EFFECT OF CYTOKINES ON TOLL-LIKE RECEPTOR 4 EXPRESSION IN ENDOTHELIAL CELLS

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This thesis entitled

EFFECT OF CYTOKINES ON TOLL-LIKE RECEPTOR 4

EXPRESSION IN ENDOTHELIAL CELLS

by

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Abstract

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Effect of Cytokines on Toll-Like Receptor 4 Expression in Endothelial Cells

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Toll-like receptors (TLRs) are a family of proteins (e.g. TLR4) widely acknowledged as the body's first line of defense against invading microbes. TLR4 induction can take place both under pro- as well as pseudo-inflammatory stimuli. This TLR4 induction has been implicated in the genesis of inflammation, which subsequently can lead to the pathogenesis of atherosclerosis and autoimmune diseases. Investigating what brings about TLR4 expression during inflammation is of fundamental importance. Hence, we sought to determine the effects of cytokines on TLR4 expression in human endothelial cells. For the limited range of conditions tested, our studies suggest that TLR4 expression is unaffected when endothelial cells are treated with pro-inflammatory Lipopolysaccharide, Interferon beta and Interferon gamma. Further studies are needed to determine if these results are valid for a wider range of conditions.

Approved:

Douglas J. Goetz

Associate Professor of Chemical Engineering

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I would like to thank my advisor Dr. Douglas Goetz for his confidence in me, providing me with valuable inputs from time to time and financially supporting me during my research. He has truly been a mentor and gem of a person I will always look up to.

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Thanks are also due to my roommates and friends who have been my family away from home providing social support. Last but not the least; I devote my thesis to my parents and sister without whose support in matters emotional, financial and all spheres of life, this thesis would not have been possible. I give them credit for instilling the qualities and values in me. What I am is a reflection of their unconditional love and immense care.

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Introduction

Immunity

The major function of the immune system is to detect and eliminate invading pathogenic microorganisms before they can cause damage to the host organism. The immune system is able to do this by distinguishing between itself and anything foreign: the non-self. In mammals, the immune system can be classified into two groups, “innate immunity” and “adaptive immunity.” The role of adaptive immunity is to detect foreign elements through recognition of peptide antigens using antigen receptors. These receptors are expressed on the surface of B and T cells. Since the number of unknown antigens that can be encountered by the host is very large, B and T cells rearrange their receptor genes to generate over 10^{11} different species of antigen receptors. Whenever antigen receptors are engaged by the cognate antigen more of those antigen-specific antibodies are produced. This intelligent and sophisticated system is observed only in vertebrates and is a very effective defense strategy against most infections found.

As opposed to adaptive immune system, the innate immune system has been phylogenetically conserved and is present in diverse species from vertebrates and invertebrates to multicellular organisms (1). The mechanism by which innate immunity recognizes non-self remained unknown until 1996 when Drosophila

protein Toll was shown to be required for flies to mount an effective immune response to the fungus *Aspergillus fumigatus*. In 1998, toll-like receptor 4 was identified as the Lipopolysaccharide (LPS) receptor required for mice to fight Gram-negative bacteria, all of which have LPS as an integral part of the outer cell membrane. This suggested that toll-like receptors play a central role in the primary recognition of infectious pathogens by mammals (2).

Toll-like receptors (TLRs)

Toll-like receptors are a family of 13 (10 in humans and 13 in mice) (3) known cell surface receptors related to IL-1 receptors described on immune cells, and form the first few chain of events in the innate immune system response. This response results in increases in genes for several inflammatory cytokines and chemokines, major histocompatibility (MHC) genes I and II as well as co-stimulatory molecules and is critical for the development of antigen-specific adaptive immunity (1). Two of the most well studied TLRs, TLR3 and TLR4 (also called Toll 4) , have been found in immune cells like human leukocytes with TLR3 selectively expressed in dendritic cells and TLR4 expressed in monocytes and neutrophils (4). TLR4 is also expressed on non-immune cells like endothelial and epithelial cells (5,6).

TLR signal transduction

Primary players involved in the TLR signal cascade are myeloid differentiation marker (MyD88), LPS-binding protein (LBP), nuclear factor–κB (NF-κB) interferon (IFN), interferon response factor (IRF), mitogen-activated protein kinase (MAPK), MyD88 adapter like (Mal), interleukin-1 receptor-associated kinase (IRAK), tumor necrosis factor receptor-associated factor (TRAF6), Toll/interleukin-1 receptor (IL-1R) homologous region (TIR), TIR-containing adapter molecule (TRIF), I-κB kinase (IKK), interferon-sensitive response element (ISRE), MAPK kinase (MKK), receptor interacting protein (RIP), TRIF-related adapter molecule (TRAM), TANK-binding kinase (TBK), transforming growth factor β-activated kinase (TAK), single immunoglobulin interleukin-1 receptor-related molecule (SIGIRR). TAK-1 binding protein (TAB) and Tollip. Uev1a and Ubc13 are TRAF6 ubiquitin ligases. Negative regulators of the process are ST2, SIGIRR, MyD88s, IRAK-M, Tollip, IRAK2c and IRAK2d (3). TLR4 mediated response to LPS can be divided into two parts: an early MyD88-dependent response and a delayed MyD88-independent response.

Figure 1 on the following page explains the process pictorially and highlights the interdependency of various tolls.

(K. Takeda, S. Akira, 2004 Seminars in Immunology, 16 3-9)

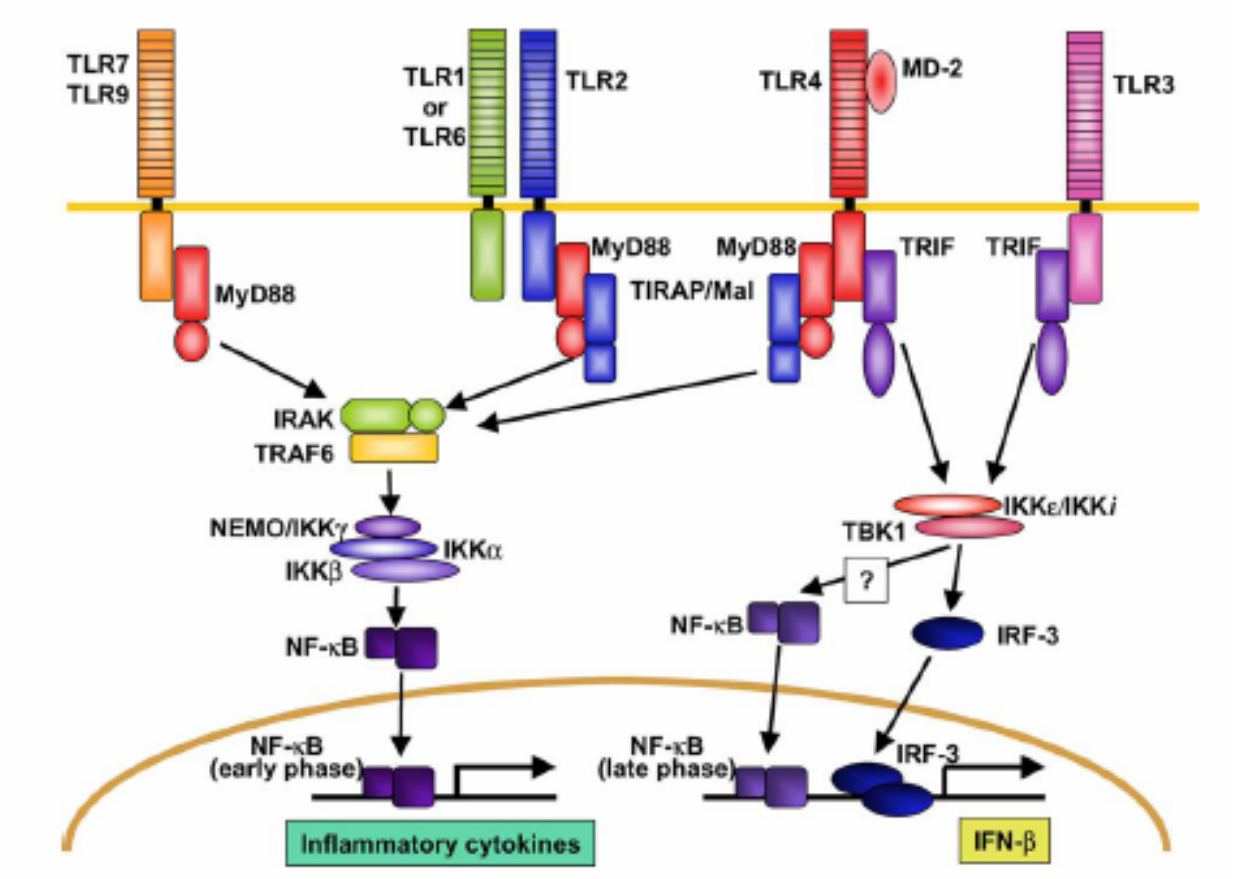

Figure 1: TLR Signal Transduction

MyD88-dependent signaling

LPS in the plasma is recognized by circulating LBP, which brings it to CD14 (Cluster of Differentiation - 14). This results in the loading of LPS onto the LPS receptor complex. This complex is composed of dimerized TLR4 receptors and two molecules of the extracellular adapter MD-2, a secreted glycoprotein

essential for LPS signaling to occur. The loading of LPS onto LPS receptor aids homodimerization of TLR4 leading to recruiting of MyD88 and Mal to the receptor complex. IRAK-1 and IRAK-4 then get associated with the receptor complex. IRAK-1 gets autophosphorylated in two substeps resulting in hyperphosphorylated IRAK-1. This leads to dissociation of IRAK-1 from the receptor complex and its subsequent association with TRAF6. TRAF6 gets activated and associates with TAB-2 which activates the MAPK kinase TAK-1. TAK-1 is constitutively associated with its adapter protein and acts as a common activator of NF-κB and of p38/c-jun N-terminal kinase (JNK) MAPK pathway. All the time, Tollip acts as a negative regulator. The first step in NF-κB activation is building of signalosome, a high-molecular weight protein complex constituted of inhibitory-binding protein κB kinase α (IKKα) and IKKβ, together with a scaffolding protein named IKKγ (also known as NEMO). Finally, a set of inhibitory-binding proteins κB (IκB) are phosphorylated and then ubiquitinated and degraded releasing NF-κB, free to translocate into the nucleus. Not only does the early MyD88-dependent response to LPS activate NF-κB, p38 and JNK MAPK, it also leads to an early activation of IRF3 and induction of IFN-β as seen by the MyD88-independent signaling (3).

MyD88-independent signaling

The role of various proteins in MyD88-independent signaling pathway has not been conclusively proved. Two models have been proposed (3). The first model

points to TRIF binding to TRAF6 via TRAF6-binding domains on TRIF. The components of signalosome are activated followed by the ubiquitination and degradation of I-κB which results in translocation of NF-κB to the nucleus. The second model proposes IKK_{ε} and TANK-binding protein (TBK) -1 act as IRF3 kinases. TBK-1 and IKK_{ε} bind to TRIF and the resulting complex results in phosphorylation and activation of IRF3. IRF3 then binds to ISRE inducing many gene subset including IFN-β (3).

TLR4 and diseases

Evidence suggests that overexpression of TLRs and TLR signaling on non-immune cells, specifically TLR3 and TLR4, is the basis for the induction of a broadly prevalent array of autoimmune and inflammatory diseases like Type 1 diabetes, colitis, inflammatory bowel disease (IBD), autoimmune thyroiditis, atherosclerosis and vascular complications of diabetes and endotoxic shock response (5-10).

C3H/HeJ mice with a point mutation in the TLR4 gene and a defect in TLR4 signaling become hyporesponsive to LPS challenge (7). Kubes et al (5) found that endothelial TLR4, as opposed to leukocyte TLR4, is a critical player in endotoxic shock. Cario et al. (8) reported that TLR4 was upregulated in intestinal epithelial cell lines isolated from patients with IBD. Ortega-Cava et al. (6) found that TLR4 is upregulated in the colon of colitic mice. Michelsen et al. (9) found

that mice deficient in TLR4 had a significant reduction in aortic plaque development in atherosclerosis-prone apolipoprotein E-deficient (ApoE-/-) mice, suggesting an important role for TLR4. They also demonstrated that lack of TLR4 signaling results in reduced monocyte adhesion to $TLR4^{-/-}$endothelium, suggesting that endothelial TLR4 may be a key player in atherogenesis.

The observations above clearly indicate that endothelial TLR4 plays an important role in many autoimmune and inflammatory diseases. These autoimmune/inflammatory diseases afflict more than 10 million Americans with enormous socioeconomic impact (11). Based on these facts, it becomes important to investigate what brings about increased TLR4 expression on endothelial cells. Once molecules that aggravate TLR4 expression or TLRs expression in general are identified, compounds that inhibit pathologic TLR4 expression and signaling could be developed to treat the wide array of diseases which fall under its domain. Given the vast number of patients that are affected by these diseases, a deeper understanding of TLR4 becomes critical.

Several molecules have been suggested as important regulators of TLR4 expression. These include LPS, IFN-β and IFN-γ. A brief overview of each is given in the following sections.

Lipopolysaccharide (LPS)

Lipopolysaccharides are important constituents of outer cell membrane of Gram-negative bacteria. The immune system utilizes this fact to mark the presence of bacteria. Septic shock caused or aggravated by chronic bacterial infection is because of LPS produced by bacteria like Moraxella catarrhalis, Staphylococcus aureus, etc (12). They are toxic to humans and lower the blood pressure to dangerous levels, a typical characteristic of the septic shock cascade (13). The host organism retaliates to the presence of LPS by expressing an entire gamut of pro-inflammatory molecules like Tumor Necrosis Factor α (TNF-α), IFN-β (cytokines) and inducible NO synthase (iNOS) (14, 15). It has been reported that LPS causes increased expression of various inflammatory molecules, which includes leukocyte adhesion molecules, cytokines and chemokines (16-18).

Interferons

Interferons belong to a large family of heat stable and low molecular weight glycoproteins known as cytokines. They are produced by various host cells in response to presence of inducers like virus, bacteria, parasites or tumors (19). They help in fighting viral infections by hampering the virus reproduction cycle in newly infected cells. They boost immune system response by enhancing macrophage and granulocyte phagocytosis, augmenting natural killer cell activity and performing other immuno-modulatory functions (20, 21). When secreted by vertebrate cells, they confer resistance against different viruses, inhibit

proliferation of normal and malignant cells and block multiplication of intracellular parasites (e.g. bacteria). Out of the many interferon signaling pathways, JAK-STAT signaling pathway has been well studied and characterized (22).

IFN-β is a Type I interferon produced by fibroblasts after infliction of a viral infection which is generally characterized by presence of live or inactivated virus or double stranded RNA. It plays an important part in anti-viral response by stimulating macrophages and natural killer cells. IFN-β is an important cofactor for LPS induced macrophage iNOS. As a cytokine, it has antiviral, antiproliferative and immunomodulating activity and plays an important part in septic shock. Interferon β binds to cell surface receptor complex known as IFNAR consisting of receptors IFNAR1 and IFNAR2 from the Interferon signaling pathway (23). It is used in treatment and control of multiple sclerosis and has been found active against tumors.

IFN-γ is a Type II interferon and is produced by T lymphocyte cells which regulate host immune response. As a cytokine, it has antiviral and antineoplastic activity in response to antigenic or mitogenic stimulation. There is only one type of interferon γ in humans and has weak anti-viral and anti-tumor effects, though it potentiates the effects of other interferons. It also stimulates macrophages to destroy engulfed bacteria. Interferon γ released by Th1 (a type of T helper) cells is important in regulating Th2 response (19- 24).

LPS and IFN-γ have been found to induce TLR4 upreguation in human dermal microvessel endothelial cells (HMEC) (25, 26). In this work, we sought to test the effect of LPS, IFN-β and IFN-γ, which are primary mediators of TLR4 signaling, on TLR4 expression in macrovascular endothelial cells, namely HUVEC.

Materials & Methods

Antibodies

Biotinylated anti-human CD-54 Domain D1 (ICAM-1) and biotinylated anti-human CD-62E (E-selectin) mouse monoclonal antibodies were obtained from Ancell Corporation (Bayport, MN). Biotin SP-conjugated Mouse IgG (whole molecule) and biotin SP-conjugated Goat IgG (whole molecule) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Biotinylated anti-human TLR-4 goat polyclonal antibody was obtained from R&D Systems (Minneapolis, MN). Streptavidin-Peroxidase polymer conjugate (ultrasensitive) was obtained from Sigma-Aldrich (St. Louis, MO).

Reagents

Medium 199, L-Glutamine, Phosphate buffered saline without calcium and magnesium (PBS), Hanks Balanced Salt Solution with calcium and magnesium (HBSS+) and Hanks Balanced Salt Solution without calcium and magnesium (HBSS-) were obtained from Cambrex Bio Science Walkersville, Inc (Walkersville, MD). Heat inactivated Fetal bovine serum (FBS), Bovine growth serum (BGS), Trypsin with EDTA without calcium and magnesium were obtained from Biomedical Technologies (Stoughton, MA). Heparin, Dimethyl Sulfoxide (DMSO), o-phenylnediamine (OPD) and Sodium Citrate were obtained from

Sigma-Aldrich (St. Louis, MO). Formaldehyde (37% w/w) and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Gelatin was obtained from Becton Dickinson (Sparks, MD). Penicillin streptomycin was obtained from Biowhittaker (Walkersville, MD). Tween-20 was obtained from Calbiochem of EMD Biosciences (San Diego, CA). Tris buffered Saline (TBS 10X pH 7.4) was obtained from Quality Biological, Inc (Gaithersburg, MD). Micro BCA™ Protein Assay kit was obtained from Pierce Biotechnology, Inc (Rockford, IL). ECL kit was obtained from Amersham Biosciences. (Buckinghamshire, UK). LDS Sample Buffer, Magic Mark XP Western Standard, NuPage Antioxidant, SDS Running Buffer, Transfer Buffer were obtained fro Invitrogen (Carlsbad, CA).

Lipopolysaccharide (LPS), Interferon beta (IFN-β), Interferon gamma (IFN-γ) and protease inhibitor cocktail were generously provided by Dr. Leonard Kohn (Ohio University, Athens, Ohio).

Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Clonetics (San Diego, CA) and maintained in culture according to existing literature (27). In order to test the expression of various proteins, HUVEC were treated with various pro-inflammatory molecules for different time periods (i.e. 4 hours and 24 hours).

Buffers and media

HUVEC were cultured in growth media consisting of heparin, FBS, penicillin streptomycin and L-glutamine in Medium 199, made sterile using microfiltration. Medium 199 with 8% FBS was used as a blocking buffer and as a dilution medium for antibody treatment during ELISA. HBSS containing 1% Bovine Serum Albumin was used as a washing buffer for washing the cells.

The lysis buffer used during western blotting was made by adding 3.5 µl protease inhibitor cocktail per ml of IP buffer, made fresh everytime before use. IP buffer solution was made with 1% 1M pH 7.5 Tris HCl, 1% NP-40 and 0.877% NaCl in distilled water. 0.1% TBSTw solution used for washing and as a base reagent during western blot was made by adding 10% TBS and 0.1% TWEEN-20 to distilled water.

Enzyme Linked Immunosorbent Assay (ELISA)

Sufficient amount of cells were placed on a 96 well plate such that the HUVEC reached 100% confluency on the day of the experiment. HUVEC were activated by LPS, IFN-β, IFN-γ. The stock concentrations of LPS, IFN-β, IFN-γ were 50 µg/ml, 10 u/µl and 1000 u/µl respectively which was diluted to a working concentration of 50 ng/ml, 100 u/ml and 1000 u/ml respectively using growth media.

Growth media or growth media with activators was added to the HUVEC and the HUVEC were then incubated at 37°C for the appropriate experimental time period in the presence of 5% CO_2.

At the end of the incubation, the HUVEC were washed 3 times with cold HBSS+ and either fixed with 1% formalin for 30 minutes (1% formaldehyde in HBSS+) or 1% formalin (20 minutes at 4°C) followed by 100% methanol (10 minutes at -20°C). After that, the cells were washed with cold HBSS+ and 220 µl of blocking buffer (Medium 199 containing 8% heat inactivated FBS). The plate was then refrigerated at 4°C for 30 minutes.

At the end of 30 minutes, the plate was washed with wash buffer (1% BSA in HBSS). Medium 199 + 8% FBS containing antibodies to TLR-4, E-selectin or ICAM-1 was added (50 µl/well, 10 µg/ml) to the appropriate wells and the plate refrigerated at 4°C for 20 minutes. mAb for anti E-Selectin and ICAM-1 were used as positive controls and HUVEC incubated with growth media alone was used as negative control. In addition, isotype matched negative control was also used in the form of mouse IgG and goat IgG.

After 20 minutes, all the wells were washed twice with wash buffer and an avidin/streptavidin polyclonal antibody (secondary antibody) was added (50 µl/well, 1:500 diluted with buffer). The plate was again refrigerated at 4°C for 20

minutes. After this, all the wells were washed at least 7 times with buffer to remove any unbound secondary antibody.

Finally, 50 µl of OPD solution (0.4 mg/ml) was added to the appropriate wells. In other wells HBSS+ was added. Working solution of OPD was prepared by dissolving one 2 mg OPD tablet in 5 ml of sodium citrate buffer. After 10 minutes of incubation at room temperature, the absorbance of the fluid was determined at 450 nm by a spectrophotometer. The level of absorbance corresponds to the amount of antigen on the HUVEC.

In each experiment 3-6 wells were subjected to the same conditions and each experiment was done at least twice. The following table shows the plate layout and experimental conditions subjected to them. Rows A and B contain unactivated cells whereas rows C and D, rows E and F and rows G and H contain cells activated with LPS, IFN-β and IFN-γ respectively.

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	a	a	a	a	b	b	b	b	c	c	c	c
B	d	d	d	d	e	e	e	e	f	f	f	f
C	a	a	a	a	b	b	b	b	c	c	c	c

D	d	d	d	d	e	e	e	e	f	f	f	f
E	a	a	a	a	b	b	b	b	c	c	c	c
F	d	d	d	d	e	e	e	e	f	f	f	f
G	a	a	a	a	b	b	b	b	c	c	c	c
H	d	d	d	d	e	e	e	e	f	f	f	f

Code	Primary Antibody (1°)	Secondary Antibody (2°)	OPD Solution
a	-	+	+
b	Mouse IgG	+	+
c	Goat IgG	+	+
d	Anti E-Selectin	+	+
e	Anti TLR-4	+	+
f	Anti ICAM-1	+	+

Western blot

Confluent cells in each of the tissue culture flasks were activated with LPS, IFN-β or IFN-γ. A flask of unactivated cells with only growth media changed was kept as a control. The cells were incubated at 37°C for the appropriate time (4h/24h) as per the experimental conditions. The concentrations were as tabulated:

Agent	Sample concentration
LPS	50 ng/ml (1:1000)
IFN-β	100 u/ml (1:100)
IFN-γ	1000 u/ml (1:1000)

After the treatment time was over the cells were washed 3 times with cold 1X PBS. Excess PBS was removed by aspiration. Approximately 1 ml of PBS was allowed to remain in the flask at the end of third aspiration. All the cells were scraped off from the walls of the flask using a cell scraper. This suspended cells-PBS solution was centrifuged at 4°C for 5 minutes at 3000 rpm. The supernatant was removed from the top without disturbing the pellet formed at the bottom. Freshly made lysis buffer was added according to size of the pellet to lyse the cells. All the samples were then sonicated for 4 seconds 3 times each.

The protein concentration of the above cell samples was determined by using Micro BCA™ Protein Assay kit following the directions of the manufacturer. The NuPage system from Invitrogen was used to run the western blot.

Based on the protein concentration the volume of the original samples was calculated so as to have 25-30 µg of total protein in them. NuPage LDS Sample Buffer (4X) and 10X Reducing Agent (NuPage - 1M DTT) were added to the sample in the required quantities. Distilled water was added to ensure all the samples had equal final volumes. These samples were then heated at 70°C for 10 minutes and spun down for a minute to ensure equal loading quantities. The samples were then loaded onto a gel. NuPage Antioxidant was added to prevent re-oxidation of reduced proteins during electrophoresis. 4-12% Bis-Tris gel in 1X MOPS NuPage SDS Running Buffer was used for running the gel at 180V for 45

minutes. At the end the proteins were transferred to a nitrocellulose membrane using the NuPage system at 30V for 1 hour. Once the transfer was complete, the proteins on the membrane were blocked for 30 minutes at room temperature using 5% powdered milk in TBSTw (0.1%). The membrane was then washed 3 times for 5 minutes each in TBSTw (0.1%). The membrane was then incubated with primary antibody (1:2000) in 1% powdered milk in TBSTw (0.1%) overnight at 4°C. Subsequently, the membrane was washed 3 times for 5 minutes each in TBSTw (0.1%) to remove any unbound primary. The membrane was then incubated with secondary antibody in 1% powdered milk in TBSTw (0.1%) for 1 hour at room temperature. Following it the membrane was washed 3 times for 5 minutes each in TBSTw (0.1%) to remove any unbound secondary.

In order to visualize the secondary antibody binding ECL Plus reagents (Amersham Pharmacia) were used according to the manufacturer's directions.

The reagents were mixed and allowed to incubate on the membrane for 3 minutes in the dark. Finally, the film was exposed to the membrane for a minute and developed. The expression of proteins can be determined by looking at the intensity of the bands formed in the various sample lanes.

Statistics

The differences between the means of different groups were calculated using analysis of variance (ANOVA). P values less than 0.01 were considered significant over the entire experiment set. For testing significance, Bonferroni correction was applied to downward adjust the p values over individual experiment sets such that p values remained less than 0.01 over the entire experiment set.

Results

Preliminary experiments were conducted with different types of experimental conditions and parameters. In that experiment set of 2-5 experiments each, mixed results for LPS, IFN-β and IFN-γ were seen on TLR4 expression. However, a systematic error was detected. This skewed data is not presented here because of the presence of systematic error during those experiments. Some of the experiments were redone after this discovery. The data from those experiments is being presented in this section.

Results for 4-hour HUVEC LPS/IFN-β/IFN-γ activated formalin fixed ELISA

As seen from figure 2 all the controls show no significant difference in detection levels between unactivated and activated HUVEC. There is no significant difference in ICAM-1 expression for LPS, IFN-β and IFN-γ activated HUVEC compared to unactivated HUVEC. We see no significant difference in TLR4 expression when HUVEC are treated with LPS, IFN-β and IFN-γ compared to untreated cells. The expression of E-selectin on LPS, IFN-β and IFN-γ activated HUVEC is not significantly different than on unactivated HUVEC.

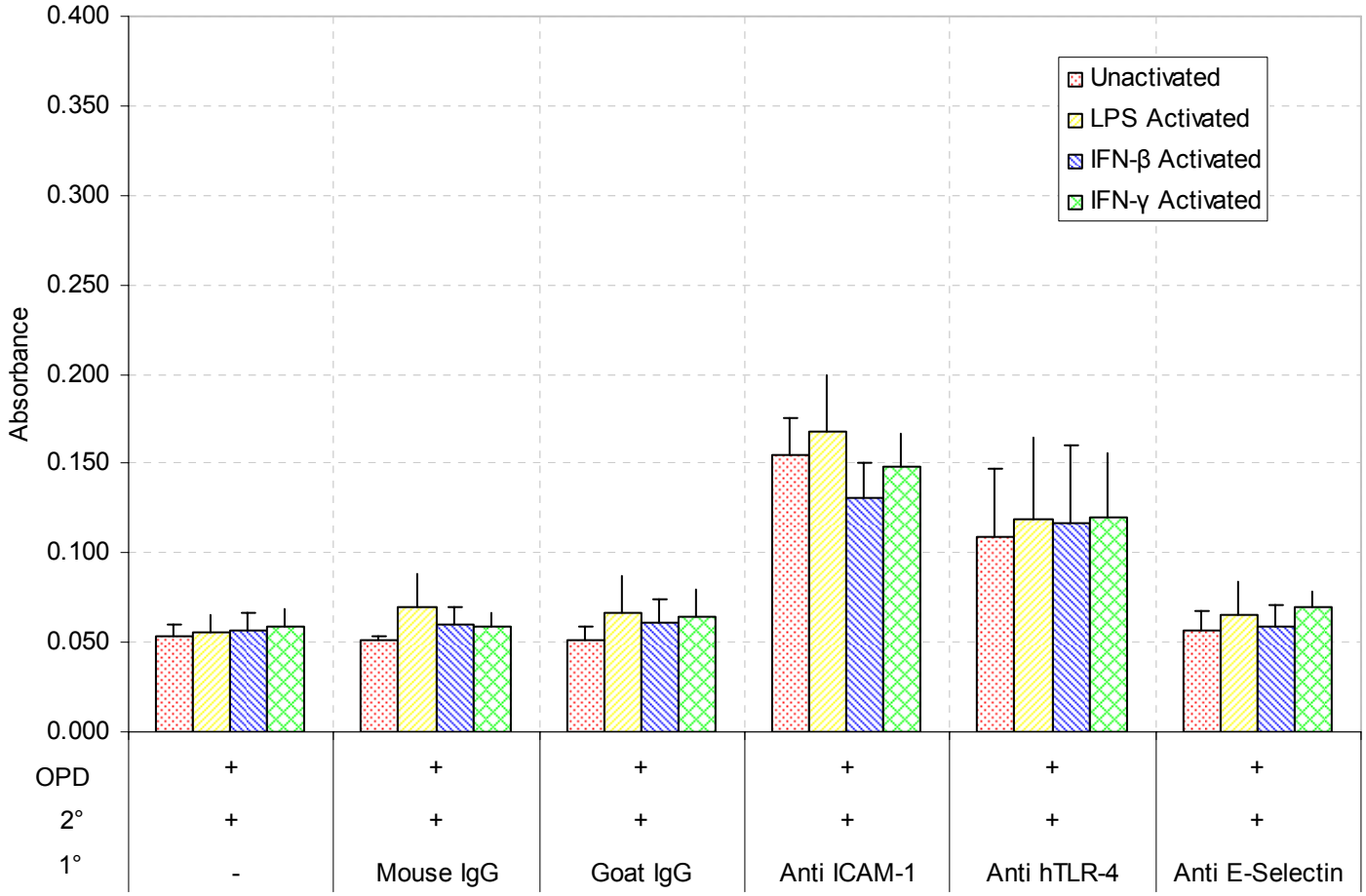

Figure 2: Results for 4-hour HUVEC LPS/IFN-β/IFN-γ activated formalin fixed ELISA. Legend: OPD = o-phenylnediamine, 2° = Secondary antibody, 1° = Primary antibody, + indicates presence, - indicates absence; * indicates $p<0.01$. Number of independent experiment sets = 2.

Results for 4-hour HUVEC LPS/IFN-β/IFN-γ activated methanol fixed ELISA

Formalin enables the detection of only extracellular proteins. However, methanol makes the cell walls more permeable which helps in detection of total protein in the cell by the antibody – extracellular as well as cytoplasmic. As seen from figure 3 all the controls show no significant difference in detection levels between unactivated and activated HUVEC. There is no significant difference in ICAM-1 expression for LPS activated HUVEC compared to unactivated HUVEC. In contrast, IFN-β and IFN-γ activated HUVEC show significantly less ICAM-1 expression compared to unactivated HUVEC. We see no significant difference in TLR4 expression for LPS, IFN-β and IFN-γ activated HUVEC compared to unactivated HUVEC. The expression of E-selectin on LPS, IFN-β and IFN-γ activated HUVEC is not significantly different than on unactivated HUVEC.

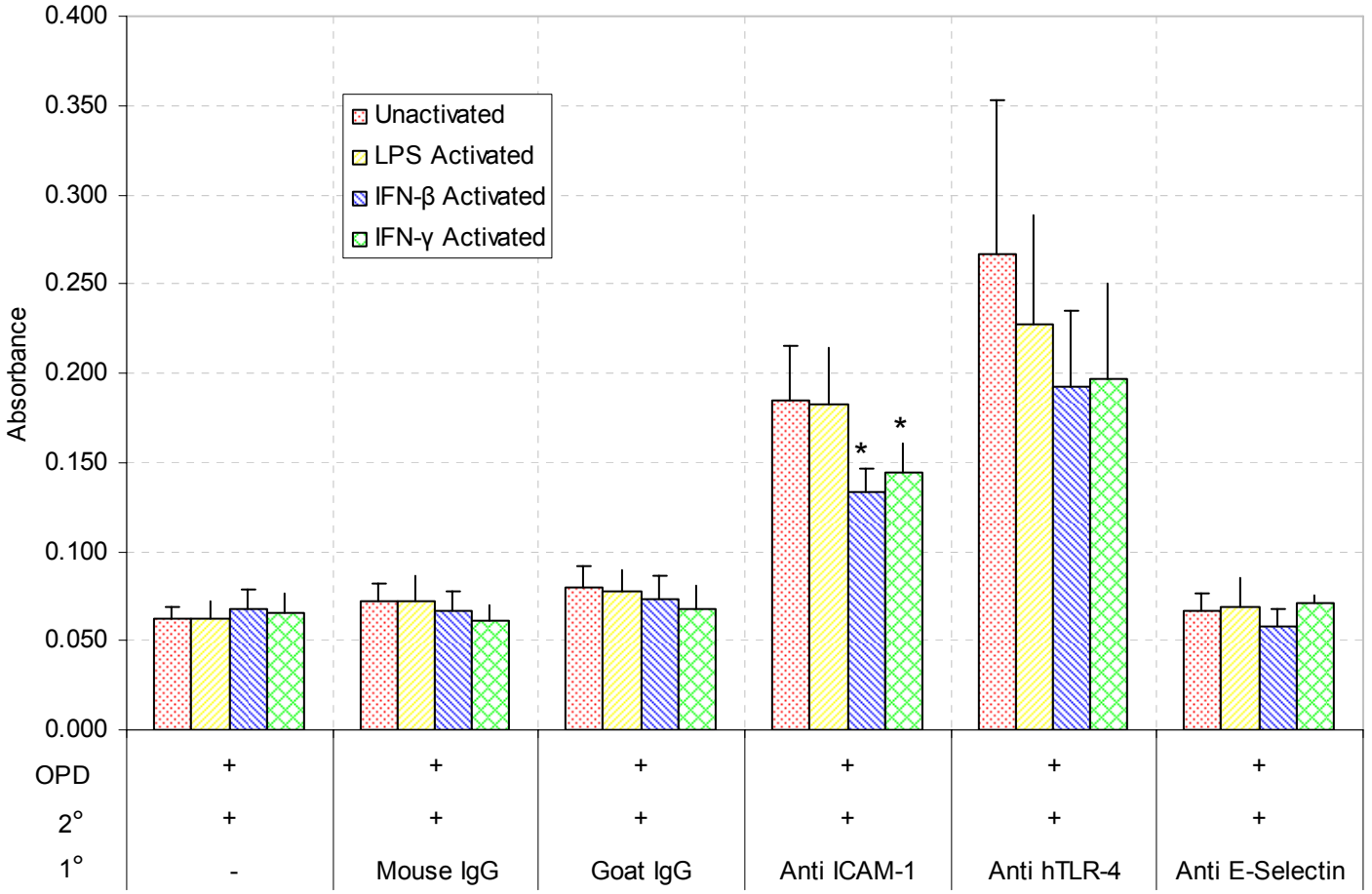

Figure 3: Results for 4-hour HUVEC LPS/IFN-β/IFN-γ activated methanol fixed ELISA. Legend: OPD = o-phenylnediamine, 2° = Secondary antibody, 1° = Primary antibody, + indicates presence, - indicates absence; * indicates $p<0.01$. Number of independent experiment sets = 2.

Results for 24-hour HUVEC LPS/IFN-β/IFN-γ activated formalin fixed ELISA

As seen from figure 4 all the controls show no significant difference in detection levels between unactivated and activated HUVEC. The level of ICAM-1 on LPS and IFN-γ activated HUVEC is greater than on unactivated HUVEC. In contrast, no significant difference in ICAM-1 expression exists between unactivated and IFN-β activated HUVEC. We see no significant difference in TLR4 expression for LPS, IFN-β and IFN-γ activated HUVEC compared to unactivated HUVEC. The expression of E-selectin on LPS, IFN-β and IFN-γ activated HUVEC is not significantly different than on unactivated HUVEC.

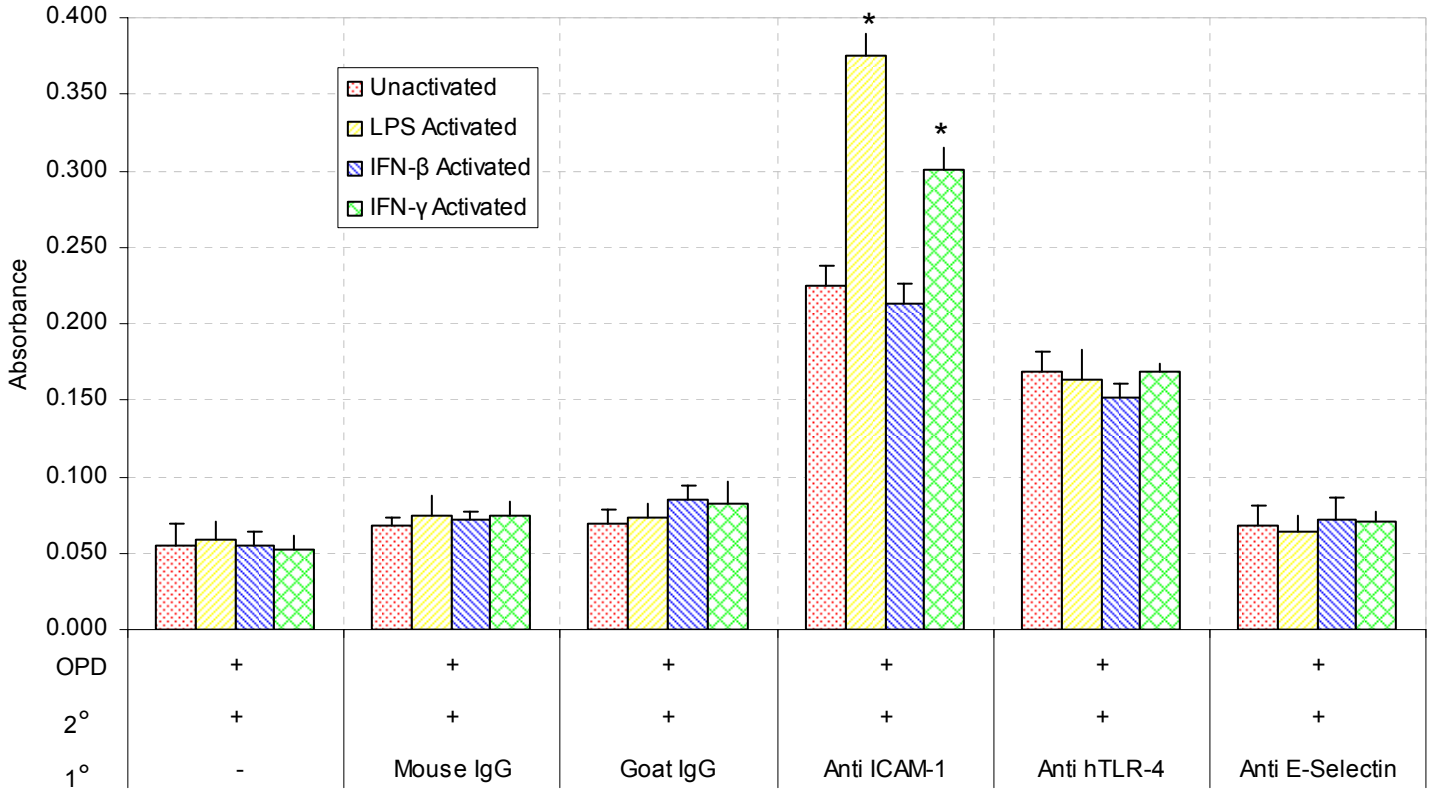

Figure 4: Results for 24-hour HUVEC LPS/IFN-β/IFN-γ activated formalin fixed ELISA. Legend: OPD = o-phenylnediamine, 2° = Secondary antibody, 1° = Primary antibody, + indicates presence, - indicates absence; * indicates $p<0.01$. Number of independent experiment sets = 1.

Results for 24-hour HUVEC LPS/IFN-β/IFN-γ activated methanol fixed ELISA

As stated earlier, methanol makes the cell walls more permeable which helps in detection of total protein in the cell by the antibody – extracellular as well as cytoplasmic. This effect is independent of original time for which cells were incubated. As seen from figure 5 all the controls show no significant difference in detection levels between unactivated and activated HUVEC. The level of ICAM-1 on LPS and IFN-γ activated HUVEC is greater than on unactivated HUVEC. In contrast, no significant difference in ICAM-1 expression exists between unactivated and IFN-β activated HUVEC. We see no significant difference in TLR4 expression for LPS, IFN-β and IFN-γ activated HUVEC compared to unactivated HUVEC. The expression of E-selectin on LPS, IFN-β and IFN-γ activated HUVEC is not significantly different than on unactivated HUVEC.

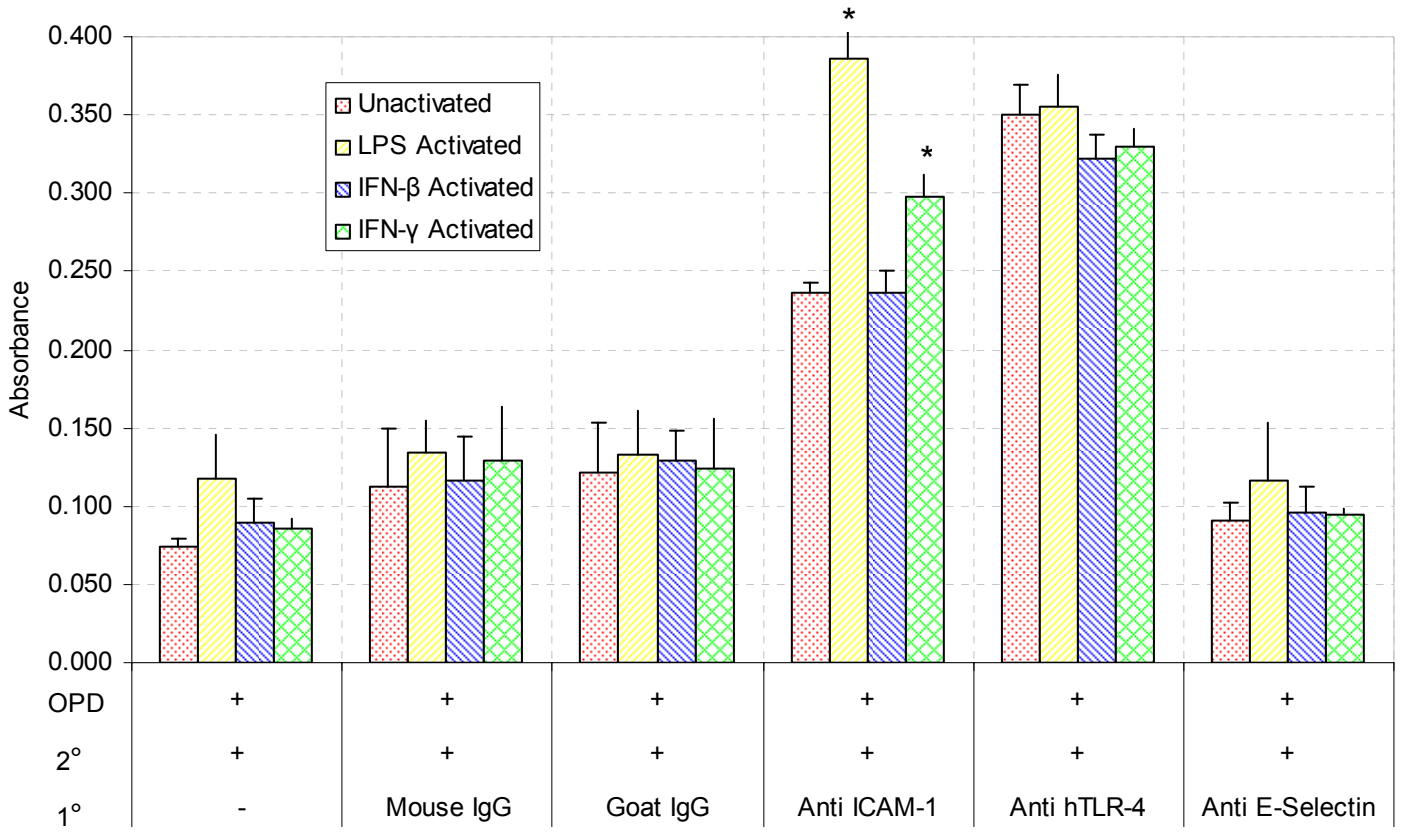

Figure 5: Results for 24-hour HUVEC LPS/IFN-β/IFN-γ activated methanol fixed ELISA. Legend: OPD = o-phenylnediamine, 2° = Secondary antibody, 1° = Primary antibody, + indicates presence, - indicates absence; * indicates $p<0.01$. Number of independent experiment sets = 1.

Results for Western blot run with 4-hour LPS/IFN-β/IFN-γ treated HUVEC

Western blots were performed to further investigate the ELISA results. Because of the delicate nature of TLR4, it was very difficult to obtain the TLR4 protein band on the gel. The bands were very faint as a result. As we can see from figure 6, there is no significant increase in the intensity of the bands for untreated cells or LPS/IFN-β/IFN-γ treatment (Representative of 4 experiments).

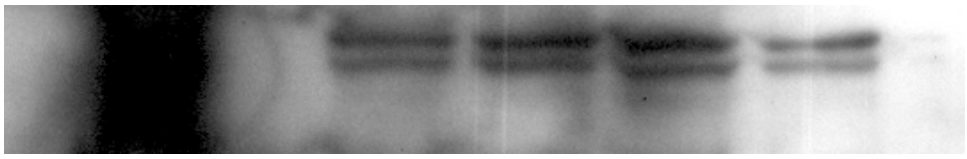

Figure 6: Results of TLR4 Western blot for 4-hour effect of LPS/IFN-β/IFN-γ on HUVEC.

Results for Western blot run with 24-hour LPS/IFN-β/IFN-γ treated HUVEC

As seen from figure 7, there is no significant increase in the intensity of the bands for cells with no treatment or treatment with LPS, IFN-β or IFN-γ (Representative of 2 experiments).

Marker Untreated + LPS + IFN-β + IFN-γ

Figure 7: Results of TLR4 Western blot for 24-hour effect of LPS/IFN-β/IFN-γ on HUVEC.

Discussion

This work was undertaken to study the effects of various inflammatory substances on expression of TLR4. On a 4-hour period with formalin as a fixative, there are no significant differences in the expression of ICAM-1, TLR4 and E-selectin in HUVEC treated with LPS, IFN-β or IFN-γ compared to untreated HUVEC. On a 4-hour period with methanol as a fixative there are no significant differences in the expression of TLR4 and E-selectin in HUVEC treated with LPS, IFN-β or IFN-γ compared to untreated HUVEC. However ICAM-1 shows significantly less expression for IFN-β and IFN-γ treated HUVEC compared to untreated and LPS treated HUVEC. On a 24-hour period irrespective of the fixative, formalin or methanol, there are no significant differences in the expression of TLR4 and E-selectin in HUVEC treated with LPS, IFN-β or IFN-γ compared to untreated HUVEC. Contrarily, ICAM-1 shows significantly higher expression for HUVEC treated with LPS and IFN-γ compared to untreated and IFN-β treated HUVEC both when formalin and methanol are used as a fixative. For 4-hour and 24-hour western blots there were no significant differences in TLR4 expression in HUVEC treated with LPS, IFN-β or IFN-γ compared to untreated HUVEC. Concluding, we found evidence that LPS, IFN-β and IFN-γ fail to upregulate TLR4 expression at 4-hour and 24-hour time period. Moreover, using methanol as a fixative enabled higher detection of TLR4 than formalin.

The limitations of the results need to be understood though. We have proof from existing literature (28-30) that there is an increased E-selectin and ICAM-1 expression in a 3 to 4-hour period on HUVEC after treatment with LPS, which we failed to detect. The fact that we saw an increased ICAM-1 expression only on a 24 hour period might be because of the lower concentration of LPS used. Also researchers have shown an increased LPS and interferon induced TLR4 expression on closely related and different cell lines to HUVEC (25, 26). We also suspect a less than optimum detection of the proteins because of use of biotinylated antibodies in the western blots that were performed.

This study will help in giving directions to future research in this area. Higher concentrations of cytokines need to be used under similar conditions to determine if they have an effect or not. The use of antibodies which are independent of biotin conjugation mechanism might help in better detection of TLR4 when performing western blots. Northern blots can be run to determine if there is an increased messenger RNA response to the cytokines even though the effect or magnitude may not transfer to the protein level.

Hence results and conclusions derived from this study cannot be conclusively stated until a clearer picture emerges by conducting more experiments to confirm the fact or find out factors responsible behind some aberrant phenomenon in this

study. With the limitations in mind, this study has commented on TLR4 expression in HUVEC.

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