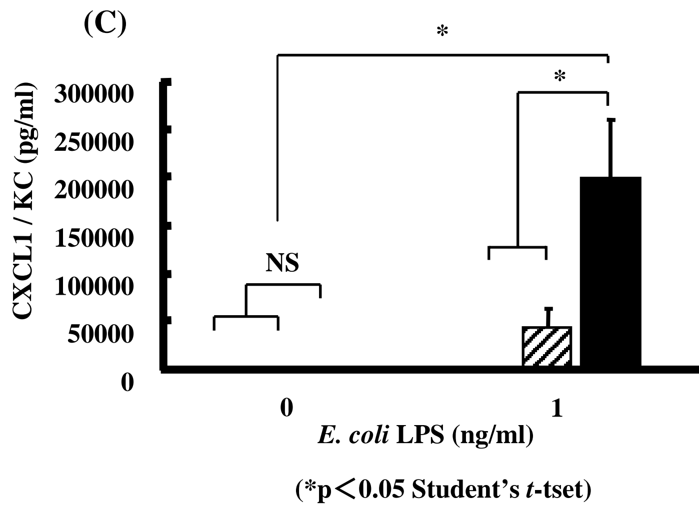
0
1
E. coli LPS (ng/ml)
(*p＜0.05 Student's t-tset)


(C)
CXCL1 / KC (pg/ml)
300000
250000
200000
150000
100000
50000
0
NS
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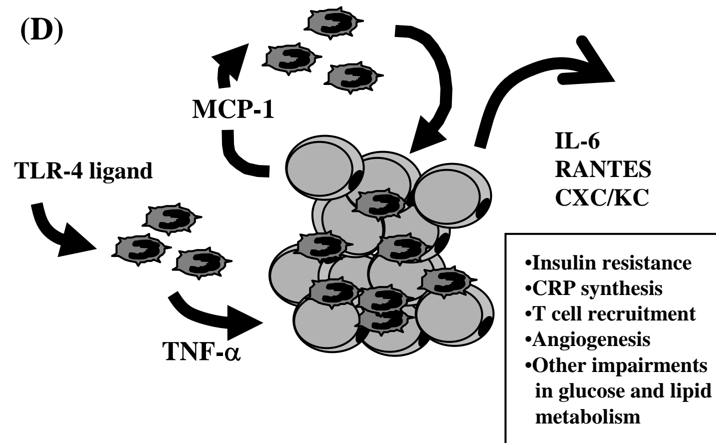
1
E. coli LPS (ng/ml)
(*p＜0.05 Student's t-tset)


(D)
MCP-1
TLR-4 ligand
TNF-α
IL-6
RANTES
CXC/KC
•Insulin resistance
•CRP synthesis
•T cell recruitment
•Angiogenesis
•Other impairments in glucose and lipid metabolism