ELSEVIER

International Immunopharmacology 6 (2006) 26–35

International Immunopharmacology

www.elsevier.com/locate/intimp

Nicotine modulation of cytokine induction by LPS-stimulated human monocytes and coronary artery endothelial cells☆

G.W. Patton [a,1], D.A. Powell [b], A. Hakki [a], H. Friedman [a], S. Pross [a,*]

[a] *Department of Medical Microbiology and Immunology, College of Medicine, University of South Florida, MDC-10, 12901 Bruce B. Downs Blvd., Tampa, FL 33612-4742, United States*

[b] *Data Management Services, National Cancer Institute-Frederick, Frederick, MD 21702, United States*

Received 8 December 2004; received in revised form 21 February 2005; accepted 18 July 2005

Abstract

Nicotine, the major addictive component of tobacco, is an immunomodulator that impacts on many cells, including immune cells involved in inflammatory processes. Nicotine also induces oxidative damage to the vascular endothelium and accentuates lipid peroxidation, resulting in vascular cell dysfunction. Furthermore, vascular endothelial cells produce growth factors, such as cytokines and chemokines capable of stimulating and recruiting immune cells to atheromatous lesions. In addition, bacterial products including lipopolysaccharides (LPS), a major component of Gram negative bacterial cell walls, activate gene expression resulting in inflammatory cytokine production causing further damage to the vasculature. In the present study, the combined effects of nicotine and bacterial LPS on the expression of IL-6, IL-8, GRO-α and MCP-1 in cell lines of human coronary artery endothelial cells (HCAEC) and pulmonary monocytes (THP-1) were examined by quantitative real-time PCR and ELISA. Results showed that nicotine suppressed the LPS induced production of IL-6 and IL-8 in both cell lines. Since cytokines which alter homeostasis of both vascular endothelial and immune cells are critical for the atherogenic process, further studies are warranted to examine in detail the role of nicotine in terms of effects on inflammatory reactions, including those induced by bacterial infection.

Keywords: HCAEC; THP-1; Nicotine; LPS; Cytokines; Chemokines

1. Introduction

Nicotine, the addictive component of tobacco, is a small organic alkaloid that can transport directly through the cell membrane and also bind to nicotinic acetylcholine receptors as an agonist [1]. Nicotinic receptors are expressed on immune cells including lymphocytes and macrophages [8,17], as well as on

☆ This research was in part supported by the training Grant # NIH/NIDA 2 T32 DA07245 12, and by the American Heart Association Grant — FL # 0051206B.

* Corresponding author. Tel.: +1 813 974 2065; fax: +1 813 974 4151.

E-mail address: spross@hsc.usf.edu (S. Pross).

1567-5769/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2005.07.005

vascular endothelial cells [15]. It is well known that nicotine's effects on endothelial cells result in production of various factors involved in development of atherosclerosis [3]. Nicotine has also been associated with alterations in immune responses through its impact on inflammation and cytokine production [26], important in regulating vascular tone and thrombogenesis. Such actions of nicotine may contribute to mechanisms whereby cigarette smoke affects the development of coronary atherosclerosis [33].

Recent studies suggest that bacterial infection, especially by organisms which contain endotoxin, increases the risk of atherosclerosis, possibly by enhancing inflammation [10]. For example, lipopolysaccharide (LPS), the endotoxin component of Gram negative bacterial cell walls, stimulates inflammatory reactions [4] and thus may contribute to the atherosclerotic process [24]. However, there is considerable controversy concerning a direct involvement of LPS in the pathophysiology of atherogenesis [9]. The present study investigated the role of nicotine and LPS, both individually and in combination, on production of immunologic mediators by two cell types related to atherosclerosis: a human monocytic leukemic cell line (THP-1) and human coronary artery endothelial cells (HCAEC). The production of cytokines important in inflammation, including interleukin 6 (IL-6), interleukin 8 (IL-8) and MCP-1, chemokines involved in the attraction of activated monocytes to arterial vessel wall injury sites, and GRO-α, which activates monocytes and endothelial cells [2,11], were studied.

The levels of these factors in cultures of stimulated cells were determined by real time RT-PCR for mRNA and by ELISA for protein. It was found that LPS enhanced expression of these inflammatory mediators in both cell lines, and treatment with nicotine alone had minimal effects. In contrast, when cells were treated with both nicotine and LPS, there was a marked inhibition of the endotoxin-induced cytokines and chemokines produced in both cell lines. This action of nicotine may limit the damage inflicted by the bacterial endotoxin, but at the same time may suppress the antimicrobial activity of the immune cells, exacerbating the effects of bacterial infection on atherosclerosis. The results of this study suggest that the combination of both nicotine and bacterial LPS can modulate inflammation and this may be one explanation concerning the controversy in regard to the impact of nicotine and infectious diseases in atherosclerosis.

2. Experimental procedures

2.1. Cells and treatments

Primary human coronary artery endothelial cells (HCAEC) (Bio-Whittaker, Inc., San Diego, CA) harvested at passages 6–8 were removed from −70 °C storage, cultured in defined medium for 3–5 days and passed into 6-well plates. After 24 h incubation at 37 °C, the cells were treated with either PBS alone as controls or with 1 μg/ml nicotine (hydrogen bitartrate salt — Sigma Chemical Co., St. Louis, MO). After 30–60 min, *Escherichia coli* LPS (Strain 0111:B4, Sigma-Aldrich) was added to the PBS controls and to the nicotine-treated cells.

2.2. Reverse transcriptase-PCR

Applied Biosystem's (ABI, Foster City, CA) Geneamp Gold RNA PCR Core Kit was used according to the manufacturer's recommendations in a two-step approach to perform the Reverse Transcription (RT) step separately from the PCR amplification step. Random hexamers were used to evaluate multiple genes from the same RT reaction product (cDNA). The cycling conditions were 10 min at 25 °C, 15 min at 42 °C, 5 min at 99 °C, and at least 5 min at 4 °C.

2.3. ELISA cytokine protein assay

The RayBiotech Human Cytokine Protein Array I (Raybiotech, Inc., Norcross, GA) is a postage stamp-sized membrane utilizing a sandwich ELISA technique. Culture supernatants were used diluted 1:1 with PBS and the membranes processed according to the manufacturer's recommendations. Amersham Pharmacia Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, England) ECL-exposed films were digitized and densitometry performed using MultiAnalyst software (Bio-Rad, Inc., Hercules, CA). The antibodies used were to IL-6, IL-8 and MCP-1, as well as to GRO proteins.

2.4. Real time quantitative PCR

Reactions were set up using SYBR Green PCR Master Mix (Applied Biosystems, Inc., Foster City, CA or Qiagen, Inc., Valencia, CA) either with published primers or ones custom-designed for human cytokines using as primer software Seqweb Wisconsin Package Version 10.3 (Accelrys, Inc., San Diego, CA),

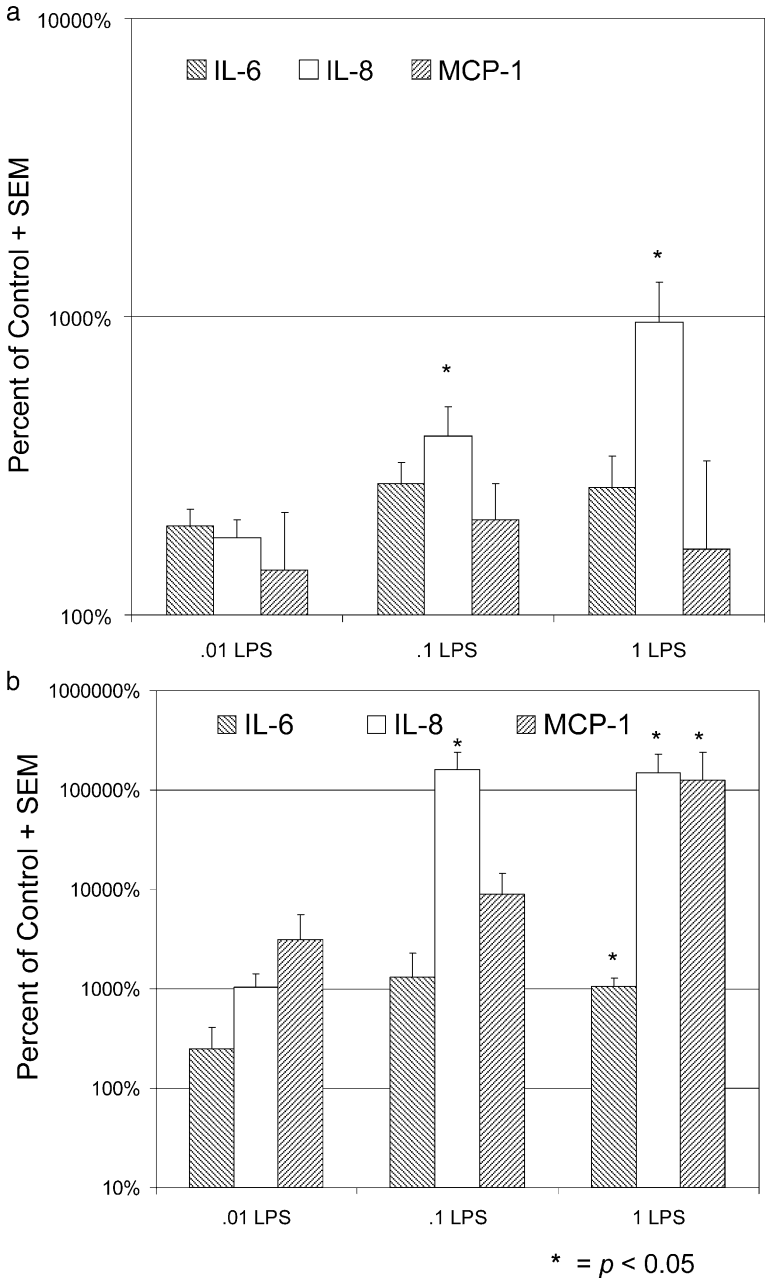

Fig. 1. LPS induced cytokine mRNA in HCAEC (a) and THP-1 cells (b). 24-h post-passage cells were treated with the indicated concentrations of LPS (all μg/ml) and their cytokine mRNA levels were determined after an additional 24 h by quantitative RT-PCR. Note the increase in cytokines, most notably in the THP-1 cells, after LPS stimulation. Bars represent mean ± SEM for 5–6 individual experiments. * $p < 0.05$.

Gene Fisher [6], or Primer 3 (Whitehead Institute for Biomedical Research) [20]. The primers were commercially synthesized by various providers. The identical RT cDNA stock for each sample was used for all amplifications within each experiment. The Bio-Rad iCycler was programmed and run per the manual. Thermocycler parameters were adjusted for each primer pair based on melting temperatures predicted from each respective sequence. These were then confirmed by gradient cycling and melt curves. A typical set of

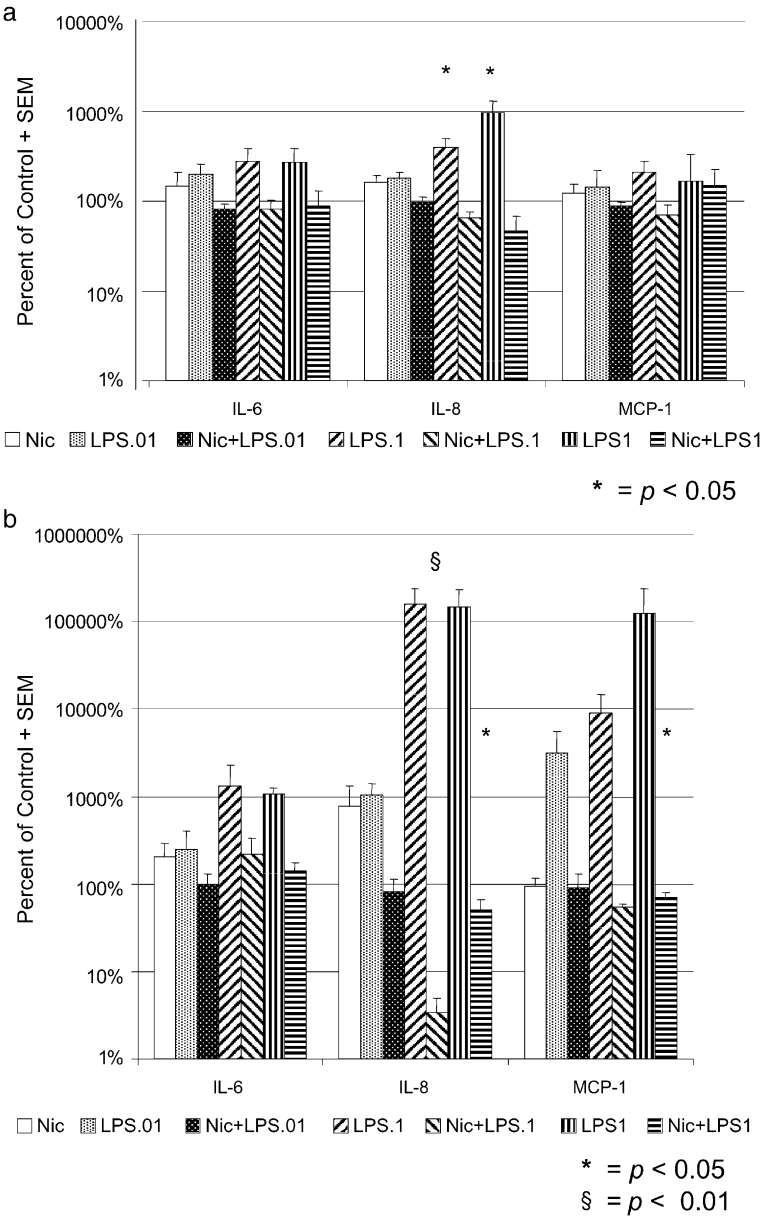

Fig. 2. Nicotine effects on cytokine RNA levels in LPS treated HCAEC (a) and THP-1 cells (b) in response to LPS stimulation. Cells were treated with nicotine (1 μg/ml) for 1 h prior to addition of LPS (concentrations are all μg/ml). Note that nicotine did decrease cytokine levels, significantly with IL-8 and MCP-1 production of THP-1 cells. Bars represent mean ± SEM for 3–6 separate experiments. * $p < 0.05$; § $p < 0.01$.

parameters was 15 min at 95 °C, 1 min at 60 °C, and 20 s at 72 °C for up to 45 cycles with a final extension period of 2 min at 72 °C, followed by the melt curve process from 50 to 96 °C in 0.5° steps. For sequencing confirmation, PCR amplification products were isolated from dimers and reagents using Qiagen MinElute spin columns. DNA sequencing was performed on an ABI Prism 377 Sequencing System using ABI PRISM BigDye™ Terminator v. 3.0 Ready Reaction Cycle Sequencing Kits with AmpliTaq® DNA polymerase following the manufacturer's protocol for cycle sequencing. Unincorporated dye terminations are removed by gel filtration using Performa DTR Gel Filtration Cartridges from Edge Biosystems (Gaithersburg, MD).

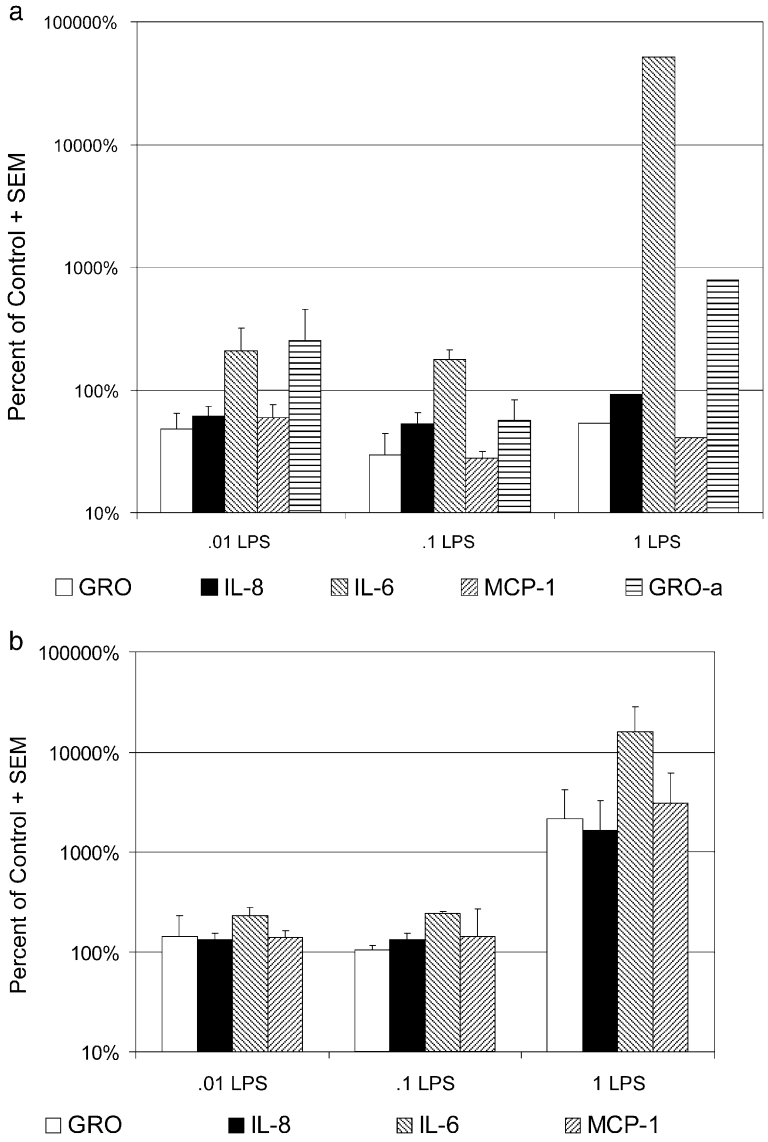

Fig. 3. LPS-Induced protein levels in HCAEC (a) and THP-1 cells (b). Cells were treated with LPS (concentrations are all μg/ml) for 48 h and cytokines were assessed by ELISA. Note that LPS stimulation of HCAEC resulted in an increase in IL-6 and GRO-α levels only whereas LPS stimulation of THP-1 cells increased the levels of all cytokines tested. Bars represent mean ± SEM for 3–4 individual experiments. * $p<0.05$; ** $p<0.01$. If no bars are shown, due to fewer time points tested.

2.5. Statistical analysis

Three or more independent experiments were conducted for each LPS treatment concentration. Protein array film densitometry results were normalized to the horseradish peroxidase spots on each membrane to control for variations in processing the membranes. Each sample's results were then normalized to the 18S rRNA value for the sample to control for anticipated differences in cell numbers between treatments since it has already been shown that nicotine and LPS can affect apoptosis and proliferation [7,30]. Normalized values of LPS treatments were compared to untreated controls by Kruskal–Wallis One Way Analysis of Variance on Ranks, followed by a multiple comparison test (Dunn's Method, $n=3$–4 experiments) using SigmaStat Statistical Analysis System Version 3.1 (Jandel Scientific Corp., San Rafael, CA). Normalized results of nicotine comparisons were tested with a one-sample *t*-test using both the expression ratio and a logarithmic transformation of the expression ratio as dependent variables. Statistical significance was accepted at $p<0.05$.

3. Results

The expression levels of mRNA for cytokines were determined in vascular endothelial cells and monocyte cultures using quantitative RT-PCR. When the cells were stimulated in vitro with LPS, there was a marked dose-dependent induction of IL-8 mRNA in both cell types, with the THP-1 cells responding more vigorously after stimulation (Fig. 1). The levels of IL-6 mRNA and MCP-1 mRNA were significantly enhanced in the THP-1 cells but only minimal changes were seen in HCAEC after LPS stimulation (Fig. 1). In additional experiments (Fig. 2), the cells were treated with nicotine (1 μg/ml) for 1 h prior to addition of graded amounts of LPS and then further incubated for 24 h. Under these conditions, there was a significant decrease in IL-8 production in the HCAEC and minimal decreases with the other cytokines (Fig. 2a). Significance could not be determined with IL-6 and MCP-1 due in part to variability between samples. The LPS induced enhancement of IL-8 and MCP-1 were significantly decreased with nicotine exposure in THP-1 cells (Fig. 2b).

Experiments were then performed to assess whether protein expression levels were consistent with the altered mRNA levels. Cells were exposed to LPS for 48 h and then analyzed by ELISA to assess the expression levels of IL-6, IL-8, MCP-1, and GRO proteins, including GRO-α (Fig. 3). LPS stimulation of HCAEC resulted in enhanced IL-6 and GRO-α levels. Stimulation of the THP-1 cells with LPS resulted in an increase in protein response for all four cytokines, consistent with mRNA induction.

Treatment of either cell type with nicotine alone had no effect on protein expression (data not shown). To determine whether nicotine modulated responses to LPS stimulation, cells were pretreated with nicotine for 1 h and then stimulated with LPS for 24 h (Fig. 4). The results with HCAEC (Fig. 4a) were variable, similar to the mRNA findings (Fig. 2a). However, nicotine pretreatment led to a suppression of LPS-stimulated cytokine protein induction in THP-1 cells (Fig. 4b), similar to the effects on message levels (Fig. 2b).

4. Discussion

Microbial infections are postulated to be risk factors involved in atherogenesis. Recent reports have suggested that bacteria, such as *Chlamydia pneumoniae* and *Helicobacter pylori*, and viruses, such as cytomegalovirus, herpes simplex virus, and hepatitis A virus, may be associated with early vascular insults that trigger atherogenesis [25,31]. However, the outcome of antimicrobial therapy on progression of atherosclerosis has been inconsistent, suggesting that additional factors may be involved. One such factor may be exposure to environmental or toxic agents. In this regard, smoking has been associated with coronary artery disease, with recent studies pointing to the effects of nicotine on vascular endothelial cells [33] as well as nicotine's role as an immunomodulatory agent associated with increased susceptibility to infections.

Cytokines are important for leukocyte/endothelial cell interactions in the development of atherosclerosis [29]. There is a close relationship between cytokine and chemokines produced by injured vascular endothelial and immune cell response [22]. Altered levels of cytokines produced by injured endothelial cells as a result of infection or exposure to toxic agents are believed to have effects on immune responses. Cigarette smoke has been reported to inhibit LPS induced inflammatory cytokines and hence contribute to the chronic colonization of bacteria [28]. In a prospective study of post-operative infections, it was reported that the infection rate for

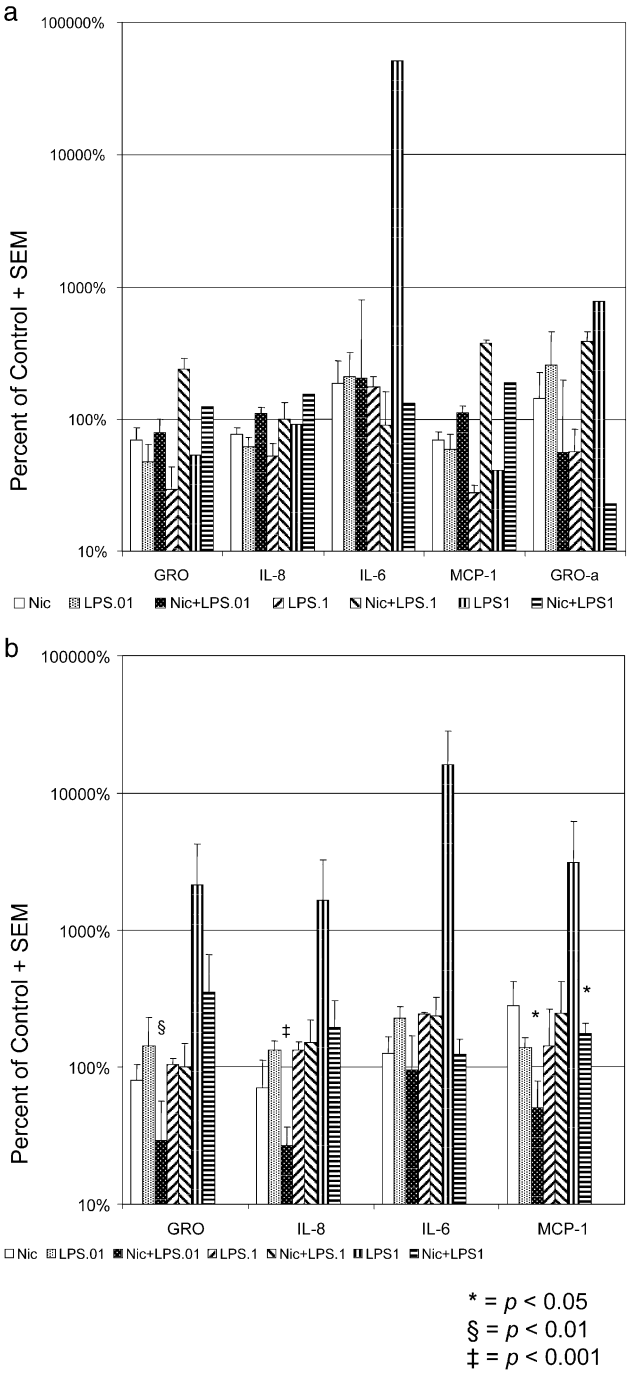

Fig. 4. Effects of nicotine and LPS on cytokine production by HCAEC (a) and THP-1 (b) cells. Cells were treated with nicotine (1 μg/ml) for 1 h prior to addition of LPS. Note that although nicotine did not affect cytokine production in the LPS (concentrations are all μg/ml) stimulated HCAEC, there was a consistent pattern of nicotine induced suppression of LPS stimulation in the THP-1 cells. * $p < 0.05$, § $p < 0.01$, ‡ $p < 0.001$. If no bars are shown, due to fewer time points tested.

heavy smokers was twice as high as for nonsmokers [27]. Furthermore, it has been reported that bacterial challenge resulting in chronic infections may be an important factor in initiation and progression of atherosclerosis due to alteration of the immune response [12].

In the present study, it was found that nicotine pretreatment alters the stimulating effects of bacterial LPS at both the mRNA and protein levels in regard to expression of certain cytokines and chemokines. The levels of the message were not always consistent with the levels of protein expression, probably related to the different processing methods of each including enzyme digestion of proteins or differential completion of message translation into protein.

Nicotine suppressed production of the IL-6 and IL-8 induced by LPS. This result with IL-6 is consistent with data reported in studies using a *Legionella pneumophila* infection model in which cigarette smoke condensate down-regulated the IL-6 response of macrophages, resulting in the enhancement of bacterial replication [16]. These results are also consistent with studies showing a decrease in IL-8 production following exposure to cigarette smoke extract in bronchial epithelial cells stimulated with LPS [13]. This down-regulation could possibly limit the inflammatory process which is associated with atherosclerosis, or conversely could limit the immune response to invasive pathogens leading to an exacerbation of infection. Interestingly, the resultant down-regulation of LPS stimulated cytokines and chemokines with nicotine treatment was often lower than with nicotine alone. This result was evidenced with IL-8 mRNA (Fig. 2b) and protein (Fig. 4b) following nicotine treatment of LPS stimulated THP-1 cells. This finding supports that nicotine's action may in fact be more profound when cells are exposed to environmental challenges than when cells are exposed to nicotine alone.

Another important factor related to the inflammatory response is production of macrophage chemotactic protein MCP-1 [18]. This chemokine induces inflammation as well as contributes to the differentiation of Th1 and Th2 cells. Injured endothelial cells express MCP-1, which recruits monocytes to the site of injury [14,32]. In this regard, MCP-1, as well as IL-8 and GRO-alpha, have been considered to be an important contributor to atherogenesis [2]. In the present study, LPS induction of MCP-1 was lower in the HCAEC than in the THP-1 cells, possibly due to higher background levels in the former. Nicotine pretreatment prior to LPS stimulation had an inhibitory effect on the induced MCP-1 production in THP-1 cells. A similar action of nicotine on MCP-1 production was reported in experiments using rat responses to spinal cord injury in which there was an overexpression of MCP-1 in response to the injury that was markedly attenuated by nicotine administration [23].

The present study shows that nicotine alone had minimal to no effect on the production of cytokines and chemokines in both cell types tested, in contrast to the nicotinic down-regulation of cytokines following LPS stimulation. The minimal effect resulting from exposing cells to nicotine alone has been reported by others [21]. This lack of a direct effect of nicotine on cytokine production has contributed to controversy as to whether nicotine in fact affects immunity. The data from this present study shows the importance of co-treatment of cells to both nicotine and another stimulatory agent, in this case a bacterial product.

In addition, this study shows that the extent of nicotine's action on LPS stimulated cells was dependent upon the specific cytokine measured and the cell type used. Recognition of this variability is important because it addresses the conflicts in the literature either supporting or disputing a role for nicotine in immunity in general and in atherosclerosis in particular. An example of the divergent nature of nicotinic action is evidenced in a study using a rat model of inflammatory bowel disease wherein chronic nicotine administration either augmented or ameliorated inflammatory responses dependent upon assessing specific areas of the bowel [5]. Other explanations for the complex interactions of nicotine exposure and varying results has to do with the role of confounding variables including gender, genetics, and environmental conditions [19].

In conclusion, nicotine decreased the production of cytokines in cells important in immune function. Not all cytokines were affected equally and there were differences based upon the cell type. Future experiments to pursue are to determine the mechanism of nicotine's action, specifically in terms of the type of nicotinic receptor subunits involved. Understanding

the role of nicotine on cytokine production with consideration of its potential impact on the pathogenic process may aid in developing therapeutic strategies for inflammatory diseases including atherosclerosis.

Acknowledgements

This study was supported in part by grant NIH/NIDA 2T310A07245 12 and by the American Heart Association Grant FL 00512063. The authors wish to express their gratitude to Drs. Hiroyuki Yamaguchi and Yoshimasa Yamamoto for providing research materials and for the many fruitful discussions. Ms. Marybeth Coulter, Moffitt Cancer Research Center, provided special expertise confirming the validity of the spin column cleanup of the QRT-PCR products for sequencing.

References

[1] Bhandari N, Sylvester SL, Rogotti NA. Source book of substance abuse and addiction;1996.
[2] Boisvert WA. Modulation of atherogenesis by chemokines. Trends Cardiovasc Med 2004;14:161–5.
[3] Cucina A, Sapienza P, Corvino V, Borrelli V, Mariani V, Randone B, et al. Nicotine-induced smooth muscle cell proliferation is mediated through bFGF and TGF-beta 1. Surgery 2000;127:316–22.
[4] Detmer K, Wang Z, Warejcka D, Leeper-Woodford SK, Newman WH. Endotoxin stimulated cytokine production in rat vascular smooth muscle cells. Am J Physiol Heart Circ Physiol 2001;281:H661–8.
[5] Eliakim R, Karmeli F. Divergent effects of nicotine administration on cytokine levels in rat small bowel mucosa, colonic mucosa, and blood. Isr Med Assoc J 2003;5:178–80.
[6] Giegerich R, Meyer F, Schleiermacher C. GeneFisher—software support for the detection of postulated genes. Proc Int Conf Intell Syst Mol Biol 1996;4:68–77.
[7] Hakki A, Hallquist N, Friedman H, Pross S. Differential impact of nicotine on cellular proliferation and cytokine production by LPS-stimulated murine splenocytes. Int J Immunopharmacol 2000;22:403–10.
[8] Hiemke C, Stolp M, Reuss S, Wevers A, Reinhardt S, Maelicke A, et al. Expression of alpha subunit genes of nicotinic acetylcholine receptors in human lymphocytes. Neurosci Lett 1996;214:171–4.
[9] Hoffmeister A, Rothenbacher D, Wanner P, Bode G, Persson K, Brenner H, et al. Seropositivity to chlamydial lipopolysaccharide and *Chlamydia pneumoniae*, systemic inflammation and stable coronary artery disease: negative results of a case-control study. J Am Coll Cardiol 2000;35:112–8.
[10] Huittinen T, Leinonen M, Tenkanen L, Virkkunen H, Manttari M, Palosuo T, et al. Synergistic effect of persistent *Chlamydia pneumoniae* infection, autoimmunity, and inflammation on coronary risk. Circulation 2003;107:2566–70.
[11] Ito T, Ikeda U. Inflammatory cytokines and cardiovascular disease. Curr Drug Targets Inflamm Allergy 2003;2:257–65.
[12] Kiechl S, Egger G, Mayr M, Wiedermann CJ, Bonora E, Oberhollenzer F, et al. Chronic infections and the risk of carotid atherosclerosis: prospective results from a large population study. Circulation 2001;103:1064–70.
[13] Laan M, Bozinovski S, Anderson GP. Cigarette smoke inhibits lipopolysaccharide-induced production of inflammatory cytokines by suppressing the activation of activator protein-1 in bronchial epithelial cells. J Immunol 2004;173:4164–70.
[14] Linton MF, Fazio S. Macrophages, inflammation, and atherosclerosis. Int J Obes Relat Metab Disord 2003;27(Suppl 3): S35–40.
[15] Macklin KD, Maus AD, Pereira EF, Albuquerque EX, Conti-Fine BM. Human vascular endothelial cells express functional nicotinic acetylcholine receptors. J Pharmacol Exp Ther 1998;287:435–9.
[16] Matsunaga K, Klein TW, Friedman H, Yamamoto Y. Epigallocatechin gallate, a potential immunomodulatory agent of tea components, diminishes cigarette smoke condensate-induced suppression of anti-*Legionella pneumophila* activity and cytokine responses of alveolar macrophages. Clin Diagn Lab Immunol 2002;9:864–71.
[17] Matsunaga K, Klein TW, Newton C, Friedman H, Yamamoto Y. *Legionella pneumophila* suppresses interleukin-12 production by macrophages. Infect Immun 2001;69:1929–33.
[18] Maus U, Henning S, Wenschuh H, Mayer K, Seeger W, Lohmeyer J. Role of endothelial MCP-1 in monocyte adhesion to inflamed human endothelium under physiological flow. Am J Physiol Heart Circ Physiol 2002;283:H2584–91.
[19] McAllister-Sistilli CG, Caggiula AR, Knopf S, Rose CA, Miller AL, Donny EC. The effects of nicotine on the immune system. Psychoneuroendocrinology 1998;23:175–87.
[20] Misener S, Krawetz SA. Bioinformatics methods and protocols. Totowa, N.J: Humana Press; 2000. xi, 500 pp.
[21] Nordskog BK, Blixt AD, Morgan WT, Fields WR, Hellmann GM. Matrix-degrading and pro-inflammatory changes in human vascular endothelial cells exposed to cigarette smoke condensate. Cardiovasc Toxicol 2003;3:101–17.
[22] Paoletti R, Gotto Jr AM, Hajjar DP. Inflammation in atherosclerosis and implications for therapy. Circulation 2004;109: III20–6.
[23] Ravikumar R, Flora G, Geddes JW, Hennig B, Toborek M. Nicotine attenuates oxidative stress, activation of redox-regulated transcription factors and induction of proinflammatory genes in compressive spinal cord trauma. Brain Res Mol Brain Res 2004;124:188–98.
[24] Schwartz Y, Dushkin MI. Endotoxin-lipoprotein complex formation as a factor in atherogenesis: associations with hyperlipidemia and with lecithin:cholesterol acyltransferase activity. Biochemistry (Mosc) 2002;67:747–52.
[25] Shen YH, Utama B, Wang J, Raveendran M, Senthil D, Waldman WJ, et al. Human cytomegalovirus causes

endothelial injury through the ataxia telangiectasia mutant and p53 DNA damage signaling pathways. Circ Res 2004; 94:1310–7.

[26] Sopori ML, Kozak W, Savage SM, Geng Y, Soszynski D, Kluger MJ, et al. Effect of nicotine on the immune system: possible regulation of immune responses by central and peripheral mechanisms. Psychoneuroendocrinology 1998;23: 189–204.

[27] Stopinski J, Staib I, Weissbach M. Do abuse of nicotine and alcohol have an effect on the incidence of postoperative bacterial infections? J Chir (Paris) 1993;130:422–5.

[28] Sugano N, Shimada K, Ito K, Murai S. Nicotine inhibits the production of inflammatory mediators in U937 cells through modulation of nuclear factor-kappaB activation. Biochem Biophys Res Commun 1998;252:25–8.

[29] Veillard NR, Steffens S, Burger F, Pelli G, Mach F. Differential expression patterns of proinflammatory and antiinflammatory mediators during atherogenesis in mice. Arterioscler Thromb Vasc Biol 2004;24(12):2339–44.

[30] Villablanca AC. Nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells in vitro. J Appl Physiol 1998;84:2089–98.

[31] Volzke H, Schwahn C, Wolff B, Mentel R, Robinson DM, Kleine V, et al. Hepatitis B and C virus infection and the risk of atherosclerosis in a general population. Atherosclerosis 2004;174:99–103.

[32] Weber KS, Draude G, Erl W, de Martin R, Weber C. Monocyte arrest and transmigration on inflamed endothelium in shear flow is inhibited by adenovirus-mediated gene transfer of IkappaB-alpha. Blood 1999;93:3685–93.

[33] Zhang S, Day I, Ye S. Nicotine induced changes in gene expression by human coronary artery endothelial cells. Atherosclerosis 2001;154:277–83.