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Porphyromonas gingivalis and *E. coli* Lipopolysaccharide Exhibit Different Systemic but Similar Local Induction of Inflammatory Markers

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Abstract

Background—*Porphyromonas gingivalis* is a gram-negative bacterium that is an important etiologic agent of human adult periodontitis. The goal of the study was to test the hypothesis that two different isoforms, $PgLPS_{1435/1449}$ and $PgLPS_{1690}$ exhibit differences in their capacity to stimulate systemic versus local responses compared to *E. coli* LPS.

Methods—Lipopolysaccharide (LPS) was inoculated into the scalp of mice and the response was measured locally at the site of site of inoculation and systemically in the heart/aorta. VCAM-1 was assessed at the protein level by ELISA and VCAM-1, E-selectin, and ICAM-1 at the RNA level of RNase protection assay. Serum TNF-α levels were also measured.

Results—*E. coli* LPS and both isoforms of *P. gingivalis* LPS groups were relatively potent in stimulating expression of inflammatory markers with *E. coli* LPS being somewhat more potent. In contrast, when the systemic response was measured in the heart/aorta, *E. coli* but not *P. gingivalis* LPS significantly induced inflammatory markers. At moderate to low doses (1 and 10 ug per injection) serum TNF–α levels were minimally induced by *P. gingivalis* LPS compared to *E. coli* LPS.

Conclusion—The results indicate that both forms of *P. gingivalis* LPS stimulate an inflammatory response when injected into connective tissue but are minimally stimulatory when a systemic response is measured. In contrast *E. coli* LPS is a potent stimulus at both the systemic and local level.

Keywords

P. gingivalis; lipopolysaccharide; inflammation; bacteria; periodontal; cytokine

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INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and a potent modulator by the innate immune system (1). The endotoxin activity of LPS is contained in the lipid region of the molecule, termed lipid A (2). Alterations in lipid A such as changing the number, position, or length of the primary or secondary acyl groups, or subtraction of phosphate or monosaccharide groups results in alteration of the biologic effects (3,4). It is now understood that bacteria may contain more than one lipid A structural type, and that environmental conditions can regulate the numbers and types of lipid A species found in a single bacterial population (5). Based on this mechanism bacteria might alter host responses to gain advantage for colonization or evading the recognition of host innate immunity. Guo et al reported that a constitutive *S. typhimurium* mutant could alter LPS-mediated gene expression by regulating structural modifications of lipid A (6). It was also found that environment could alter the lipid A species of *Pseudomonas aeruginosa* strains isolated from the lungs of individuals with cystic fibrosis, which resulted in more potent stimulation of E-selectin, TNF–α and IL-8 production (7).

Porphyromonas gingivalis is a gram-negative bacterium that is an important etiologic agent of human adult periodontitis, a major cause of tooth loss (8). This bacterium releases copious amounts of outer membrane vesicles containing LPS, which can penetrate periodontal tissue and thus participate in destruction associated with activation of the innate immune response. Compared with enterobacterial LPS, *P. gingivalis* LPS has variable potency in stimulating biologic activity depending on the cell type stimulated. Several studies demonstrated that the for some cell types such as endothelial cells the biologic activity of *P. gingivalis* was low compared to that of LPS isolated from enterobacteria (9,10). However, when injected *in vivo*, *P. gingivalis* induces the expression of inflammatory molecules and formation of an inflammatory infiltrate (11,12). It is possible that some of the variability in response to *P. gingivalis* or *P. gingivalis* LPS is due to the predominant forms of LPS produced (13,14). In this regard, the major isoforms of *P. gingivalis* LPS have been characterized with lipid A mass ions at m/z 1,435/1,450 and at 1690 (5). It has been predicted that these lipid A modifications will elicit specific alterations in innate host response, with potential pathogenic significance.

The goal of this study was to determine whether *P. gingivalis* induced both a strong local and systemic inflammatory response and to compare the induction of selected inflammatory markers with that of *E. coli* LPS. The secondary goal was to compare whether two major isoforms of *P. gingivalis* LPS exhibited similar inflammatory responses both locally and systemically.

MATERIALS and METHODS

Animals

8 week male CD-1 mice were purchased from the Charles River Laboratories in Wilmington, MA and were housed in the Laboratory Animal Science Center (LASC) at Boston University Medical Campus. All animal procedures were approved by the Institutional Animal Care and Use Committee, Boston University Medical Center. For each group there were six animals per data point. Each assay was carried out at least three separate times. Statistical differences between different groups were determined by One-Way ANOVA.

Preparation of $PgLPS_{1435/1449}$, $PgLPS_{1690}$, and *E. coli* LPS

$PgLPS_{1435}$ was prepared as described in (8)and $PgLPS_{1690}$ was prepared as described in(15) and both were prepared from *P. gingivalis* strain 33277. These LPS preparations contained less

than 0.1 % protein contamination, the appropriate fatty acid composition, and were highly enriched for the their respective lipid A structures (8,15). 2. E. coli O111:B4 LPS was obtained from Sigma-Aldrich (St Louis, MO). This preparation was further purified by the method of Manthey and Vogel to remove trace contaminating proteins (16).

LPS treatment and samples collection

$PgLPS_{1435/1449}$, $PgLPS_{1690}$, and *E. coli* LPS were injected subcutaneously in the mid scalp as previously described (9). Following injection mice were euthanized at the indicated time points and the scalp was immediately harvested and frozen in liquid nitrogen. The heart and aorta were obtained by dissection and opened with a scissor so that blood could be removed by rinsing thoroughly with PBS after which they were frozen in liquid nitrogen. To obtain blood, cardiac puncture was performed just prior to sacrifice.

Measurement of serum TNF-α and VCAM-1 in scalp and heart/aorta

Serum TNF-α protein levels were measured by ELISA. Protein was extracted from the heart/aorta or scalp by pulverization of frozen tissue followed by extraction with lysis buffer containing protease inhibitors. Total protein was determined using a BCA Protein Assay kit (Pierce, Rockford, Illinois). VCAM-1 was measured by ELISA of 100ug of protein extract.

RNA analysis

Total RNA from the scalp and heart/aorta were extracted using Trizol Reagent from pulverized frozen tissue following the manufacturer's instructions. mRNA levels were measured by the RNase protection assay (RPA). P^{32} labeled probes were incubated with 6 μg total RNA for VCAM-1, ICAM-1 and E-selectin. Samples were subjected to RNase digestion. Following electrophoresis on 6% polyacrylamide gels and the optical density of radiolabeled bands was measured using a PhosphoImager. Each value was then normalized by the value of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the same lane. The mean densitometric values ± SEM from three separate RNase protection assays are shown.

RESULTS

$PgLPS_{1435/1449}$, $PgLPS_{1690}$ and *E. coli* LPS were injected into connective tissue and the response was determined at the local and systemic levels by assessing the expression of adhesion molecules that support inflammation. Vascular-cellular adhesion molecules (VCAM-1), an adhesion molecule that is selectively expressed on vascular cells was measured at the protein level by ELISA. At the site of inoculation all three isoforms of LPS were highly stimulatory inducing a 6 to 8 fold increase at 12 hours and 5 to 10 fold increase at 24 hours compared to baseline ($P<0.05$) (Fig 1A). Although the difference was relatively small, $PgLPS_{1435/1449}$ induced levels that were statistically greater than $PgLPS_{1690}$ at 24 and 48 hours ($p< 0.05$). The results observed systemically in the heart/aorta differed considerably (Fig 1B). Only *E. coli* LPS induced VCAM-1 protein levels, which peaked at 12hours when they were five fold higher than baseline ($P<0.05$). In contrast *P. gingivalis* LPS had no effect.

VCAM-1 expression was also assessed at the mRNA level. $PgLPS_{1435/1449}$, $PgLPS_{1690}$ and *E. coli* at a high dose, 50ug, induced a similar expression of VCAM-1 in the scalp, which was approximately 7.5 times higher than baseline ($p<0.05$) (Fig 2A). In contrast, only *E. coli* LPS increased mRNA levels of VCAM-1, which were 2.5 fold over baseline in the heart/aorta ($p<0.05$). At low dose, 1ug, $PgLPS_{1690}$ and $PgLPS_{1435/1449}$ stimulated approximately a 2 fold increase while *E. coli* LPS stimulated a 3 fold increase in VCAM-1 mRNA levels at the site of inoculation. The somewhat higher level of expression stimulated by *E. coli* LPS compared to *P. gingivalis* LPS was significant ($p<0.05$). Only *E. coli* LPS stimulated VCAM-1 expression in the heart/aorta, which was significantly different from baseline (Fig 2B)($p<0.05$).

mRNA levels of E-selectin, another pro-inflammatory adhesion molecule was assessed (Fig 3). Ahigh dose of $PgLPS_{1690}$ and $PgLPS_{1435/1449}$ at the inoculation site, 50ug, stimulated approximately a 3 fold increase in E-selectin while *E. coli* LPS stimulated a 4.5 fold increase compared to baseline ($p<0.05$) (Fig 3A). The differences between *E. coli* and *P. gingivalis* LPS was significant ($p<0.05$), while the difference between $PgLPS_{1690}$ and $PgLPS_{1435/1449}$ was not ($P>0.05$). In the heart/aorta, only *E. coli* LPS induced E-selectin expression ($p<0.05$). For the low dose at the inoculation site $PgLPS_{1690}$ and $PgLPS_{1435/1449}$ stimulated a 1.7 to 1.8 fold increase and *E. coli* LPS a 2.1 fold increase ($p<0.05$) (Fig 3B). The differences among three LPS groups was not significant ($p>0.05$). In the heart/aorta E-selectin was not induced by any of the LPS groups at the low dose ($P>0.05$) (Fig 3B).

$PgLPS_{1690}$ and $PgLPS_{1435/1449}$ high dose, 50ug, induced an approximately 3 fold increase in mRNA levels of ICAM-1, while *E. coli* LPS induced a 7.5 fold enhancement ($p<0.05$) (Fig 4A). The differences between *E. coli* and *P. gingivalis* LPS were significant ($p<0.05$). In the heart/aorta only *E. coli* LPS induced ICAM-1 expression ($p<0.05$). mRNA levels of ICAM-1 induced by a low dose of each LPS isoform stimulated a 1.8 to 2.1 fold increase ($P<0.05$)(Fig 4B). The difference between *E. coli* and *P. gingivalis* LPS was not significant ($P>0.05$). In the heart/aorta only *E. coli* LPS was stimulatory ($P<0.05$).

The systemic response to the different forms of LPS was also assessed by measuring TNF–α protein levels in serum at different time points (Fig 5). There was no TNF–α detected at baseline. At 12 hours TNF–α levels induced by *E. coli* LPS were approximately 140% higher than $PgLPS_{435/1449}$ and 370% greater than that stimulated by $PgLPS_{1690}$. The difference between each was significant ($P<0.05$). At 24hours both *P. gingivalis* LPS groups returned to baseline while TNFα was still elevated in mice injected with *E. coli* LPS ($P<0.05$). At 48 hours all groups were at or close to baseline. When a dose response was measured *E. coli* LPS induced significantly higher level of TNF-α compared to both isoforms of *P. gingivalis* LPS at all doses (Fig 6) ($p<0.05$). A difference between $PgLPS_{435/1449}$ and$PgLPS_{1690}$ was found only at the highest dose, 50ug.

DISCUSSION

In vitro experiments indicate that the response to *P. gingivalis* LPS varies considerably (8, 17). This may be dependent upon the cell type examined or the type of LPS produced. In order to investigate this issue we examined two major isoforms of *P. gingivalis* LPS in a connective tissue setting where complex cell to cell interactions would be maintained. The scalp model was chosen for this purpose as we and others have previously described (11,18). *P. gingivalis* LPS and *E. coli* LPS were potent inducers of VCAM-1 measured at the protein level at the site of inoculation. *E. coli* LPS had a small but statistically significant increase compared to *P. gingivalis* LPS, with $PgLPS_{1435/1449}$ being slightly more stimulatory than $PgLPS_{1690}$. In contrast only *E. coli* LPS was a potent inducer when measuring VCAM-1 protein at a distant site, in the heart/aorta. This general pattern was supported by expression of the mRNA levels of VCAM-1, E-selectin and ICAM-1. Both isoforms of *P. gingivalis* LPS were potent stimulators of inflammation although somewhat less than that of *E. coli* at the local site of inoculation. In the heart/aorta only *E. coli* LPS was stimulatory. The systemic response was also assessed by measuring TNF–α in the serum. At a high dose both $PgLPS_{1435/1449}$ and $PgLPS_{1690}$ were stimulatory but less so compared to *E. coli* LPS with $PgLPS_{1435/1449}$ somewhat more potent that $PgLPS_{1690}$. At a moderate dose, 10μg per injection, only *E. coli* LPS induced high levels of serum TNF-α Thus, these studies indicate that the local response to *P. gingivalis* LPS is relatively strong whereas the systemic response is relatively weak. In contrast *E. coli* LPS is a strong stimulus of both a local and systemic inflammatory response.

It has previously been shown that in endothelial cells *P. gingivalis* LPS is a weak inducer of adhesion molecule expression (1). However, *in vivo* under conditions where multiple cell types are present, studies shown here demonstrate that highly purified *P. gingivalis* LPS stimulates expression of adhesion molecules associated with endothelial cells, VCAM-1 and E-selectin. Thus it is likely that other cells, which respond to *P. gingivalis* LPS, induce a cascade leading to indirect activation of endothelial cells. For example, isolated microphages are highly reactive to *P. gingivalis* LPS (17) and may mediate *P. gingivalis* induction of endothelial cells. Alternatively, it is possible that minor contaminants of LPS at high concentrations had some activity affecting the local inflammatory response. However, it should be noted that even at high concentrations *P. gingivalis* LPS was weak at inducing a systemic inflammatory response.

Porphyromonas gingivalis has been shown to cause septic shock-like symptoms and even animal death (19). However, *P. gingivalis* LPS is considerably less potent in inducing septic shock when applied intravenously compared to LPS derived from enteric bacteria (13). Results presented here are consistent with the latter findings and suggest that when endothelial cell responses predominate, as reflected in serum TNF-α levels and the short term response of cardiovascular tissue, *P. gingivalis* LPS is much less potent than LPS from *E. coli*.. This may reflect A TLR2 response to $PgLPS_{1435/1449}$ and $PgLPS_{1690}$ that is specifically associated with endothelial cells (7,15,20). Furthermore the results given insight into how *P. gingivalis* may contribute to a systemic disease such as atherosclerosis. It has been reported that invasion of *P. gingivalis* is required for accelerated progression of atherogenesis in an animal model. This may be necessary since *P. gingivalis* does not elicit a strong systemic inflammatory response and may require local invasion to promote atherogenesis (21). However, when the cellular environment is more complex and multiple cellular interactions occur, the response to *P. gingivalis* LPS is more pro-inflammatory and more similar to that of *E. coli* LPS.

Acknowledgments

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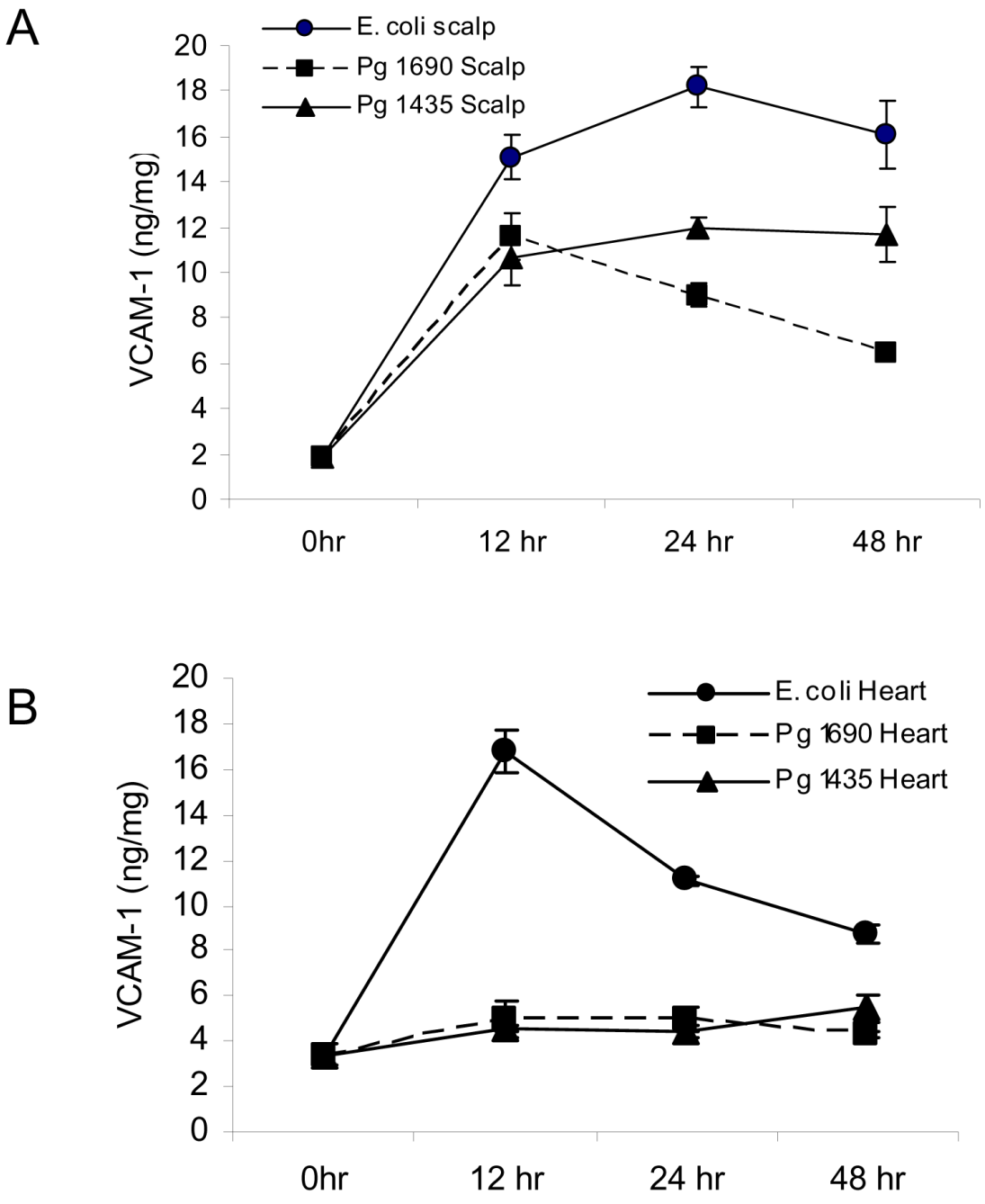

Figure 1.

Local and systemic induction of VCAM-1 protein by $PgLPS_{1435/1449}$, $PgLPS_{1690}$ LPS and *E. coli* LPS. 50ug of each LPS was injected adjacent to the periosteum of the murine scalp and animals were euthanized 0, 12, 24 and 48 hours later. (A) Total protein was extracted from scalp. Compared to baseline (0hr) all three isoforms of LPS were significant at each time point ($P<0.05$). (B) Total protein was extracted from the heart/aorta after thorough rinsing. Only E. coli LPS at 12, 24 and 48 hrs was significant compared to baseline ($P<0.05$). Each value represents the mean of three separate ELISAs +/− SEM.

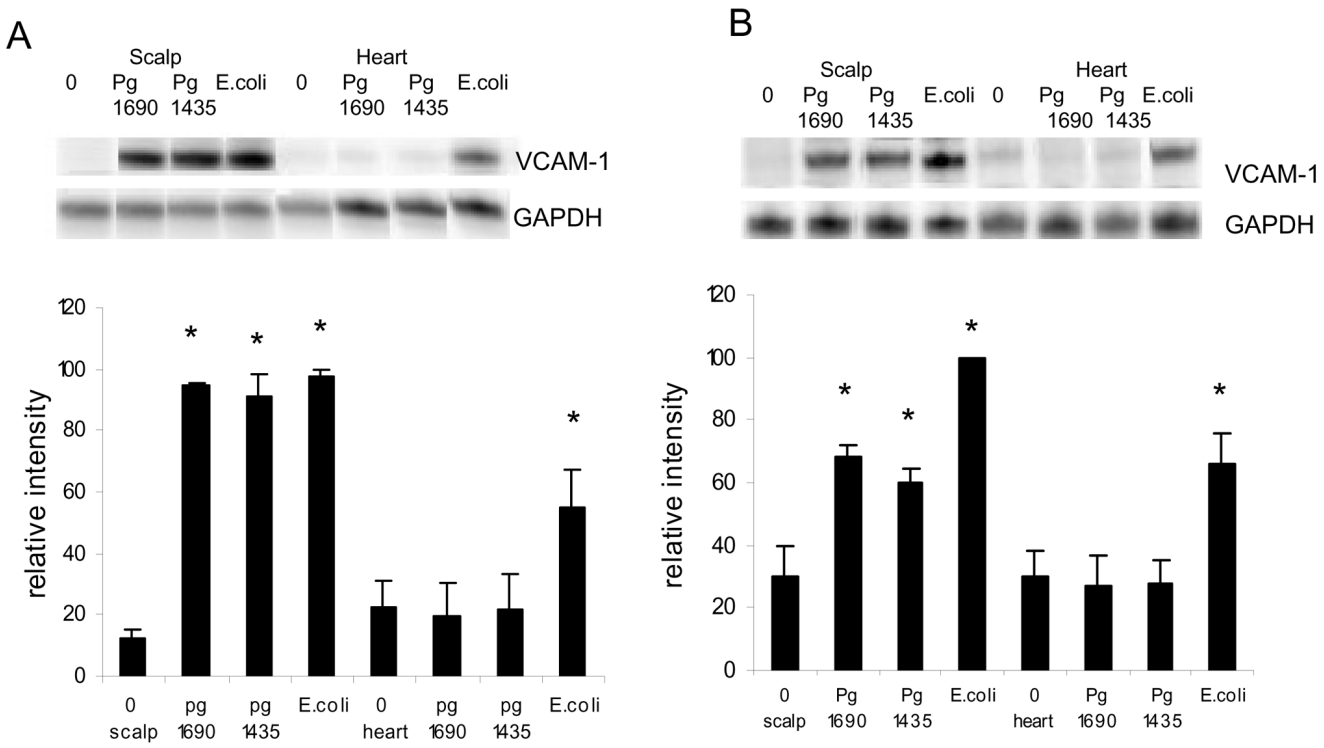

Figure 2.

Early local and systemic induction of VCAM-1 mRNA by $PgLPS_{1435/1449}$, $PgLPS_{1690}$ LPS and *E. coli* LPS. (A) Low dose (1ug) of each LPS was injected adjacent to the periosteum of the murine scalp. (B) High dose (50ug) of each LPS was injected. Animals were euthanized 0 or 12 hours later and total RNA was isolated from the scalp and heart/aorta. VCAM-1 was assessed by RNase protection assay. The resulting autoradiograms and densitometric values normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in the same lane are shown. Each value represents the mean of three separate RPAs +/− SEM. * indicates significantly different from baseline (O hr) ($P<0.05$).

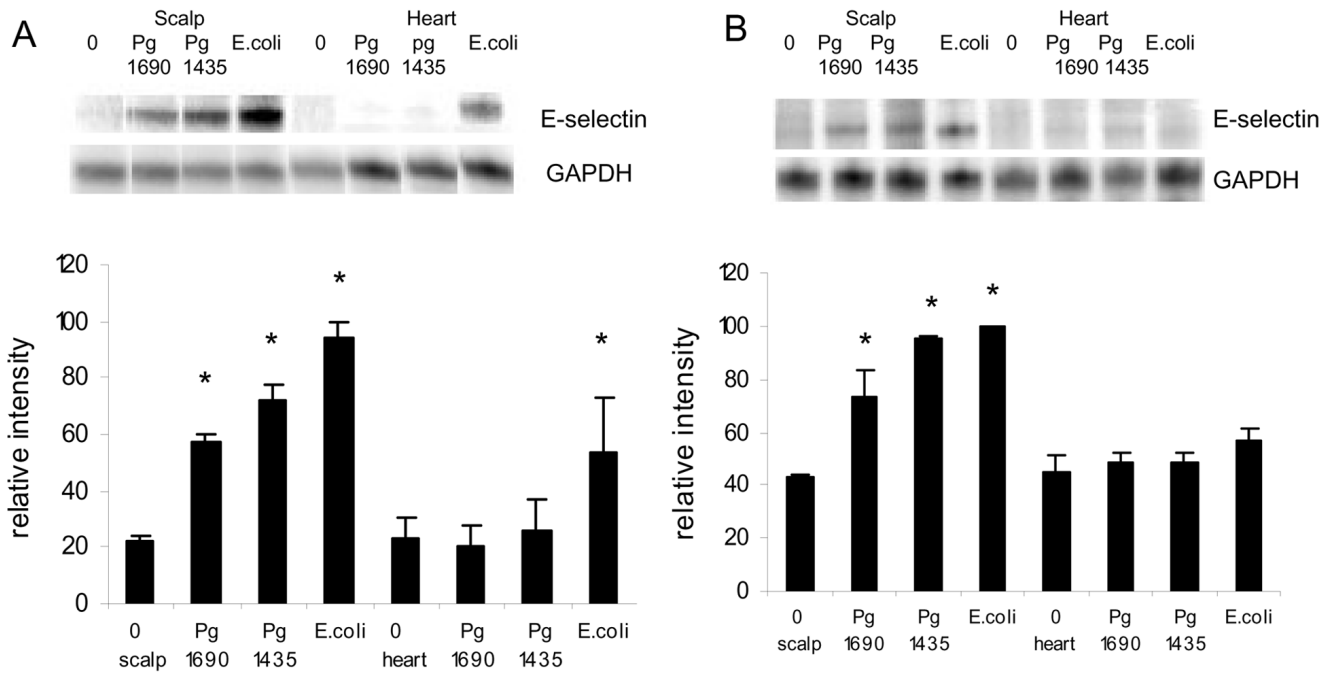

Figure 3.

Early local and systemic induction of E-selectin mRNA by $PgLPS_{1435/1449}$or $PgLPS_{1690}$ LPS and *E. coli* LPS. (A) Low dose (1ug) of each LPS was injected adjacent to the periosteum of the murine scalp. (B) High dose (50ug) of each LPS was injected. Animals were euthanized 0 or 12 hours later and total RNA was isolated from the scalp and heart/aorta. E-selectin was assessed by RNase protection assay. The resulting autoradiograms and densitometric values normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in the same lane are shown. Each value represents the mean of three separate RPAs +/− SEM. * indicates significantly different from baseline (O hr) ($P<0.05$).

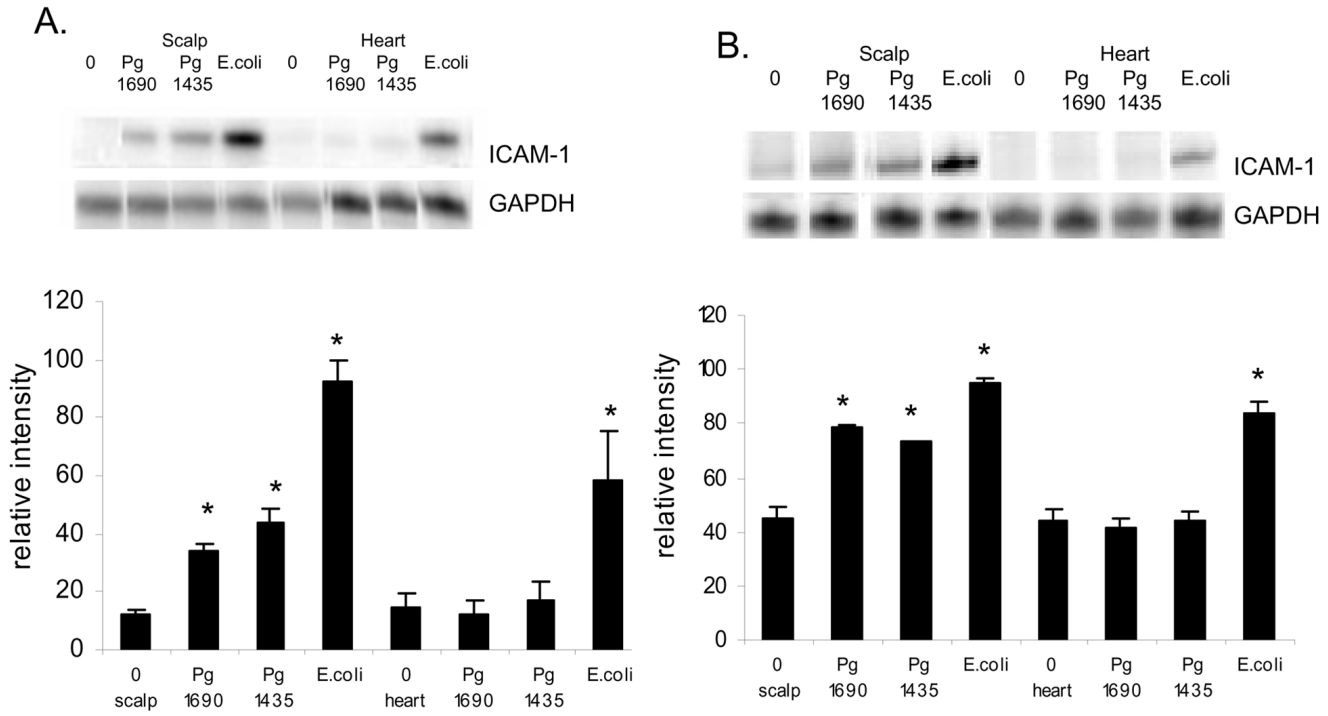

Figure 4.

Early local and systemic induction of ICAM-1 mRNA by $PgLPS_{1435/1449}$or $PgLPS_{1690}$ and *E. coli* LPS. (A) Low dose (1ug) of each LPS was injected adjacent to the periosteum of the murine scalp. (B) High dose (50ug) of each LPS was injected. Animals were euthanized 0 or 12 hours later and total RNA was isolated from the scalp and heart/aorta. ICAM-1 was assessed by RNase protection assay. The resulting autoradiograms and densitometric values normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in the same lane are shown. Each value represents the mean of three separate RPAs +/− SEM. * indicates significantly different from baseline (O hr) ($P<0.05$).

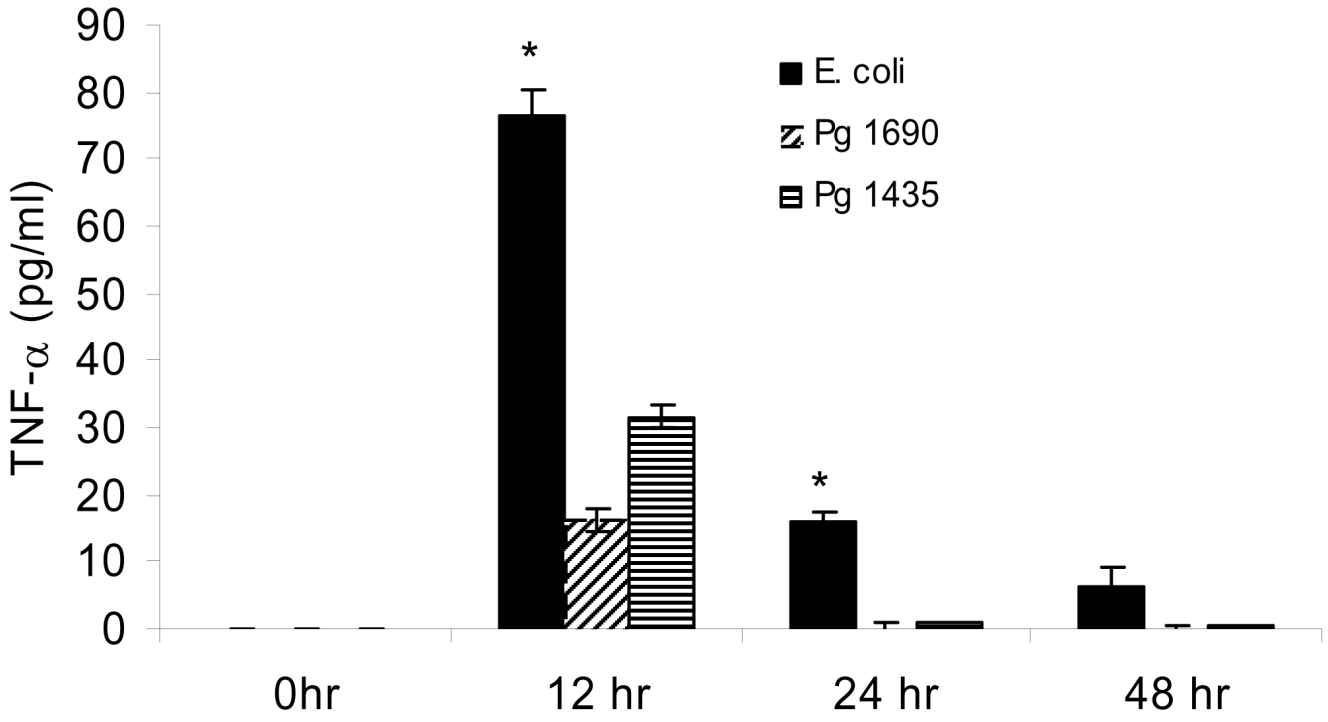

Figure 5.

Serum TNF-α protein levels induced by local injection of $PgLPS_{1435/1449}$, $PgLPS_{1690}$ and *E. coli* LPS. 50ug of each LPS was injected adjacent to the periosteum of the murine scalp and animals were euthanized 0, 12, 24 and 48 hours later. Serum TNF-α levels were measured by ELISA. Each value represents the mean of three separate experiments +/− SEM. * indicates *E. coli* LPS was significantly greater than *P. gingivalis* LPS under the same conditions ($P<0.05$).

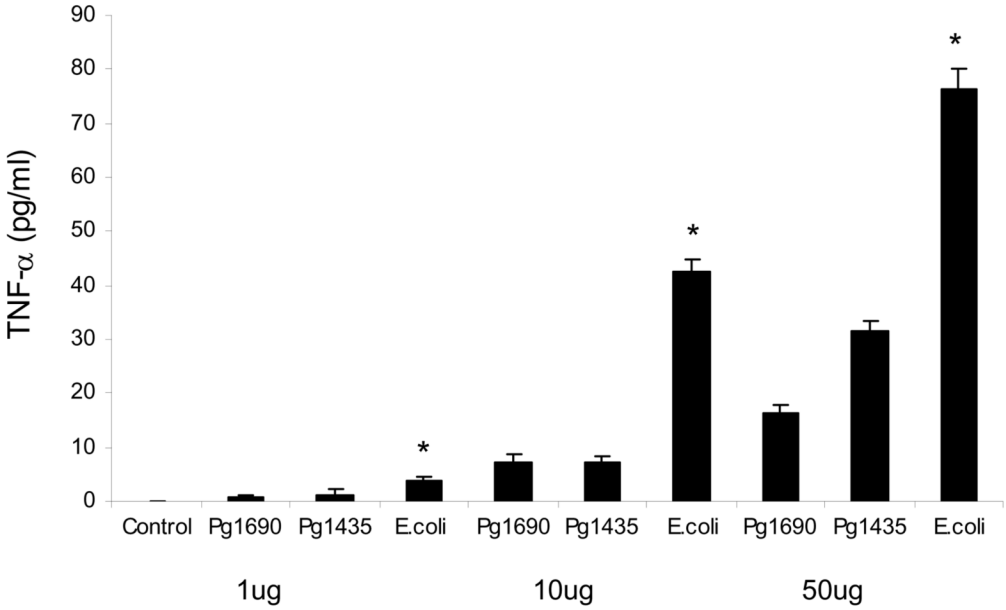

Figure 6.

Dose-dependent induction of serum TNF–α protein levels by local injection $PgLPS_{1435/1449}$, $PgLPS_{1690}$ and *E. coli* LPS. Low (1ug) medium (10ug) and high dose (50ug) of each LPS was injected and animals were euthanized 0 or 12 hours later. Serum TNF-α levels were measured by ELISA. Each value represents the mean of three separate experiments +/− SEM. * indicates *E. coli* LPS was significantly greater than *P. gingivalis* LPS under the same conditions ($P<0.05$).