The Influence of Nicotine on DMSO-induced Promyelocytic HL-60 Cells Expressing Nicotinic Acetylcholine Receptors and Associated Effector Function

by

Minqi Xu

A Thesis/Practicum submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirement of the degree of

MASTER OF SCIENCE

Department of Oral Biology

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ABSTRACT

Background: Neutrophils leave the bone marrow as terminally differentiated cells, yet little is known of the influence of tobacco smoke on neutrophil differentiation and subsequent effector function. Of the more than 4000 constituents of tobacco smoke, nicotine is primarily responsible for addiction and is the best studied.

Aims: To examine the influence of nicotine on HL-60 promyelocytes that were induced to differentiate into the neutrophilic lineage with 1.3 % (v/v) dimethylsulfoxide (DMSO).

Methods: HL-60 cells were differentiated into neutrophils by 1.3% (v/v) DMSO with and without nicotine (10^{-7} to 10^{-4} M). Expression of nicotinic acetylcholine receptors (nAChRs) was determined by western blot and immunofluorescence. Cell differentiation was evaluated over 5 days by measuring (a) the expression of the terminal myeloid differentiation marker, CD11b, (b) cell viability, (c) cell growth kinetics (cellular density), and by assessing (d) cellular morphology and ultrastructure, (e) growth phase and apoptosis, (f) conformational changes of major cellular components by Fourier-transform infrared spectroscopy, and (g) the ability to mount an oxidative burst. Effector functions of DMSO-differentiated HL-60 cells were also examined, i.e. phorbol 12-myristate 13-acetate (PMA)-triggered respiratory burst, and lipopolysaccharide (LPS)-induced matrix metalloproteinase (MMP) secretion.

Results: Both promyelocytic and neutrophil-differentiated HL-60 cells expressed the α7-acetylcholine nicotinic receptor subunit (55 kDa). Expression of the α7-acetylcholine

nicotinic receptor was up-regulated upon the addition of DMSO, irrespective of nicotine exposure. Nicotine increased apoptosis-associated cellular lipid content and DNA-protein ratios, but did not significantly elevate the percentage of DMSO-undifferentiated or -differentiated HL-60 cells committed to apoptosis as shown by propidium iodide staining in flow cytometry assays. Furthermore, nicotine exposure during differentiation suppressed the oxidative burst in HL-60 cells, inhibited bacterial killing, and increased the release of MMP-9, but not MMP-2, in an α7-acetylcholine nicotinic receptor-dependent manner. There were no statistically significant differences in any other markers of neutrophil differentiation between treated and control HL-60 cells (all $p > 0.05$).

Conclusions: Nicotine induces apoptosis-associated alterations to the biochemical profiles of α7-nicotinic acetylcholine receptor-expressing HL-60 cells. However, physiological relevant doses of nicotine do not affect gross markers of neutrophil differentiation or induce commitment to apoptosis in this cell model. Exposure to nicotine during cellular differentiation alters effector function in neutrophils which may partially explain the increase in susceptibility of tobacco smokers to bacterial infection.

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LIST OF ABBREVIATIONS

ATCC -- American Type Culture Collection

BaF_2 -- Barium fluoride

B lymphocyte -- Bone marrow lymphocyte

$CaCl_2$ -- Calcium chloride

cAMP -- Cyclic adenosine monophosphate

C-C -- Carbon-carbon bond

CD -- Clusters of differentiation

C-H -- Carbon-hydrogen bond

C-MYC-- Myelocytomatosis cell oncogene

C-O -- Carbon-oxygen bond

C=O -- Carbon-oxygen double bond

CO_2 -- Carbon dioxide

DNA -- Deoxyribonucleic acid

EDTA -- Ethylenediaminetetraacetic acid

ELISA -- Enzyme-linked immunosorbent assay

FITC -- Fluorescein isothiocyanate

HL-60 -- Human promyelocytic leukemia cell line

H_2O_2 -- Hydrogen peroxide

HRP -- Horseradish peroxidase

IFN -- Interferon

IgG -- Immunoglobulin G

IL -- Interleukine

kDa – Kilodalton

LPS -- Lipopolysaccharide

mRNA -- Messenger ribonucleic acid

NaCl -- Sodium chloride

NADPH oxidase -- Nicotinamide adenine dinucleotide phosphate-oxidase

N-H -- Nitrogen-hydrogen bond

·OH -- Hydroxide

O_2^- -- Superoxide anion

PBS -- Phosphate buffered saline

PC-12 -- Rat pheochromocytoma cell line

PE -- Phycoerythrin

PI-3-K -- Phosphatidylinositol-3-kinase

PKC -- Protein kinase C

P=O -- Phosphate bond

RNAase A -- Ribonuclease-A

RPMI media -- Roswell Park Memorial Institute media

RT -- Room temperature

SDS-PAGE -- Sodium dodecyl sulfate polyacrylamide gel electrophoresis

T lymphocyte -- Thymus lymphocyte

TNF -- Tumor necrosis factor

Tris -- Trishydroxymethylaminomethane

Tris-HCl -- Trishydroxymethylaminomethane hydrochloride

ν_{as}**CH$_2$** -- Asymmetric stretching vibration of CH_2 group

ν_{as}**PO$_2^-$** -- Asymmetric stretching vibration of PO_2^- group

ν_s**CH$_2$** -- Symmetric stretching vibration of CH_2 group

ν_s**PO$_2^-$** -- Symmetric stretching vibration of PO_2^- group

CHAPTER 1

INTRODUCTION -- PART ONE

Tobacco Use

Tobacco use is a highly pervasive, addictive social behavior and a major cause of morbidity and mortality (Scott and Thliveris, 2005). The World Health Organization recently estimated that there are 1.3 billion current cigarette smokers (http://www.who.int/tobacco). This is equivalent to one third of the world's entire population older than 15 years of age, with 650 million people expected to die directly from tobacco-associated diseases (http://www.who.int/tobacco). Health Canada estimates that more than 37,000 people will die this year in Canada due to smoking (http://www.hc-sc.gc.ca/hl-vs/tobac-tabac/fact-fait/fs-if/index_e.html). In addition to active smokers, more than 300 non-smokers will die of lung cancer and at least 700 non-smokers will die of coronary heart disease caused by exposure to second-hand smoke.

1.1 Tobacco-associated Diseases

Exposure to tobacco smoke induces and / or exacerbates a multitude of diseases related to almost every system in the human body.

Many studies have provided convincing evidence that tobacco users have a much

higher risk of developing tumors in multiple organs, including lung, mouth, nasopharynx, oesophagus, stomach, bladder, kidney and liver (Wang & Scott, 2005). Smokers are also more likely to develop various vascular disorders, such as stroke, heart attack, peripheral vascular disease. Additionally, smokers appear to have elevated risk for developing periodontitis, osteoporosis, diabetes mellitus, chronic obstructive pulmonary disease, rheumatoid arthritis, pancreatic disorders, peptic ulcers, psoriasis, erectile dysfunction, responding poorly to surgery, and having low birth weight or developmentally abnormal infants (Wang & Scott, 2005). Multiple tobacco-associated diseases or disorders are listed in Table 1.

1.2. Addiction to Tobacco

Tobacco use causes or exacerbates various diseases, often in a dose-dependent manner. Theoretically, such health threats could be avoided through tobacco cessation. However, the highly addictive properties of nicotine make cessation extremely difficult for many individuals, with only about 10% of long-term smokers who are desperate to quit smoking actually achieving the goal of permanently quitting smoking (Hajek *et al.*, 2005). Furthermore, ex-smokers who have successfully quit smoking for years are prone to restarting tobacco use when triggered by certain factors. More depressingly, there is lack of solid evidence to support any currently available therapies as being efficient in prohibiting such relapses (Hajek *et al.*, 2005).

Tobacco smoke is highly complex, containing more than 4000 different chemicals, including toxic free radicals, polycyclic aromatic hydrocarbons, tobacco specific nitrosamines, nicotine, bacterial endotoxin, and nitric oxide (Pryor and Stone, 1993; Cerami *et al.*, 1997 and Hasday *et al.*, 1999). Considering the plethora of toxic components in tobacco smoke, many of which are further metabolized by the body system, the exact mechanisms of tobacco-induced disease processes are varied and intricate. However, with respect to addiction, a single, major constituent of tobacco smoke – nicotine is primarily responsible (Benowitz, 1992a, 1992b and 1996; Graul and Prous, 2005).

Table 1: Tobacco-associated diseases

Smoking causes and / or exacerbates:		
• Addiction	• Cancer, stomach	• Infant death
• Aging	• Cancer, thyroid	• Inflammatory bowel disease
• Bone marrow damage	• Cancer, sinonasal	• Lung emphysema
• Cancer, bladder	• Cardiac infarction	• Lung function impairment
• Cancer, blood	• Chronic Bronchitis	• Metabolic disorders
• Cancer, brain	• Coronary heart disease	• Neurological, brain disorders
• Cancer, breast	• Cough & phlegm production	• Ocular disease
• Cancer, cervix	• Decreased physical fitness	• Osteoporosis
• Cancer, colorectal	• Dental disease	• Premature babies
• Cancer, esophagus	• DNA damage	• Preterm deliveries
• Cancer, head & neck	• Edema of vocal cords	• Psychiatric, behavioral disorders
• Cancer, kidney	• Endocrinological disorders	• Renal disease
• Cancer, lips	• Gastrointestinal disease	• Reproductive disorders
• Cancer, liver	• Gingival inflammation	• Sensory neural disorders
• Cancer, lung	• Hearing loss	• Stroke
• Cancer, oral cavity	• Hyperlipidemia	
• Cancer, ovary	• Hypertension	
• Cancer, pancreas		
• Cancer, pharynx		
• Cancer, prostate		

Smoking has been linked to:	
• Alcoholism	• Illicit drug use
• Allergy	• Infertility
• Arthritis	• Interaction with medicines
• Asthma	• Menstrual cycle disorders
• Craniosynostosis in the child	• Peptic ulcer
• Diabetes	• Periodontal bone loss
• Earlier onset of menopause	• Pneumococcal infection
• Ewing's sarcoma	• Psoriasis
• Fetal malformation	• Slow healing of bone fracture
• Folicular non-Hodgkin lymphoma	• Slow healing of wounds
• Hypertension in the child	• Suicide

The table is reproduced from the website of **International Society for the Prevention of Tobacco Induced Diseases (ISPTID)** http://isptid.globalink.org/smoking.html.

CHAPTER 2

INTRODUCTION – PART TWO

Nicotine & Nicotine Signaling

2.1. Chemistry and Pharmacology of Nicotine

Nicotine (3-(1-methyl-2-pyrrolidinyl) pyridine; $C_{10}H_{14}N_2$, *see* Figure 1), a major component of tobacco, is of considerable medical significance because of its toxic and addictive characteristics.

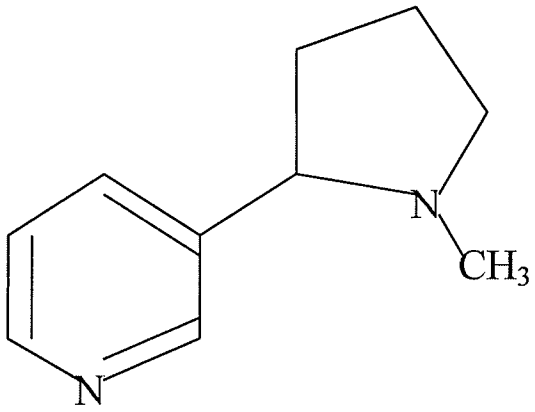

Figure 1: The sketch of nicotine ($C_{10}H_{14}N_2$) molecular structure

Nicotine composes 0.5% ~ 3.0% dry weight of tobacco plant. Each cigarette contains nicotine on average ranging from 10^{-3} to 10^{-2} g (Benowitz, 1996). Nicotine is one of the few natural liquid alkaloids. At room temperature and exposure to air, nicotine exists as brown, oily liquid which is highly miscible with water. Nicotine can easily penetrate through many biological barriers, such as the skin, lung, and blood-brain barriers. At a pH of 7.4, 31% of nicotine is non-ionized and can readily cross cell

membranes (Benowitz. 1996). Although most nicotine in tobacco is burned during the process of smoking, the amount of nicotine inhaled and absorbed by smokers is sufficiently high to induce multiple pathogenetic effects.

The mean lethal dose of nicotine is estimated to be 30 - 60 mg for adults and 10 mg for children (Okamoto *et al.*, 1994). Nicotine itself is not directly carcinogenic, although it may promote tumor cell development by inhibiting cell apoptosis (Wright *et al.*, 1993 and Landais *et al.*, 2005). However, tobacco-specific nitrosamines - nicotine metabolites are highly carcinogenic (Hoffmann *et al.*, 1996).

Nicotine enters the body through exposed surfaces, i.e. the oral cavity, nasal cavity, respiratory tract, and lung. Once absorbed into the bloodstream, nicotine is distributed rapidly and systemically within 8 minutes. It takes only 20 seconds for nicotine to cross the blood-brain barrier and reach the central nervous system (CNS) after tobacco smoke inhalation (Benowitz, 1996; Benowitz and Jacob, 1997). During the first 2 minutes of nicotine absorption, the arterial concentration is 6.0 to 10.0 fold of the venous counterpart (Benowitz, 1996; Benowitz and Jacob, 1997). Furthermore, nicotine is found in diverse biological fluids, such as saliva, tear, urine, and breast milk, and enters fetal circulation and the surrounding amniotic fluids (Scott and Thliveris, 2005). Nicotine distribution in the multiple human tissues and organs is summarized in Table 2.

Table 2: Nicotine distribution in the multiple tissues and organs of smokers

Sites	Nicotine Concentrations	References
Blood (Venous)	25-444nM, mean: 203nM	Russell *et al.*, 1980
Blood (Venous)	10-50ng/ml	Benowitz, 1988
Blood (Venous)	40ng/ml (Plateau Con. at day)	Benowitz & Jacob, 1993
Serum (Venous)	6-52ng/ml	Moyer *et al.*, 2002
Plasma (Venous)	6-498ng/ml (smokers & non-smokers)	Shin *et al.*, 2002
Serum (Venous)	Light smokers: 13.4±8.4μg/l Moderate smokers: 20.6±7.2μg/l Heavy smokers: 23.7±10.3μg/l	Lawson *et al.*, 1998
Urine (24h secretion)	Light smokers: 2.02±1.26mg/24h Moderate smokers: 2.14±1.28mg/24h Heavy smokers: 3.93±2.79mg/24h	Lawson *et al.*, 1998
Urine	Nonsmokers: 46.2nM (mean) Passive smokers: 133.2nM (mean) Non inhaling smokers: 2455.6nM (mean) Slight inhaling smokers: 7786.2nM (mean) Moderate inhaling smokers: 8329.2nM (mean) Deep inhaling smokers: 9426.6nM (mean)	Feyerabend *et al.*, 1982
Urine	100-6780ng/ml	Moyer *et al.*, 2002
Urine	0-1590ng/ml (smokers & non-smokers)	Shin *et al.*, 2002
Saliva	Nonsmokers: 36.4nM (mean) Passive smokers: 62.3nM (mean) Non inhaling smokers: 939nM (mean) Slight inhaling smokers: 2600.5nM (mean) Moderate inhaling smokers: 2800.2nM (mean) Deep inhaling smokers: 5589.4nM (mean)	Feyerabend *et al.*, 1982
Saliva	0-207ng/ml (smoker & non-smoker)	Shin *et al.*, 2002
Breast milk	Light smokers: 18±16ng/ml Moderate smokers: 28±21ng/ml Heavy smokers: 48±25ng/ml	Luck and Nau, 1984 and 1987
Placentas	3.3-28ng/g	Luck *et al.*, 1985
Amniotic fluid	1.5-23ng/ml	Luck *et al.*, 1985
Fetal serum	0.5-25ng/ml	Luck *et al.*, 1985

* Nicotine: 1M ≈ 164g/l

Significant concentrations of nicotine also exist in the other organs and tissues of smokers, such as liver, brain, kidney, spleen, lung, adipose, skeletal muscle and hair. Interestingly, nicotine concentrations are higher in specific tissues than in blood. (Janoff *et al.*, 1987; Urakawa *et al.* 1994; Al-Delaimy, 2002; Torano and van Kan, 2003)

Nicotine is predominantly metabolized in the liver by cytochrome P450 isoenzymes, aldehyde oxidase, flavin monooxygenase and glycosylation, and has a short elimination half time of approximately 2 hours with 10% ~ 15% of nicotine secreted into urine (Moyer *et al.* 2002). 70% ~ 80% of circulating nicotine is metabolized to cotinine, which has a substantially longer half life of 20 ~ 40 hours and is mainly eliminated by the kidney. Compromised liver and/or kidney function, not infrequent in heavy smokers, can result in reduced nicotine clearance (Moyer *et al.* 2002). Indeed, the clearance of nicotine, when normalized for body weight, is significantly slower in smokers than in nonsmokers (Benowitz and Jacob, 1993).

Multiple types of nicotine receptors, and particularly the nicotinic acetylcholine receptors (nAChRs), are widely distributed. Acute and chronic stimulation of different nicotine receptors, and different doses of nicotine, may induce differential effects. Accordingly, the pharmacological actions of nicotine on the body are complex and often unpredictable (Hardman and Limbird, 2001). Additionally, it is now known that neutrophils express multiple functional nAChRs (Benhammou *et al.*, 2000; Villiger *et al.*, 2002; Cormier *et al.*, 2004 and De Rosa *et al.*, 2005).

2.2. Nicotinic Receptors & Signaling

2.2.1. Nicotinic Acetylcholine Receptors

The best studied nicotine receptors are the nAChRs. The nAChRs are a family of

ligand-gated, pentameric ion channels whose endogenous ligand is acetylcholine (Hardman & Limbird, 2001). These receptors, widely expressed in the nervous system and in the skeletal muscles, traditionally function as transmembrane cationic channels that mediate and modulate ligand-induced signal transmission on the basis of changing the cell membrane's permeability to Na^+, Ca^{2+} and K^+ (Hardman & Limbird, 2001). Increasing evidence now demonstrates that nAChRs are also expressed in the other types of cells, such as leukocytes (Benhammou *et al.*, 2000; Villiger *et al.*, 2002; Wang *et al.*, 2003; Cormier *et al.*, 2004; Skok *et al.*, 2005 and 2006; Yang *et al.*, 2005), epithelial and endothelial cells (Grando, 1997; Mihovilovic *et al.*, 1997 and Maus *et al.*, 1998) and may signal through ion channel-independent mechanisms. nAChRs are composed of a combination of five homo- or heterogenous subunits (Albuquerque *et al.*, 1997). Five major types of nAChR subunits are known (α, β, δ, ε & γ) along with multiple subtypes. The α subunit (other than the α5-subunit) is necessary for functional ligand binding (Dougherty and Lester, 2001). The typical structure of heterogenous nAChRs is a clockwise arrangement of α-γ-α-β-δ or α-γ-α-β-ε, providing two ligand binding sites, as illustrated in Figure 2. Of the homo-pentameric receptors, only the α subunits are functional (Unwin, 1998 & 2003). The α and β subunits can be divided into different subtypes which are distinguished by Arabic numbers (Hogg *et al.*, 2004 and Jensen *et al.*, 2005). At least 14 distinct nAChR α and β subunits (α1 ~ α10, β1 ~ β4) have been cloned (Peng *et al.*, 2004). All subunits are comprised of four transmembrane spanning domains, named as M1-M4, of which the five M2 domains from each individual subunit form the

ion channel (Bertrand *et al.*, 1993). In addition to nicotine and acetylcholine, there are other modulatory binding sites on nAChRs, i.e. for specific steroids, calcium, and acetylcholinesterase inhibitors (Pereira *et al.*, 2002). Thus, members of the nAChR family are functionally diverse.

2.2.2. Alpha-7 nAChRs

Of the multitudinous nAChR superfamily, the α7 subunit demonstrates several unique characteristics (Villiger *et al.*, 2002), including (1) the formation of functional homopentamers (Gopalakrishnan *et al.*, 1995; Chen and Patrick, 1997), (2) a higher membrane permeability of Ca^{2+} over Na^{+} (Bertrand *et al.*, 1993a and 1993b), (3) a wide distribution in non-neuronal and non-muscle cells, such as leukocytes and lung fibroblasts (Lindstrom, 1997; Villiger *et al.*, 2002; Wang *et al.*, 2003; Skok *et al.*, 2004 and 2005; Scott and Thliveris, 2005 and De Rosa *et al.*, 2005), (4) functionality in neuronal and non-neuronal cells (McGehee *et al.*, 1995; Gray *et al.*, 1996; Zhang *et al.*, 1996; Ullian *et al.*, 1997; Wang *et al.*, 2003 and Rosa *et al.*, 2005), (5) and a partly duplicated human α7 gene on the same chromosome, which expresses gene product (Gault *et al.*, 1998 and Villiger *et al.*, 2002).

Growing evidence shows that leukocyte α7 pentamers are important in regulating multiple cellular functions, including proliferation, apoptosis, cytokine release, and other aspects of the immune- and inflammatory responses (Wang *et al.*, 2003; Rosa *et al.*, 2005;

Skok *et al.*, 2005, Agenes and Changeux, 2006).

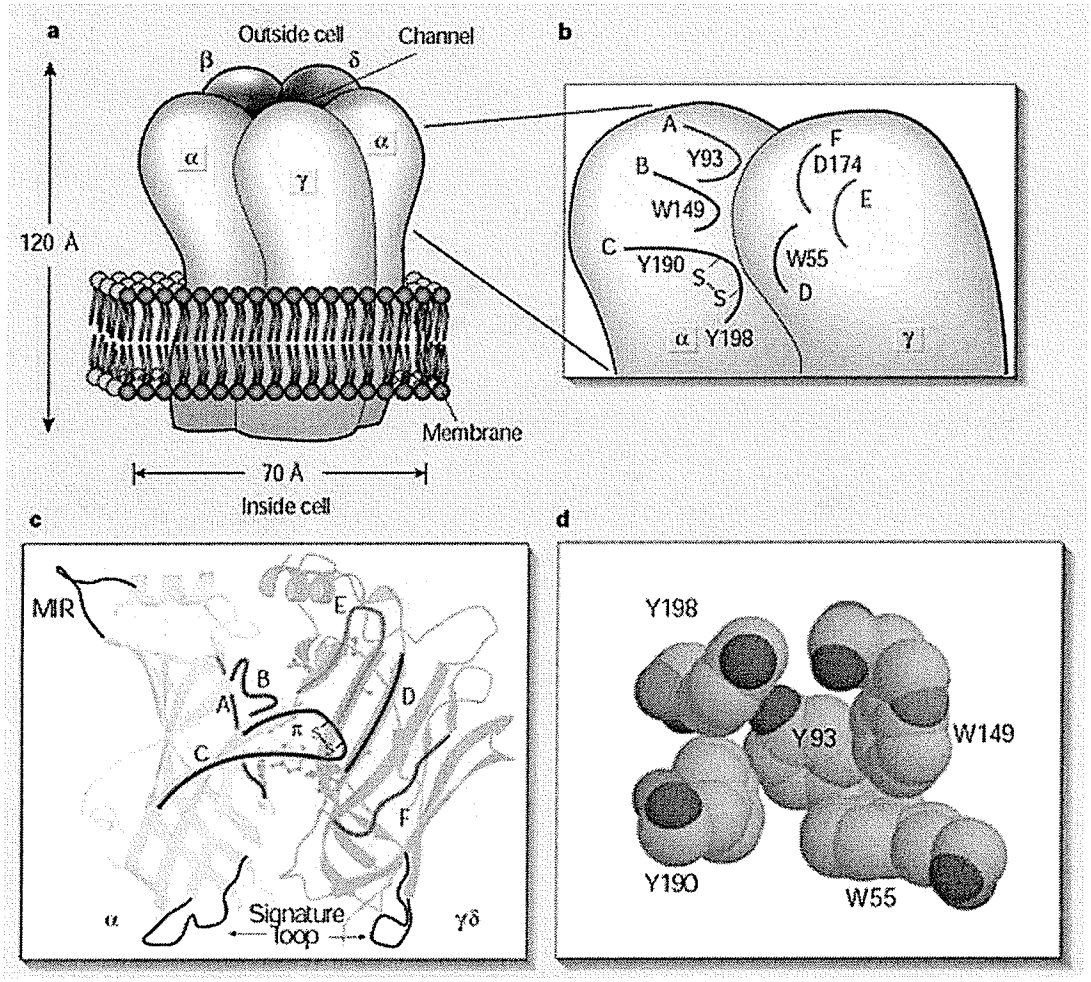

Figure 2: The structure and ligand-binding sites of nAChRs:

(a) The overall layout of the receptor, based on cryoelectron microscopy studies by Unwin and colleagues, showing the α, β, γ and δ subunits. This is the receptor found in postsynaptic muscle cells; some neuronal receptors consist of just one type of subunit. **(b)** Biochemical results mapped onto the structure shown in a. The agonist-binding site consists of the interface between the α and γ subunits (or the other α and the subunit δ; not shown). One key disulphide bond is shown (labelled S–S), along with the six loops (A–F) that contribute to the agonist-binding site, and the key aromatic residues (Y, tyrosine; W, tryptophan). D174, aspartate residue 174. **(c)** The agonist-binding site is denoted by π, emphasizing the importance of aromatic residues in general and tryptophan 149 in particular. Also highlighted are the signature loops of both subunits, and the main immunogenic region (MIR). **(d)** The open box, formed by five aromatic residues, that probably defines much of the agonist-binding site. The view is derived from c by a 90° rotation clockwise around the vertical axis. **(Reprinted by permission from Macmillan Publishers Ltd: *Nature* 411, 252-255 (17 May 2001) | doi:10.1038/35077192, Neurobiology: Snails, synapses and smokers, Dennis A. Dougherty1 and Henry A. Lester)**

2.2.3. Nicotine-mediated Signaling

Specific neuronal signaling pathways activated on nAChR engagement are presented in Figure 3 (Dajas-Bailador and Wonnacott, 2004). Na^+ influx following nAChR activation in postsynaptic muscle cells results in a localized cell membrane depolarization leading to muscle contraction. Recent studies have provided initial insight into the nicotine-induced signaling events in non-neuronal cells, such as lung cancer cells (Heusch and Maneckjee, 1998, Konu *et al.*, 2004; Mai *et al.*, 2003 and Chen and Shields, 2005), and leukocytes (Kawashima and Fujii, 2000). Signaling pathways similar to the neuronal environment may be widespread, i.e. the induction of a dramatic increase in intracellular Ca^{2+}, which functions as a secondary messenger to evoke the subsequent activation of PKC, PI-3-K and cAMP, triggering the downstream activation of mitogen-activated protein (MAP) kinase cascade, which finally induces the activation of transcription factors, such as the cAMP response element binding protein (CREB) (Tang *et al.*, 2004), refer to Figure 3.

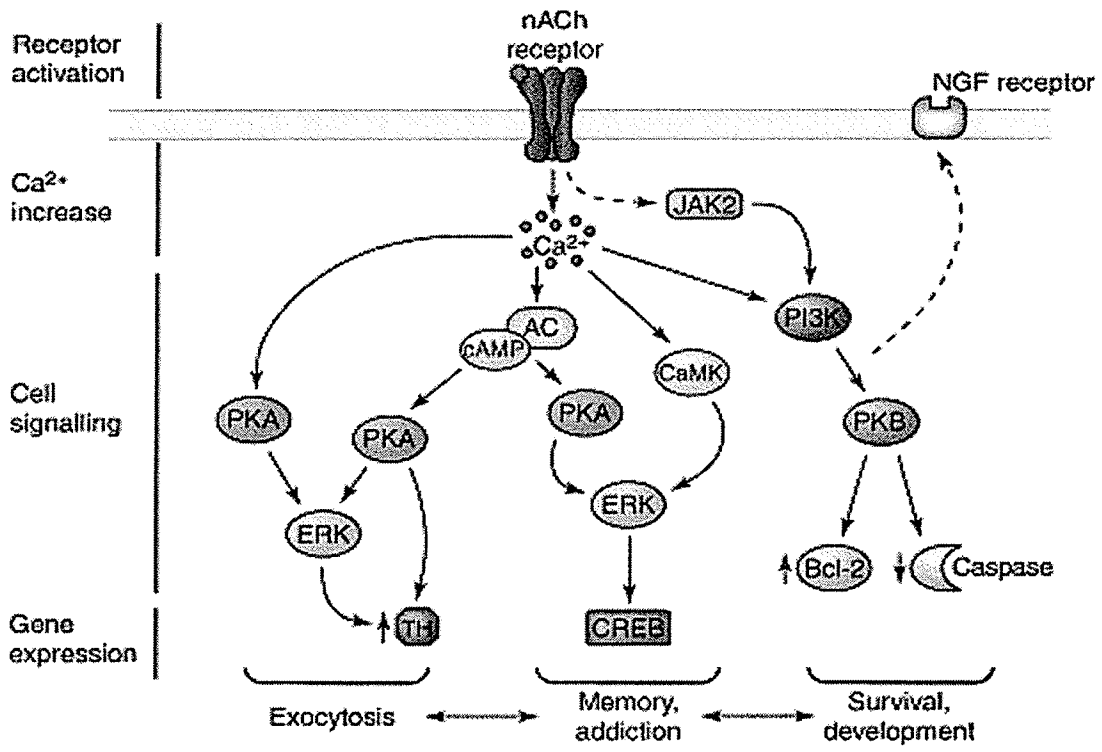

Figure 3: nAchRs signaling

Key signaling molecules in Ca^{2+}-dependent, nicotinic acetylcholine (nACh) receptor-mediated neuronal processes (for clarity, some intermediate steps are not shown). The capacity of nACh receptors to regulate diverse neuronal functions depends on the activation of specific signaling cascades. Thus, the increase in intracellular Ca^{2+} that arises from nACh receptor activation can activate adenylyl cyclase (AC), protein kinase A (PKA), PKC, Ca^{2+}-calmodulin-dependent protein kinase (CaMK) and phosphatidylinositol 3-kinase (PI3K). In turn, these phosphorylate downstream targets, such as extracellular signal-regulated mitogen-activated protein kinase (ERK), which leads to the activation of transcription factors such as the cAMP response element-binding protein (CREB) and increases in expression of genes that encode, for example, tyrosine hydroxylase (TH) and nerve growth factor (NGF) receptors. The lipid signaling cascade that is initiated by PI3K, through phosphorylation of PKB (Akt), is credited with modulating the relative activities of neuroprotective and apoptotic factors, such as Bcl-2 and caspases, respectively (Shaw *et al.*, 2002; Kihara *et al.*, 2001). In addition, Ca^{2+}-dependent phosphatases are also likely to be recruited (Stevens *et al.*, 2003), but little is known about nACh receptor-regulated phosphatase activity. Thus, nACh receptors can exert a wide range of influences through Ca^{2+} signals, from changes in synaptic plasticity, which is pertinent to many situations including cognition, memory and addiction, to the life-and-death events involved in development and neuroprotection. Abbreviation: JAK, janus-activated kinase. **(Reprinted from *Trends in Pharmacological Sciences*, 25, Federico Dajas-Bailador and Susan Wonnacott, NAChRs and the regulation of neuronal signalling, 317-324, Copyright (2004), with permission from Elsevier.)**

2.3. Roles of Nicotine in Tobacco-related Diseases

Nicotine exposure promotes addiction to smoking, causing smokers to subject themselves to frequent and high doses of tobacco smoke over many years.

Of the metabolites of nicotine, tobacco-specific nitrosamines, are highly carcinogenic (Hecht and Hoffmann, 1989). Furthermore, Wright and coworkers (1993) reported that nicotine inhibits diverse stimuli-induced apoptosis of multiple tumor cell lines, including tumor cell types related to tobacco use. Heusch and Menechjee (1998) indicated that nicotine activation of MAP kinase and PKC signaling pathway has been involved in inhibition of the apoptosis of human lung cancer cells. Landais *et al.* (2005) found that nicotine induces a series of phenotype changes correlated to tumor promotion in HL-60 human leukemia cell line, such as chromatin decondensation, histone H3 acetylation, and up-regulation of the c-Jun transcription factor mRNA. Taken together, nicotine exposure may play an indirect role in stimulating the development of tobacco-related tumors.

Nicotine exposure may decrease the risk of developing ulcerative colitis, Parkinson's and Alzheimer's disease in smokers has been widely postulated (Lindstrom, 1997; Green *et al.*, 2000; Fratiglioni and Wang, 2000; Scott and Martin, 2006), although the exact mechanisms are poorly understood. However, nicotine exposure is more usually associated with increased disease risk through multiple mechanisms, for example,

dysfunction of the neutrophil oxidative burst (Dunn *et al.*, 2005), facilitation of atherosclerosis (Di Luozzo *et al.*, 2005), dysregulation of B lymphocyte development (Skok *et al.*, 2005 and 2006), and negatively impacting the generation of pulmonary surfactant (Scott and Thliveris, 2005).

Studies on the influence of nicotine on the human body may provide some essential clues to better understand the intricate mechanisms of tobacco-related diseases. However, one must appreciate that tobacco smoke is highly complex and comprises thousands of different components, just one of which is nicotine.

CHAPTER 3

INTRODUCTION -- PART THREE

Neutrophils

3.1. Physiology of Neutrophils

Neutrophils are the most abundant leukocytes in blood, accounting for 50~70% of the total (Ross and Pawlina, 2006). These innate immune cells are central to the inflammatory response and fulfill both anti-infectious and pro-inflammatory functions (Witko-Sarsat *et al.*, 2000).

The primary physiological functions of neutrophils include: (1) vascular cell adherence, extravasation, and subsequent migration along cytokine and other chemoattractant gradients that are produced or released in response to tissue injury or infection, mediated by a series of signal integration of ligand and receptor binding, such as the interaction of β2 superfamily (CD11a, CD11b, CD11c/CD18) with intracellular adhesion molecule - 1 (ICAM-1); (2) phagocytosis of microbes, with opsonization abetted by Fcγ and complement receptors; (3) killing of engulfed microbes, primarily through the production of radical oxygen species (ROS), including O_2^-, H_2O_2 and $^{\cdot}OH$; (4) degranulation of pre-formed antimicrobial enzymes, for example, bactericidal/permeability increasing protein (BPI); (5) synthesis and release of diverse immune modulating cytokines, including the pro-inflammatory mediators TNF-α, IL-1,

IL-12, and particularly IL-8 (Witko-Sarsat *et al.*, 2000).

3.2. Development and Kinetics of Granulopoiesis

Neutrophils originate from the multipotent colony-forming unit-granulocyte, erythrocyte, and monocyte (CFU-GEMM) stem cells, which differentiate into colony forming unit-granulocyte and monocyte (CFU-GM) by the stimulation of cytokines such as granulocyte-monocyte colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and IL-3. In the process of maturation, the neutrophil undergoes six morphologically identifiable stages, known as myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and segmented neutrophil. The myeloblast is the earliest recognizable neutrophil precursor cell in the bone marrow (Ross and Pawlina, 2006).

The mitotic phase in granulopoiesis, referred to the differentiating stage in cells earlier than metamyelocytes, lasts about one week. The postmitotic phase, i.e. from metamyelocyte to segmented neutrophil, lasts another one week. The lifespan of mature neutrophils released to systemic circulation is short. It takes only 6 ~ 8 hours for 50% of the circulating segmented neutrophils to randomly leave the peripheral blood system and enter surrounding tissues or lumens. In connective tissue, neutrophils live for another 1 ~ 2 days before they die by apoptosis, if unactivated, and are subsequently cleared by macrophages (Ross and Pawlina, 2006).

CHAPTER 4

INTRODUCTION -- PART FOUR

Nicotine & Neutrophil Physiology and Pathology

4.1. Overview

Some common tobacco-induced and/or exacerbated diseases, including COPD (Shapiro *et al.* 2003; Churg and Wright *et al.*, 2005 and Drost *et al.*, 2005), atherosclerosis and other vascular diseases (Blann, 1997; Dunn *et al.*, 2005 and Kougias *et al.*, 2005), asthma (Cundall *et al.*, 2003 and Wenzel, 2003), and periodontitis (Deas *et al.*, 2003; Scott and Singer, 2004; Palmer and Scott *et al.*, 2005), have been associated with neutrophil influx, cytokine release, and degranulation events. Also tobacco smoke can affect the diverse important aspects of neutrophil function, including neutrophil chemotaxis and recruitment (Seow *et al.*, 1994; Sorensen *et al.*, 2004 and van der Vaart *et al.* 2005), oxidative burst (Dunn *et al.*, 2005), bacterial killing and clearance (Pabst *et al.*, 1995 and Drannik *et al.*, 2004), apoptosis (Finkelstein *et al.*, 2001), and the clearance of apoptotic neutrophils by macrophages (Kirkham *et al.*, 2004). Furthermore, smoking causes a generalized neutrophilia by an unknown mechanism (Pitzer *et al.*, 1996 and Terashima *et al.*, 1999).

Neutrophils leave the bone marrow as terminally differentiated, transcriptionally quiescent, and functionally mature cells. Neutrophils express multiple functional nAChRs

(Villiger *et al.*, 2002 and Cormier *et al.*, 2004), yet the effect of nicotine, a major component of cigarette smoke, on neutrophil differentiation is still unknown, while the available data on how nicotine influences effector function in fully differentiated granulocytes are scarce. Potential mechanisms by which nicotine could act on neutrophils, directly or indirectly, hence influencing disease processes in smokers, are illustrated in Figure 4.

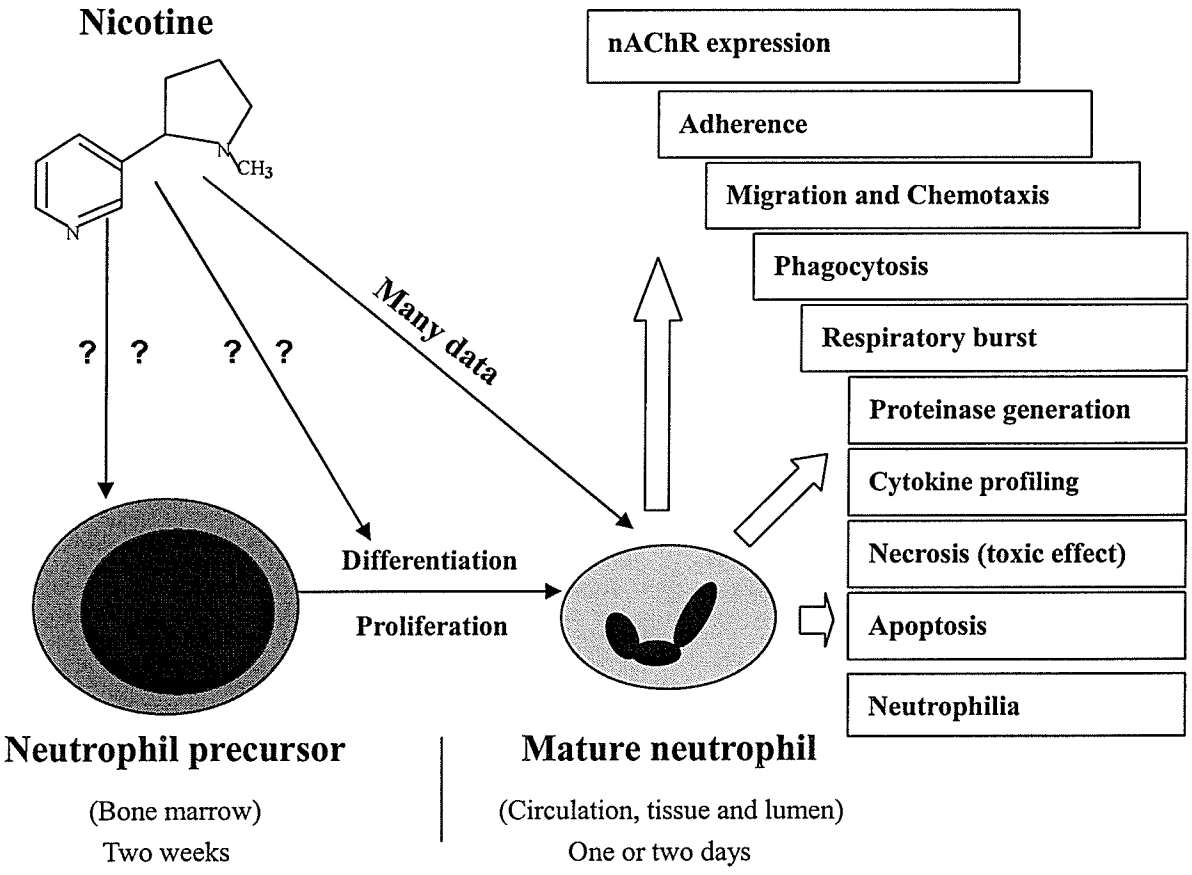

Figure 4: The potential effects of nicotine on neutrophil functions. Nicotine may have an impact on neutrophil function by interfering with normal neutrophil differentiation or by adversely affecting effector function in mature neutrophils. The effects of nicotine on neutrophil differentiation remains, essentially, unknown and thus represents the focus of this research project.

4.2. Nicotine & Profiles of Mature, Circulating Neutrophils

4.2.1. Expression of nAChRs by neutrophils

Nicotinic acetylcholine receptors are traditionally considered to be found on nerve and muscle cells. However, over the last ten years it has become increasingly clear that the expression of nAChRs by other cell types is more common than previously appreciated, although the physiologic role of those non-neuron and non-muscle cell derived nAChRs remains largely unknown (Lebargy *et al.*, 1996; Mihovilovic *et al.*, 1997; Benhammou *et al.*, 1998; Sato *et al.*, 1999; Villiger *et al.*, 2002 and Cormier *et al.*, 2004).

Human neutrophils express functional nAChRs. Benhammou *et al.* (2000) reported that polymorphonuclear leucocytes (PMNs) expressed both α4β2 and α3β4 nicotinic receptor subunits at the mRNA and protein levels, and [^{3}H] nicotine binding was increased in PMNs isolated from smokers and correlates with tobacco consumption. In 2002, Villiger's research group detected the expression of the α7 subtype in human PMNs at both the mRNA and protein levels (Villiger *et al.*, 2002). Also, multiple specific nAChR-agonists and -antagonists have been used to show that nAChRs control the specific inflammatory functions of neutrophils (Iho *et al.* 2003). More recently, Cormier *et al.* found that human and mouse PMN membrane both expressed heteromeric nAChRs and homomeric α7 nAChRs. The same authors also pointed out that a three-day exposure to nicotine upregulated the expression of the α4 and/or β2 nicotinic receptor subunits in human and mouse neutrophils, but not the α7 subtype (Cormier *et al.*, 2004).

4.2.2. Neutrophil adherence, chemotaxis, migration and phagocytosis

The migration of neutrophils is mainly determined by three major processes: cell adherence, vascular transmigration, and chemotaxis. An early study suggested that nicotine ($5 \times 10^{-6} \sim 5 \times 10^{-4}$ M) has no effect on human PMN migration and chemotaxis towards n-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sasagawa *et al.*, 1984). In contrast, Gillespie *et al.* have reported that acute or subchronic exposure to 0.02mg/kg nicotine by i.p. augmented fMLP-induced rat PMN chemotaxis, but that higher nicotine doses (0.2 mg/kg) might inhibit chemotaxis (Gillespie *et al.*, 1987). Nowak and co-workers subsequently observed that 10^{-6} M nicotine increased the PMN chemotactic response to zymosan-activated serum (ZAS) and fMLP, whereas the concentrations above 10^{-4} M had an adverse influence on PMN chemotaxis. The same authors also reported that nicotine enhanced PMN influx to inflammatory regions in the murine pleural cavity (Nowak *et al.*, 1990). Later, Ryder examined the effect of nicotine on neutrophil motility *in vitro*. Nicotine ($10^{-2} \sim 10^{-3}$ M) significantly increased two essential events in neutrophil motility, i.e. F-actin formation and intracellular calcium release, while neutrophil phagocytosis was somewhat suppressed by nicotine (Ryder, 1994). Impaired neutrophil phagocytosis was also observed by Seow *et al.*, who treated neutrophils *in vitro* with nicotine at the concentrations achievable in the lung and oral tissues after cigarette smoking. Seow *et al.* (1994) also found that nicotine suppressed neutrophil chemotaxis. Razani-Boroujerdi *et al.* (2004) recently investigated the chronic influence of nicotine, released by a miniosmotic pump, on leukocyte migration in rats and mice. Nicotine was

found to attenuate the migration of neutrophils and lymphocytes to the site of inflammation, perhaps explained by a suppression of calcium-mediated chemotactic signaling. In another study, nicotine (10^{-7} M) was shown to promote rabbit myocardial neutrophil infiltration (Owasoyo *et al* 1998). Thus, the available data on the influence of nicotine on neutrophil chemotaxis is highly conflicted.

The expression and activation of a series of adhesion molecules is essential for neutrophil adherence and subsequent migration. Higher serum concentrations of specific soluble adhesion molecules involved in neutrophil transmigration and chemotaxis have been reported in smokers (Scott *et al.*, 2000a; Palmer & Scott *et al.*, 2000 and 2002). In 2000, Scott *et al.* examined the acute effects of cigarette smoking on key serum and cellular adhesion molecules in heavy, light and non-smokers. sICAM-1, a critical adhesion molecule in neutrophil tethering to the vascular endothelium during inflammation, was increased in the serum of smokers in a dose-dependent manner that was rapidly reversed on smoking cessation (Scott *et al.* 2000a, 2000b and 2000c, Fraser and Scott *et al*, 2001). No significant difference in the surface expression of ICAM-1, CD18, platelet/endothelial cell adhesion molecule-1 (PECAM-1) or L-selection on peripheral neutrophils was observed over a one-hour period after smoking, suggesting that the elevated concentration of sICAM-1 in smokers is not due to an immediate effect of smoking (Scott *et al.*, 2000a). Earlier, Ryder *et al.* (1998) found that the expressions of L-selectin and CD18 in neutrophils were not affected by exposure to $10^{-7} \sim 10^{-2}$ M

nicotine *in vitro*. Recently, Speer *et al.* (2002) have demonstrated the dose-dependent inhibition of the integrin (CD62L, CD11a, and CD11b) expression by nicotine in neutrophils, *in vitro*. However, these results remain controversial (Scott and Palmer, 2002) and the available data on the influence of nicotine on neutrophil adhesion molecule expression are conflicted.

4.2.3. Neutrophil respiratory burst

While neutrophils can kill microbes by non-oxygen-dependent mechanisms, the most efficient antimicrobial activity of neutrophils is the generation of multiple radical oxygen species (ROS) and reactive nitrogen species (RNS), known as the respiratory burst. Indeed, respiratory burst deficiencies can have serious consequences, as exemplified by the recurrent and often life-threatening infections noted in patients with chronic granulomatous disease (CGD), whose phagocytes fail to mount a respiratory burst due to a defect in the NADPH oxidase gene (Gallin *et al.*, 1991; Segal and Abo, 1993).

Several studies have indicated that nicotine administration may have an inhibitory effect on the respiratory burst (Sasagawa *et al.*, 1985; Srivastava *et al.*, 1989; Nowak *et al.*, 1990; Pabst *et al.*, 1995 and Maity *et al.*, 2003). Sasagama *et al.* (1985) observed the suppression of the production of superoxide anions by neutrophils on fMLP or cytochalasin B-stimulation following nicotine treatment. This inhibition was not affected by atropine, hexamethonium, or acetyl-beta-methylcholine, suggesting a direct negative

effect of nicotine on neutrophils. Srivastava *et al.* (1989) have also examined the effect of nicotine on the synthesis of oxygen free radicals by neutrophils. Nicotine (10^{-5} M) blocked oxygen free radical production by over 90%. Similarly, Pabst *et al.* (1995) found the impaired production of superoxide anions and hydrogen peroxide in fMLP- or lipopolysaccharide (LPS)-stimulated neutrophils exposed to 0.01 ~ 0.1% nicotine, *in vitro*. They speculated that the compromised ROS production results from the interruption of neutrophil oxygen uptake as well as the direct superoxide absorption by nicotine. In addition, nicotine-enhanced neutrophil oxidative damage to gastrointestinal mucosa has been summarized by Maity and coauthors (2003).

On the other hand, Jay *et al.* (1986) reported that nicotine exposure resulted in a dose-dependent potentiation of the superoxide release by neutrophils responding to fMLP or PMA stimulation. These results are supported by Gillespie *et al.* (1987), whose data revealed an increased superoxide anion generation by the neutrophils of rats receiving nicotine administration. Recently, Maity *et al.* (2003) found that nicotine potentiated the generation of reactive oxygen intermediates by PMNs gathering in gastrointestinal wall in a cAMP-protein kinase A-dependent manner, and hypothesized that this exacerbates oxidative mucosal damage and promotes the formation of gastroduodenal ulcers.

The results of Seow *et al.* (1994), who found that nicotine had no significant impact on the superoxide production of neutrophils, confuse the overall picture further.

Thus, the true effects of nicotine on the neutrophil respiratory burst remain uncertain. However, it should be remembered that the generation of reactive oxygen and reactive nitrogen species by neutrophils is dependent on multiple factors, including cell activation state, the characteristics of inducers (selectively acting on NADPH oxidase, mitochondrial electron transport chain, xanthine-xanthine oxidase or cytochromes P-450, etc.), oxygen concentrations in the surrounding environment (aerobic or anaerobic), and the media used (with or without cations and/or glucose). Therefore, it is perhaps not astonishing to have such diverse findings. Indeed, this diversity demonstrates that nicotine may have a profound and extensive influence on neutrophil respiratory burst and that this influence varies depending on the specific neutrophil environment.

4.2.4. Neutrophil-derived proteases

The many proteases secreted by inflammatory leukocytes, principally serine-proteases and matrix metalloproteinases (MMPs), cause the degradation of extracellular proteins. Tissue remodeling is a normal part of the inflammatory process but, when unbalanced, it contributes to the development of many diseases, including COPD (Churg and Wright, 2005; Elkington and Friedland, 2006), periodontitis (Palmer and Scott *et al.*, 2005), vascular diseases (Murphy *et al.*, 1998) and even back pain causing by intervertebral disc degeneration (Fogelholm and Alho, 2000). The importance of neutrophil recruitment and subsequent release of proteolytic enzymes, particularly elastase and specific MMPs in tobacco-induced destruction of pulmonary vessels and tissues has been established

(Wright *et al.*, 2003 and 2004; Churg *et al.*, 2004 and Palmer *et al.*, 2005). Tobacco smoking may enhance the proteolytic activity of neutrophils (Rennard *et al.* 1990; Donaldson *et al.*, 1991; Sewo *et al.*, 1994 and Murphy *et al.*, 1998). For example, Sewo *et al.* (1994) reported that physiological doses of nicotine induced the release of elastase and inflammatory mediators from neutrophils. Murphy *et al* (1998) looked at the effects of nicotine on the development of aortic aneurysms and suggested that nicotine-induced neutrophil elastase activity plays an active role in the pathogenesis of tobacco-associated vascular diseases.

Recently, nicotine has also been shown to modify the synthesis and release of specific MMPs by rat macrophages (Valenca *et al.*, 2004), human osteoblastic saos-2 cells (Katono *et al.*, 2006), and mouse choroidal vascular smooth muscle cells (Suner *et al.*, 2004). The neutrophil is the primary source of MMP-8 in periodontal tissues and gingival crevicular fluid (GCF) (Liu and Scott *et al.*, 2006). MMP-8 levels in GCF remain unchanged following the surgical treatment of periodontitis in smoking patients, but decrease in non-smoking peers (Persson *et al.*, 2003). More recently, March *et al.* (2006) found that cigarette smoke exposure *in vivo* dramatically elevated the numbers of neutrophils, macrophages, lymphocytes, and the activity of MMP-2 and MMP-9 in the bronchoalveolar lavage fluid (BALF) collected from a murine pulmonary emphysema model. The data are consistent with an earlier study by An *et al.* (2003). To the best of our knowledge, the mechanisms by which tobacco and specific tobacco components, such as

nicotine, influence neutrophil MMP release remain unreported.

In conclusion, tobacco-related inflammatory damage to cells and tissues is caused at least partially through protease synthesis and release by neutrophils.

4.2.5. Neutrophil-derived cytokines

The human neutrophil is not only a target of multiple cytokines, such as, IL-1b, TNF-α, IL-6 and IL-8 but also a source of cytokines, chemokines, and growth factors, including TNF-α, IL-1, IL-8, IL-12, and IFN-α, β (Cassatella *et al.*, 1999 and Witko-Sarsat *et al.*, 2000).

Tobacco smoke induces lung fibroblasts and bronchial epithelial cells to release IL-8, a prototypic chemokine which is a potent chemoattractant and activator of neutrophils (Mio *et al.*, 1997; Sato *et al.*, 1999). Furthermore, nicotine-induced endogenous IL-8 production in neutrophils is associated with nAChR-mediated peroxynitrite generation and subsequent NF-kB activation (Iho *et al.*, 2003). Currently, Scott's group is examining the influence of cigarette smoke extraction (CSE) on cytokine release by human neutrophils. Preliminary data shows that CSE significantly enhances IL-8 release by neutrophils with or without LPS co-stimulation. Other cytokines are unaffected (personal communication).

4.2.6. Apoptosis and necrosis of neutrophils

Blockade of apoptosis may result in tumor development (Wright *et al.*, 1993 and Landais *et al.*, 2005). Additionally, apoptosis is critical in maintaining an inflammatory balance. In order to enhance the host defense against microbial infection, activated neutrophils survive longer than naïve neutrophils through the inhibition of apoptosis mediated by some inflammatory mediators and neutrophil activators, such as GM-CSF, G-CSF, and LPS (Haslett, 1997; Condliffe *et al.*, 1998 and Murray *et al.*, 2003). Once the microbial challenge is eliminated, the resolution of inflammatory process predominantly depends on the rapid apoptosis of neutrophils and other inflammatory cells. Therefore, regulation of neutrophil apoptosis is closely associated with inflammatory control.

Aoshiba *et al.* (1996) found that nicotine prolonged human neutrophil survival *in vitro* in a dose-dependent manner, with the survival rate of neutrophils exposed to 10^{-6} M nicotine for 72 hours approximately two fold that of the control cells. The authors suggested that nicotine suppresses apoptosis by a noncholinergic nicotine receptor-mediated mechanism.

Mariggio *et al.* (2001) have presented contrary results. Their data showed that the apoptotic effects of PMNs isolated from adult healthy volunteer were enhanced by 0.3% nicotine, using two standard methods for apoptosis detection (DNA fragmentation ladder by gel electrophoresis and the expression of apoptosis-signaling receptor Fas/Apo-1). It

should be noted that Mariggio *et al.* used very high nicotine doses (approximately 1.8 x 10^{-2} M) compared to the *in vivo* situation and to the experiments of Aoshiba *et al.* (10^{-9} to 10^{-6} M).

In contrast to apoptosis, necrosis is a more passive, disorganized form of cell death. Cell necrosis typically is associated with the early loss of plasma membrane integrity, resulting in the release of pro-inflammatory factors from damaged cells, which further leads to the potential inflammatory damage to surrounding tissues (Janeway *et al.*, 2005). Sasagawa *et al.* (1984) reported that nicotine, at concentrations of 10^{-5} M to 10^{-4} M, did not affect the neutrophil viability, later confirmed by Pabst *et al.* (1999).

4.3. Nicotine & Neutrophil Development

4.3.1. Neutrophilia

Neutrophilia (neutrophil leukocytosis) is defined as circulatory neutrophil concentrations higher than the normal upper limit (7.5 x 10^9/L). The common pathological conditions accounting for neutrophilia include acute bacterial infection, and other inflammatory stimuli (such as tissue necrosis), metabolic disorders, neoplasm of all cell types, corticosteroid therapy, and myeloproliferative disease (Hoffbrand *et al.*, 2001).

Leukocytosis, and particularly granulopoiesis, reflects an active, systemic inflammatory response (Hubl *et al.*, 1997) which is thought to contribute to increased

morbidity and mortality (Friedman *et al.*, 1974; Grimm *et al.*, 1985; Weiss *et al.* 1995; Furman *et al.*, 1996 and Suwa and Hogg *et al.*, 2001). As early as the 1980s, some epidemiological investigations revealed that peripheral leukocyte numbers were higher in smokers, compared to non-smokers (Chan-Yeung *et al.*, 1981 and Vanuxem *et al.*, 1984). The increased peripheral white blood cells counts have been related to the plasma concentrations of nicotine (Taylor *et al.* 1986). Later on, others have confirmed that leukocytosis, including neutrophilia, lymphocytosis, and in some cases, monocytosis, are correlated with tobacco smoking, and that leukocytosis can be remedied by smoking cessation (Schwarts and Weiss, 1996 and Freedman *et al.*, 1996). More recently, tobacco smoking has been consistently shown to lead to an increase in the numbers of neutrophils in the systemic circulation (Van Eeden & Hogg, 2000; Iho *et al.*, 2003 and Sorensen *et al.*, 2004) as well as the pulmonary microvascular system (Chalmers *et al.*, 2001 and Seagrave *et al.*, 2004).

Factors thought to be responsible for neutrophilia include increased levels of neutrophil chemoattractants and activators resulting in a prolonged neutrophil life span, and/or enhanced neutrophil differentiation and proliferation in the bone marrow, as shown in Figure 5. Interestingly, nicotine itself at low concentrations may be a weak chemotactic stimulus for human neutrophils, and may exacerbate the response of neutrophils to chemotactic peptides (Totti *et al.* 1984). It has been shown that IL-8, induces mild and moderate neutrophilia in animals (Jagels and Hugli, 1992; Terashima *et*

al., 1998) and that nicotine stimulate neutrophils to produce IL-8 in a time-, dose-, and nAChRs-dependent manner (Iho *et al.* 2003) which may contribute to leukocytosis in tobacco smokers. Another potential explanation for the neutrophilia in smokers is extended neutrophil lifespan due to the suppression of apoptosis. The essential effects of tobacco smoke, nicotine included, on the apoptosis of circulating neutrophils have been examined by several research groups (Aoshiba *et al.*, 1996; Yoshida *et al.*, 1998 and Mariggio *et al.*, 2001), whereas no conclusive and consistent data have been obtained. A third assumed mechanism is the susceptibility to infections, especially chronic infections, in smokers. Infection-induced inflammatory mediators may stimulate granulopoiesis and/or suppress neutrophil apoptosis. However, there is lack of solid evidence confirming this hypothesis. Therefore, the mechanisms underlying neutrophilia in tobacco users remain in need of clarification. In fact, it leaves a fourth possibility unaddressed, i.e. tobacco smoke or specific constituents of smoke may directly promote the development of neutrophil precursor cells in the bone marrow, contributing to more and /or accelerated release of mature neutrophils into the peripheral circulation. However, there is a dearth of data relating to the effects of tobacco smoke, including nicotine, on neutrophil differentiation.

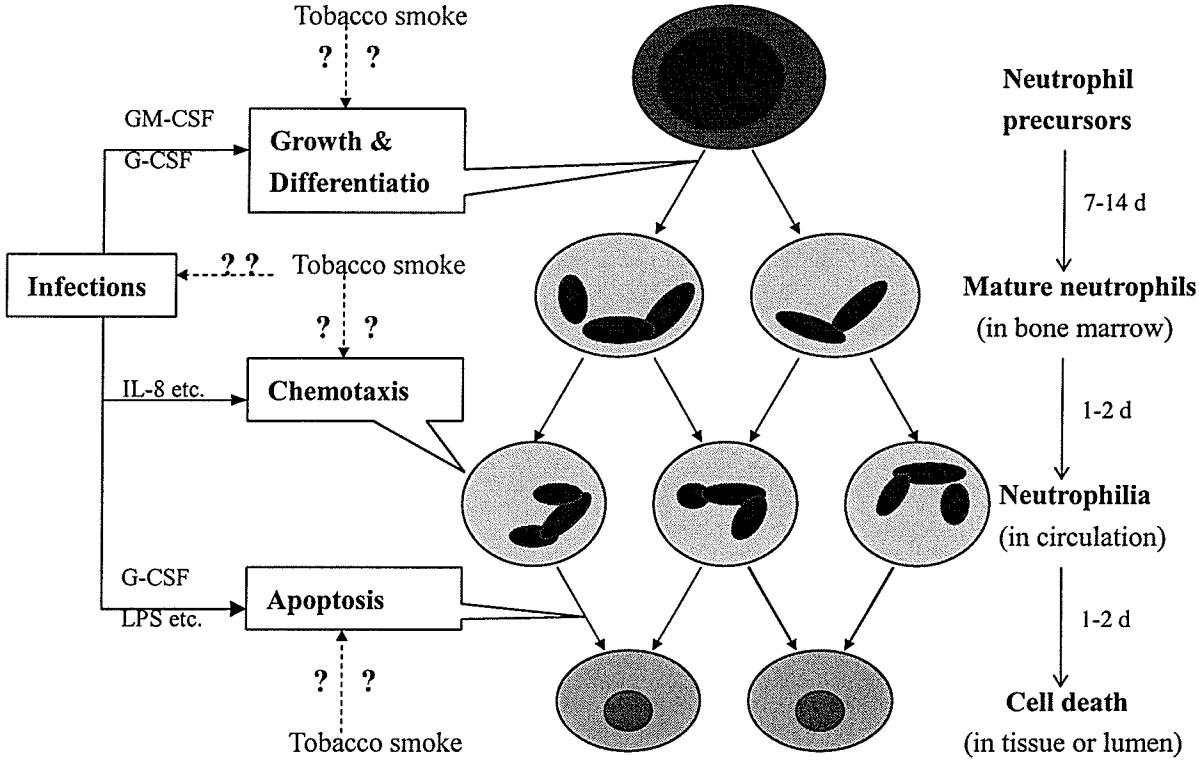

Figure 5: The potential mechanisms of tobacco-related neutrophilia

Neutrophilia may be caused by increased levels of neutrophil chemoattractants and activators resulting in a prolonged neutrophil life span, and/or enhanced neutrophil differentiation and proliferation in the bone marrow. The currently available data on the mechanisms by which tobacco smoke induce neutrophilia are scarce and conflicting. Data of the impact of tobacco smoke on neutrophil differentiation is essentially lacking.

4.3.2 Hypothesis

Neutrophils leave bone barrow as terminally differentiated cells with low transcriptional activity. Without appropriate inflammatory stimuli, neutrophils normally will have a short life span of one to two days (Borregaard, 1997 and Witko-Sarsat *et al.*, 2000). Indeed, many biologically active effectors of mature neutrophils, such as multiple granules

containing various proteinases and bacteriostatic/bactericidal agents, are pre-synthesized even before neutrophils are liberated to peripheral blood from the bone marrow (Ross and Pawlina, 2006). Also some essential phenotypes and functions of neutrophils, such as Fc receptor expression, integrin molecule expression, respiratory burst, cell phagocytosis and chemotaxis, are well developed in the course of cell differentiation in the bone marrow (Glasser and Fiederlein, 1987). Therefore, many important tobacco-induced alterations to neutrophil physiology, including neutrophilia, may occur before neutrophils even reach the systemic circulation. However, to the best of our knowledge, there are essentially no data available on the influence of tobacco smoke, nicotine included, on neutrophil differentiation and associated effector function development. In rabbits, *in vivo* cigarette smoke exposure has been associated with the increased numbers of both mature and banded neutrophils liberated form the bone marrow into the circulation, resulting in preferential neutrophil sequestration in the microvasculature (Terashima *et al.*, 1999). High doses of nicotine can induce the macrophage/monocyte-like differentiation of a human promyelocytic leukemia cell line (Maturana *et al*, 1991). The supernatant from alveolar macrophages exposed to air pollution particles smaller than 10 μm, perhaps reflecting the high particulate content of tobacco smoke, can promote myeloid line differentiation in HL-60 cells (Suwa *et al.*, 2002). Also, it has been reported that nicotine affects the proliferation of other leukocytes, i.e. T and B lymphocytes, in the bone marrow and in a nAChR-mediated manner (Skok *et al.*, 2005 and 2006). Growing evidence reveals the expression of functional nAChRs by both bone marrow cells and

neutrophils (Villiger *et al.*, 2002; Iho *et al.* 2003 and Cormier *et al.*, 2004). Importantly, of some 4000 compounds in tobacco smoke, pharmacological concentrations of nicotine have been proved the existence in the multiple organs, tissues and body fluids of smokers. Since it is difficult to establish a human neutrophil differentiation model *in vivo*, we employed a well-characterized human leukemia cell line, HL-60, which is commonly used as a model for neutrophil differentiation and inflammation. All these above evidences have led us to urgently verify the hypothesis that nicotine affects neutrophil differentiation and correlated effector functions.

CHAPTER 5

INTRODUCTION -- PART FIVE

HL-60 Cell Line

HL-60 cells are a well-characterized human promyelocytic cell line established from the peripheral leukocytes of a patient with acute promyelocytic leukemia (Collins *et al.*, 1978 and 1987). As characterized by morphological and histochemical examinations, this cell line is predominantly neutrophilic promyelocytes (>70%). Less than 20% of HL-60 cells still are blocked at an earlier differentiation stage, i.e. myeloblasts. Up to 10% cultured HL-60 cells spontaneously differentiate into more mature myeloid cells, having very similar morphological and functional characteristics of normal myelocytes, metamyelocytes and neutrophils. The terminal neutrophil-like differentiation of HL-60 cells can be dramatically enhanced by the addition of dimethylsulfoxide (DMSO, 1% to 1.5%), butyrate, hypoxanthine, actinomycin D, retinoic acid, etc. Mature HL-60 cells demonstrate the functional characteristics of phagocytes, including phagocytosis and chemotaxis (Gallagher *et al.*, 1979).

HL-60 cells express features of differentiated granulocytes, including receptors for Fc fragments and complement (C3). The HL-60 cell line is positive for myc oncogene expression and able to form subcutaneous solid myeloid tumors in nude mice (Collins *et al.* 1978 and 1987; Gallagher *et al.*, 1979). Current studies also reveal that HL-60

leukemia cell line expresses nAChRs, such as α-7 subunits (Villiger *et al.*, 2002; Gimonet *et al.*, 2003). In addition, increasing evidence shows that this cell line releases multiple biomolecules involved in the inflammatory response, such as MMPs, elastase, TNF, and IL-8 (Devy *et al.* 2002; Armstrong *et al*, 1996 and Fang *et al.*, 2005). Otherwise, HL-60 cells retain a consistent morphological phenotype even after 85 passages (Gallagher *et al.*, 1979). Therefore, the HL-60 cell line is considered an excellent model for studies of neutrophil differentiation.

CHAPTER 6

INTRODUCTION -- PART SIX

Infrared Spectroscopy

The Infrared (IR) region, which lies to the low energy side of visible light, is the last region of the electromagnetic spectrum to have been applied to the practice of medicine and biomedical science.

The absorption of infrared radiation induces vibrations within covalently bonded molecules, and for this reason IR spectroscopy is sometimes referred to as vibrational spectroscopy. Each molecular vibration corresponds to a discrete energy and therefore contributes a unique IR absorption band. The specific absorption frequencies can be remarkably informative, revealing qualitative characteristics of the nature of the chemical bonds and their molecular environment (Jackson & Mantsch, 1996). For example, in complex biological materials such as cells or biological fluids, the major IR absorption bands arise from the N-H, C=O, C-H, and P=O bonds found in proteins, lipids, nucleic acids, and carbohydrates (Figure 6) (Jackson & Mantsch, 1996). Infrared spectroscopy, therefore, holds great promise as an analytical tool to assess tissues and cells because it can probe chemical compositions and compositional and structural changes in complex systems at the molecular and sub-molecular levels (Liu *et al.* 2006).

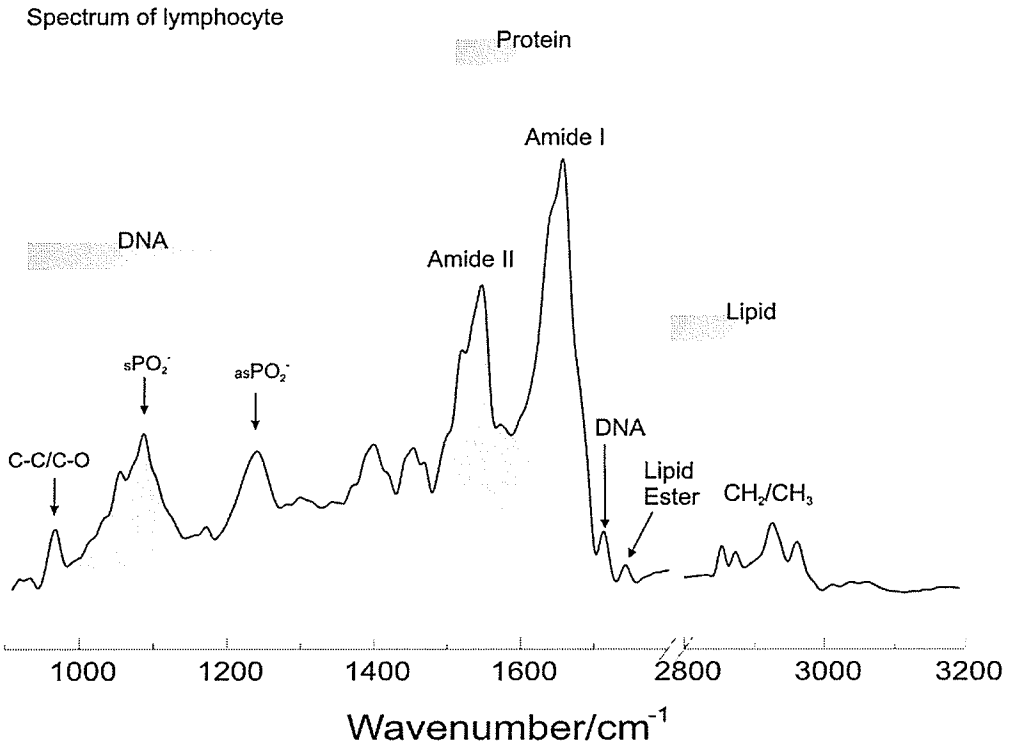

Figure 6: The infrared spectrum of normal lymphocytes. In complex biological materials such as cells or biological fluids, the major infrared absorption bands arise from chemical bond vibrations in submolecular components of proteins, lipids, nucleic acids, and carbohydrates. Here, an infrared spectrum representative of normal lymphocytes is shown. **Reprinted from *Blood cells, Molecules, and Diseases*, Volume 35 (3), Kan-Zhi Liu, Min-Hua Shi and Henry H. Mantsch. "Molecular and chemical characterization of blood cells by infrared spectroscopy: a new optical tool in hematology", 404-412, Copyright (2005), with permission from Elsevier.**

Essentially, the IR spectrum of a tissue or cell sample can be regarded as molecular fingerprint of the tissues or cells (Liu *et al.*, 2006). Advantages include reagent-free processing, time efficiency, minimum sample preparation, and easy automation. IR spectroscopy has been successfully employed to investigate several aspects of leukocyte physiology and pathology, including the determination of differentiation and apoptotic processes in leukocytes and leukemia cells (Liu *et al.*, 2005 and 2006). In this study, we use IR spectroscopy to detect molecular and sub-molecular alterations to the biochemical profile of neutrophils during differentiation.

CHAPTER 7

Research Significance, Objectives and Experimental Approaches

7.1. Significance

It is known that tobacco smoking has a comprehensive and profound influence on neutrophil physiology and render smokers susceptible to multiple inflammatory diseases. Yet many basic science studies on the effects of smoking on human neutrophils have generated controversy. Furthermore, no research has been published to allow us to understand the potential effects of nicotine on neutrophil differentiation. This research project represents the first major study of the influence of nicotine on differentiating neutrophils.

7.2. Objectives and Experimental Approaches

The main hypothesis of this work is that pharmacologically relevant nicotine doses exert influence on neutrophil differentiation and associated effector functions.

I. To investigate the influence of nicotine on neutrophil differentiation.

We will:

a. Use DMSO-stimulated HL-60 cells to model neutrophil differentiation

b. Determine the optimal time course of cell differentiation.

c. Investigate whether pharmacologically relevant nicotine doses affect neutrophil differentiation at the morphological, biochemical and molecular

levels.

II. To establish the impact of nicotine on phenotypic changes in differentiating neutrophils.

We will:

a. Confirm the expression of nAChRs by HL-60 cells and examine the regulation of nAChR expression by nicotine.

b. Determine whether pharmacological nicotine concentrations affect several important aspects of differentiating HL-60 cell, including apoptosis, necrosis, cell cycle, MMP release and oxidative burst.

c. Examine alterations to the molecular profile of differentiating neutrophils exposed to nicotine using infrared spectroscopy.

CHAPTER 8

MATERIALS & METHODS

Materials

HL-60 cells (CCL-220) were purchased from ATCC (Manassas, VA, USA). RPMI-1640 medium, penicillin, streptomycin, nicotine, cotinine, α-bungarotoxin, dimethyl sulfoxide (DMSO), nitroblue tetrazolium (NBT), trypan blue, RNAase A, phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) derived from *Escherichia coli* 0111:B4 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) and Hema 3 staining kit were bought from Fisher Scientific (Nepean, ON, Canada). Serum-free UltraCULTURE media was ordered from Cambrex Co. (Walkersville, MD, USA). Propidium iodide (PI) and ProLong Gold antifade reagent were bought from Molecular Probes (Eugene, OR, USA). Quantikine Human MMP-9 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Anti-α7 nicotinic acetylcholine receptor antibodies (AChRsα7 [H-302]), HRP-conjugated or FITC-conjugated goat anti-rabbit IgG secondary antibodies and α7 protein positive controls (mouse frontal brain extractions & mouse cerebellum extractions) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Nitrocellulose membranes were from BIO-RAD Laboratories (Mississauga, ON, Canada). Amersham Biosciences Corp (Piscataway, NJ, USA) enhanced chemiluminescence (ECL) western detection kits and Kodak brand x-ray films were used for visualization. PE-conjugated

anti-CD11b (clone ICRF44) and Mouse, κ IgG1 (MOPC-21) isotype control antibodies were ordered from BD Biosciences Pharmingen (San Diego, CA, USA).

Growth of HL-60 cells

HL-60 cells were cultured in RPMI-1640 medium with 0.3 g L^{-1} *L*-glutamine and 2.0 g L^{-1} sodium bicarbonate supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (50 U ml^{-1}) and streptomycin (50 μg ml^{-1}). The cells were grown at 37 °C in an atmosphere of 5% CO_2 and 100% humidity. Cell density was maintained between 1.0 ~ 10.0 x 10^5 ml^{-1} by three subcultures per week. All HL-60 cells used for this research project were obtained from cell cultures with 4 - 30 passages. HL-60 cells were grown at an initial cell density of 2.0 x 10^5 ml^{-1} for all experiments, unless otherwise mentioned.

Time course of HL-60 cell differentiation

Granulocytic differentiation of HL-60 cells was initiated by the addition of 1.3% (v/v) DMSO (final concentration) (Gallagher *et al.*, 1979; Collins *et al.* 1978 and 1987). We found that the maximum proliferation (hemocytometer, Figure 14a) and differentiation (morphological examination, Figure 14b) of DMSO-induced HL-60 cells occurred after 5 days. These data are consistent with earlier studies by Collins *et al.* (1978). Therefore, the effects of nicotine on HL-60 differentiation were determined over 5 days from initiation with DMSO.

Establishment of Nicotine Dosing Regimens

In the venous blood of tobacco smokers, nicotine concentrations are normally 0.5 x 10^{-7} M to 5.0 x 10^{-7} M (Table 2). Arterial or tissue nicotine concentrations are six to ten-fold or two to three-fold higher, respectively (Urakawa *et al.*, 1993; Benowitz *et al.*, 1996; Benowitz and Jacob, 1997). Levels of nicotine in the oral and pulmonary environments are higher still. Therefore, the HL-60 cells were exposed to nicotine at a concentration range of 10^{-7} M to 10^{-4} M.

Differentiation of HL-60 cells

Granulocytic differentiation was initiated by the addition of 1.3% (v/v) DMSO (final concentration) for 5 days with or without various concentrations of nicotine (10^{-7} M to 10^{-4} M). Differentiation was monitored by (a) cell density (hemocytometer) and viability (0.4% (w/v) trypan blue staining), (b) morphological examination [Hema 3 staining (Choi *et al.*, 2005 and Lieber *et al.*, 2004)], (c) ability to reduce nitro blue tetrazolium (NBT) (Hua *et al.*, 2000), (d) expression of the terminal differentiation marker - CD11b (Trayne *et al.*, 1998), and (e) alteration of the key cellular components (infrared spectroscopy), such as proteins, lipids, and nucleic acids (Zhou *et al.*, 2002 and Liu *et al.*, 2006).

Cell proliferation & viability

Cell proliferation and viability were monitored by 0.4% (w/v) trypan blue exclusion coupled with cell counting using a hemocytometer. Cells stained by trypan blue were

counted as dead cells. Cell viability was quantitatively assessed as the percentage of viable cells. Cell proliferation was indicated as viable cell density.

Morphological examination (Hema 3 staining)

Cells were forced to adhere to pre-cleaned glass slides by centrifugation (50 *g*, 2 minutes, RT) in a Shandon Cytospin® 3 Cytocentrifuge. Slides were dried, fixed, and stained using Hema 3 staining kits, following the instructions provided by the manufacturer. The cells were examined by light microscopy using an Olympus BX41 microscope. The morphology of 400 stained cells selected randomly from each slide was examined. Using the distinct morphological characteristics, described in Table 3, the cells were classified as belonging to different subtypes, i.e. myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and segmented neutrophil. Cell differentiation was measured as the percentage of morphologically differentiated cells vs. total counted cells.

Nitro blue tetrazolium reduction test

Cells were harvested and incubated with NBT (0.5 mg ml^{-1}, final concentration) and PMA (200 ng ml^{-1}, final concentration) at 37 °C for 30 minutes in the dark. The cells were cooled to RT over 15 minutes, and pipetted onto a hemocytometer for observation by light microscopy using an Olympus BX41 microscope. Cells containing dark blue or purple intracellular formazan deposits, as shown in Figure 7, were considered differentiated, NBT positive cells (Hua *et al.*, 2000). Cell differentiation was evaluated as the percentage

of NBT positive cells.

Table 3: Distinct morphological characterization of myeloid neutrophilic differentiation

Cell type	Size (μm, diameter)	Nucleus & Mitosis	Nucleoli	Cytoplasm	Granules	Nucleus / Cytoplasm
Myeloblast	12-14	Round or oval; fine diffuse immature chromatin; Mitosis (+)	2-5	Basophilic; cytoplasmic blebs at cell periphery	None	> 3:1
Promyelocyte	16-24	Round to oval; slightly coarse chromatin; Mitosis (+)	1-2	Basophilic; No cytoplasmic blebs at cell periphery	Azurophilic granules	3:1 to 2:1
Neutrophilic myelocyte	10-12	Flattened, acentric; coarse chromatin; mitosis (+)	0-1	Less basophilic	Azurophilic & specific granules	1:1
Neutrophilic metamyelocyte	10-12	Kidney-shaped; dense; coarse chromatin; mitosis (-)	None	Less basophilic	Azurophilic & specific granules	1:1 to 1:2
Neutrophilic band cell	9-12	Horseshoe-shaped; curled rod; very coarse and clumped chromatin: mitosis (-)	None	Less basophilic	Azurophilic & specific granules	<1:2
Neutrophilic segmented cell	9-12	Multilobed; very coarse and clumped chromatin; mitosis (-)	None	Less basophilic to neutrophilic	Azurophilic & specific granules	<1:2

Differentiated cells refer to those cells that are morphologically more mature than the promyelocytes, including myelocytes, metamyelocytes, band- and segmented neutrophil. Based on the similarities of function and morphology, cells are classified as early phase differentiating cells with the ability of mitosis (myeloblasts, promyelocytes, and myclocytes), and late phase differentiating cells lack of mitosis (metamyelocytes, banded-, and segmented neutrophils). (Collin *et al.*, 1978; Ross and Pawlina 2006)

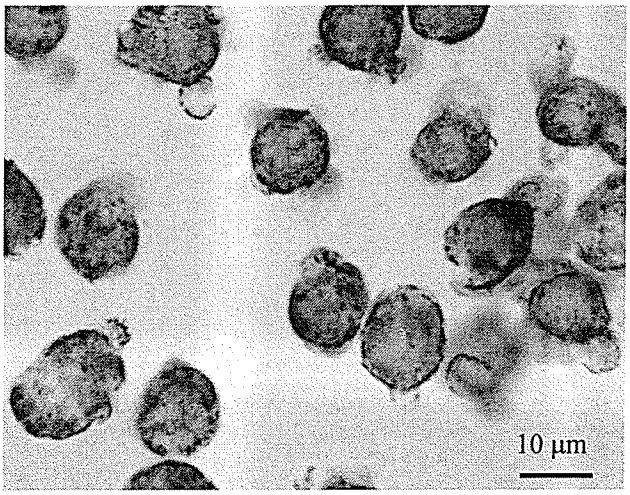

Figure 7: NBT positive (differentiated) HL-60 cells: Over a five-day incubation with 1.3% (v/v) DMSO, most cells were differentiated enough to exhibit the ability to reduce NBT, indicated by numerous intracellular purple deposits. (The image was taken on an Olympus BX41 microscope by a SONY 4.1MP digital camera. Magnification: x 1000).

Expression of CD11b (integrin-αM)

Cells were centrifuged (300 *g*, 5 minutes, and 4 °C) and washed twice in ice-cold PBS (pH 7.4) before being re-suspended in 1 ml ice-cold PBS (pH 7.4). The cells were stained with PE-labeled anti-CD11b monoclonal antibody (clone ICRF44, 20 µl antibody per 1 million cells) for 30 minutes with gentle shaking at 4 °C in the dark. The samples were centrifuged (300 *g*, 5 minutes, and 4 °C) and gently washed with ice-cold PBS (pH 7.4) twice in order to remove unbound antibody. An isotype–matched antibody (Mouse IgG1) was used as a negative control. Data from 10,000 cells were collected using flow cytometry (EPICS Altra-Fluroscence-Activated Cell Sorter; Beckman Coulter Canada Inc, Mississauga, Canada).

Conformational changes in differentiating HL-60 cells

Fourier transformed-infrared (FT-IR) spectroscopy can monitor structural alterations to key cellular components, including membranes, proteins, and nucleic acids. Therefore, conformational changes in differentiating HL-60 cells exposed to nicotine, or not (control cells), were examined by FT-IR spectroscopy, essentially as described by Zhou *et al.* (2002). Briefly, HL-60 cells were washed in ice-cold 0.9% NaCl, loaded onto infrared transparent BaF_2 windows, and vacuum dried at 25 Torr. Infrared spectra were recorded using a Bio-Rad FTS-40A IR spectrometer (Bio-Rad Laboratories, Cambridge, MA) at a nominal resolution of 2 cm^{-1}. Two separate films from each subject sample were measured, each spectrum consisting of 256 co-added interferograms, apodized with a triangular smoothing function before Fourier transformation. All IR spectra were baseline corrected and area normalized between 900-1800 cm^{-1} using WIN-IR software (BIO-RAD Laboratories, Cambridge, MA) and analyzed using WIN-IR and in-house FT-IR spectroscopy software (Molecular Spectroscopy Group, National Research Council, Winnipeg, Canada). Alterations to DNA were determined by analysis of the DNA-specific spectral bands at 965 cm^{-1} (C-C/C-O stretching vibration), 1087 cm^{-1} ($\nu_s PO_2^-$), 1240 cm^{-1} ($\nu_{as} PO_2^-$), and 1713 cm^{-1} (characteristic of base-paired DNA strands). Lipid changes were assessed by analysis of the spectral bands at 1399 cm^{-1}, 2850 cm^{-1} ($\nu_s CH_2$), 2958 cm^{-1} ($\nu_{as} CH_2$), 1455 cm^{-1} (CH_2 deformation band), and 1740 cm^{-1} (lipid ester C=O stretching vibration). Membrane protein changes were assessed by analysis of the spectral bands at 1649 - 1660 cm^{-1} (the α–helix peak), 1615 - 1637 cm^{-1} (the β–sheet peak), and 1638 -

1648 cm^{-1} (the random coil peak).

Expression of Nicotinic Acetylcholine Receptors (nAChRs) by HL-60 Cells

I. Immunoblotting for α-7 nAChR Expression:

Whole cell lysate preparation

Cell suspensions were centrifuged (300 *g*, 5 minutes, 4 °C). Cell pellets were resuspended and washed two times in ice-cold PBS (pH 7.4). 1ml lysis buffer [50mM Tris-HCl, 7.4, 150 mM NaCl, 1% (v/v) IGERAL (NP-40), 2 mM EDTA and protease inhibitor cocktail (Roche Diagnostics GmabH, Germany)] was used to lyse 1 x 10^7 cells by incubation on ice for 30minutes with gentle shaking. Cell debris was removed by centrifugation (14000 *g*, 10 minutes, 4°C). The supernatants were transferred into 1.5 ml microcentrifuge tubes and stored at -80 °C until needed.

Protein concentration assay

Sample protein concentrations were determined spectrophotometrically using the BIO-RAD DC Protein Assay Kit, based on the Coomassie blue dye-binding protocol described by Bradford (1976). Bovine serum albumin (BSA) was used as a relative protein standard for all assays. The optical density of protein samples at 595 nm was measured using a Beckman DU Series 640 spectrophotometer. Protein concentrations of each sample were extrapolated from BSA standard curves.

Protein electrophoresis: SDS-PAGE system

Sample proteins in crude extracts were size-fractioned by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the buffer system described by Laemmli (1970). A BIO-RAD mini-gel vertical electrophoresis apparatus was used for all applications. 12% resolution gels [12% (w/v) acrylamide, 375 mM Tris-HCl, pH 8.8, and 0.1% (w/v) sodium dodecyl sulfate (SDS)] with 4% stacking gels [4% (w/v) acrylamide, 125 mM Tris-HCl, pH 6.8, and 0.1% (w/v) SDS] were employed.

Sample protein preparation

Sample proteins were prepared by diluting crude cell extracts in sample buffer [finial concentration: 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 12.5% (v/v) glycerol, 2.5% (v/v) β-mecraptoethanol, 0.01% (w/v) bromphenol blue] which were then heated to 95 °C for 5 minutes. 20 - 40 μg of protein equivalents for each crude extract was loaded onto SDS-PAGE gels which were fractionated at a constant voltage of 100 mV for 2 hours at RT. The electrode running buffer used was composed of 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. Pre-stained low range protein standards (BIO-RAD) were used to indicate relative molecular weight in all Western Blots.

Immunoblotting of whole cell lysate proteins

SDS-PAGE fractionated proteins were electroblotted onto nitrocellulose membrane as described by Towbin and Gordon (1984). Transfer of protein from gel onto nitrocellulose

membranes (0.45 μm) was achieved overnight at a constant voltage of 25 mV at 4 °C, using transfer buffer comprising of 25 mM Tris, 192 mM glycine, 0.05% (w/v) SDS and 20% (v/v) methanol. Blotted membranes were extensively washed with distilled water before the subsequent process of immunoblotting. Otherwise, the blots were stored at 4 °C in Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH 7.5) and immunostained within 24 hours.

Immunostaining for α7 nAChR

Nitrocellulose membrane blots were blocked with 5% (w/v) milk casein in TBS-T buffer (0.1% (v/v) Tween-20 in TBS) for 60 minutes at RT with constant shaking. The membranes were added to 5% (w/v) milk casein in TBS-T buffer containing a 1:200 dilution of a primary polyclonal rabbit antibody (anti-nAChRα7), which recognizes amino acids 367-502 mapping to the C terminus of α7 subunit of the human receptor. After incubation with the primary antibody for 120 minutes at RT on a horizontal shaker, the membranes were washed 6 times for 5 minutes each with TBS-T buffer. A secondary antibody (HRP-conjugated goat anti-rabbit IgG) was used at a dilution of 1:2000 in 5% milk casein/TBS-T buffer for blotting for 60 minutes with constant shaking. After repeating the washing step described above, primary antibody binding sites were visualized using enhanced chemiluminescence (ECL) western detection kits, according to the manufacturer's instructions (Amersham Biosciences Corp, Piscataway, NJ, USA).

Images of ECL films (Epson Perfection 1250 Scanner with Epson Smart Panel Software) were entered into Quantiscan v3.0 (Biosoft, Cambridge, UK) for quantitative densitometric analysis of the protein bands of interest, with backgrounds calibrated as an interpolated minimum.

II. Fluorescent Immunocytochemistry

Cells were forced to adhere to pre-cleaned glass slides by centrifugation (50 *g*, 2 minutes, RT) in a Shandon Cytospin® 3 Cytocentrifuge, and fixed with 4% (w/v) paraformaldehyde (PMF) in PBS (pH 7.4) for 30 minutes at RT and washed with PBS (pH 7.4). The cells were immersed in 0.1% (v/v) Triton X-100 in PBS (pH 7.4) for 15 minutes at RT prior to repeating washing with PBS (pH 7.4). The fixed cells were incubated with a 1:100 dilution of rabbit polyclonal antibodies against α7 nAChR in PBS (pH 7.4) containing 1.0% (w/v) BSA for 90 minutes at RT. Control cells were incubated without the primary antibodies. After extensive washing in PBS (pH 7.4), cells were incubated with a 1:1000 dilution of FITC-conjugated goat anti-rabbit antibodies in cyto-buffer [1.0% (w/v) BSA and 1.0% (v/v) goat serum in PBS (pH 7.4)] for 1 hour, in the dark and at RT. After repeating the extensive washing, the cells were immersed into ProLong Gold antifade reagent and mounted on glass coverslips for observation using an inverted fluorescence microscope (Nikon Eclipse TS100, equipped with Nikon Plan Fluor objectives and an Omega Optical Fluor filter set) and an inverted confocal microscope (Olympus IX70, equipped with Nomarski DIC optics). Images were captured by InCytIm 1 software and

Fluo View software, respectively.

Ultrastructural Examination (Transmission Electron Microscopy)

Ultrastructural characteristics of HL-60 cells were determined by transmission electron microscopy, essentially as described by Karim *et al.* (1995). 1 x 10^7 cells of each culture were pipetted into a sterile culture tube and washed, centrifuged (300 *g*, 10 minutes, RT) and re-suspended twice in PBS (pH 7.4). The cells were centrifuged again (300 *g*, 10 minutes, RT) to form a loose pellet which was fixed with 2.5% (w/v) PBS (pH 7.4)-buffered glutaraldehyde for 2 hours and post-fixed for another 2 hours with 1% (w/w) osmium tetroxide. During post-fixation, the pellet was dislodged from the bottom of the tube prior to progressive dehydration in acetone for 5 minutes in each of 30, 50, 60, 70, 80, 90, and 100% (v/v) concentrations respectively. After the dehydration procedure, the pellet was cut into smaller pieces and progressively infiltrated with acetone/Epon mixture for 4 hours each at the ratio of 3:1, 1:1, 1:3, followed by the submergence in pure Epon overnight. Embedded cells were polymerized in a 60°C oven over 48 hours and sectioned (1μm) for light microscopy. Ultra-thin sections (30 nm) were prepared, stained for 1 hour with uranyl acetate, then 5 minutes with lead citrate prior to being examined using a Philips 300 electron microscope.

Cell Cycle Analysis

The percentage of apoptotic cells was determined by propidium iodide (PI) DNA staining and flow cytometry. Briefly, cells were washed in ice-cold PBS (pH 7.4) before centrifugation (200 *g*, 5 minutes, 4 °C) and resuspension in 0.5 ml of ice-cold PBS (pH7.4). The cells were fixed in 5 ml ice-cold 70% (v/v) ethanol buffered with PBS (pH7.4) and kept at 4°C overnight. After two washings with ice-cold PBS (pH7.4), fixed cells were centrifuged (300*g*, 5 minutes, 4 °C) and resuspended in 1.0 ml ice-cold PBS (pH 7.4) with 100 U ml^{-1} RNase A (final concentration) and incubated at RT for 30 minutes. The cells were further incubated with 25 µg ml^{-1} PI (final concentration) for 30 minutes at RT in the dark. 10,000 cells stained by PI were analyzed by flow cytometry (EPICS Altra-Fluroscence-Activated Cell Sorter; Beckman Coulter Canada Inc, Mississagua, Canada). Cells sorting into the area of pro-G_0/G_1 were considered as apoptotic cells.

Oxidative Burst Analysis (Qualitative NBT Assay)

The oxidative burst capacity of HL-60 cells was estimated by quantifying the ability to reduce NBT, essentially as previously described by Pompéia *et al.* (2003) with little modification. Briefly, cells (2 x 10^6 in 200 µl for each sample) were treated for 1 hour with 0.5 mg ml^{-1} NBT (final concentration) and 200 ng ml^{-1} PMA (final concentration), at 37°C in the dark. The reaction was stopped by adding 100 µl acetic acid. After removal of the supernatant through centrifugation (12000 *g*, 2 minutes, RT), reduced NBT formazan

in cell pellets were extracted with 200 µl 50% (v/v) acetic acid and further dissolved by a Heat Systems Microscon Ultrasonic Ultrasonic Cell Disrupter setting at level 7 (3 five-second pulses). The cell debris was pelleted by centrifugation (12000 *g*, 5 minutes, RT) and the supernatant was transferred into 96-well plates. Reduced-NBT was quantified spectophotometrically (relative optical densities at 560 nm; Bio-RAD microplate reader model 550) by extrapolation from a standard curve.

Quantitative Analysis of MMP-2 and MMP-9

Sample preparation

HL-60 cells were maintained in complete RPMI-1640 media, as described above. Cells were washed twice with PBS at RT, seeded at 2.0 x 10^5/well (6-well plates), and cultured with 5 ml serum-free UltraCULTURE media containing 1.3% (v/v) DMSO for 5 days. Concurrently, the cells were treated with or without various concentrations of nicotine (10^{-7} to 10^{-4} M). In some experiments, 200 µg L^{-1} (final concentration) α-bungarotoxin was added to cell cultures for a 30 minutes-pretreatment prior to the exposure of nicotine. Following differentiation in the presence and absence of nicotine, the 5-day HL-60 cells were stimulated with 1.0 mg L^{-1} LPS (final concentration). Conditioned cell culture media were harvested at one hour and 24 hours after the LPS stimulation, centrifuged (3000 *g*, 10 minutes, 4 °C) to remove the cell debris, and stored in -80°C until needed for MMP quantification.

ELISA

MMP-9 release by HL-60 cells was quantified using a 96-well sandwich ELISA, according to the manufacturer's instructions (R&D Systems). Optical densities were determined within 30 minutes, using a Bio-RAD model 550 microplate reader set to 450 nm coupled with wavelength correction set to 570 nm. MMP-9 concentrations were extrapolated from standard curves.

Gelatin zymography

Gelatinolytic activities in the conditioned media were evaluated by discontinuous SDS-PAGE using non-reducing buffer system (Laemmli, 1970) according to the procedure described by Heussen and Dowdle (1980). Briefly, 20 µl of the conditioned culture media mixed with 10 µl of zymogram sample buffer [62.5 mM Tris-HCl, pH 6.8, 25% (v/v) glycerol, 4% (w/v) SDS & 0.01% (w/v) bromophenol blue] were applied without boiling to 8.0% (w/v) SDS-polyacrylamide gels complemented with 1mg ml^{-1} (final concentration) gelatin type I (Sigma-Aldrich). After electrophoresis, SDS was removed from the gels by two 30-minute long soakings in 2.5% (v/v) Triton X-100, in order to allow proteins to renature. Subsequently, the gels were immersed into TCS buffer (50 mM Tris-HCl, pH 7.4, 0.2 M NaCl & 5 mM $CaCl_2$) overnight at 37 °C prior to a 60-minute staining with 0.25% Coomassie blue solution [30% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.25% (w/v) Coomassie brilliant blue R 250]. MMP-2 and MMP-9 activity was indicated by clear bands, representing degraded gelatin type I, against the

blue background of intact gelatin following destaining [30 minutes in 10% (v/v) glacial acetic acid, 30% (v/v) methanol]. Proteolytic activity was quantified by densitometry, as described above.

Statistical Analysis

Data were analyzed statistically with Student's t-test or, where appropriate, a one-way analysis of variance test followed by Duncan's new multiple range post hoc test (Ott, 1977), assuming a significant difference at $p < 0.05$. All figures, except for photographic images, were prepared with the software of SigmaPlot 10.0 (SPSS Incorporated, Chicago, ILL),

CHAPTER 9

RESULTS

9.1. Time Course of Cell Differentiation Assessment

DMSO-induced HL-60 cells obtained the maximum morphological differentiation [82.2 ± 2.9 %, (mean, s.d.), Hema 3 staining] and proliferation [9.8 ± 0.6 x 10^5 ml^{-1} (mean, s.d.), viable cell density] after five days of cell culture, as demonstrated in Figure 8. Thus, the influence of nicotine on HL-60 cell differentiation was established over five days from incubation with DMSO.

9.2. Expression of α7 nAChR by HL-60 cells

To investigate the expression of the nAChR α7 subunit protein by HL-60 cell line, we carried out Western blot and immunocytochemistry assays. Mouse frontal brain extract served as a positive control for the α7 protein (Villiger *et al.*, 2002). The results (Figure 9a) showed that an antibody specific against the nAChR α7 subunit bound to a protein band with the relative molecular mass of 55 kDa, the accepted molecular mass for α7 subunit (Want *et al.*, 2003; Huynh *et al.*, 2001 and Cox *et al.*, 2001), in both undifferentiated HL-60 cells and five-day, DMSO-induced HL-60 cells. The expression of the 55 kDa protein was up-regulated in DMSO-treated HL-60 cells over five days, irrespective of nicotine (10^{-6} M or 10^{-4} M) exposure, as determined by densitometric analysis (Figure 9b). Furthrmore, α7 nAChR-specific immunofluorescence was clearly observed in both undifferentiated HL-60 cells and five-day differentiated HL-cells, as illustrated in Figure 10.

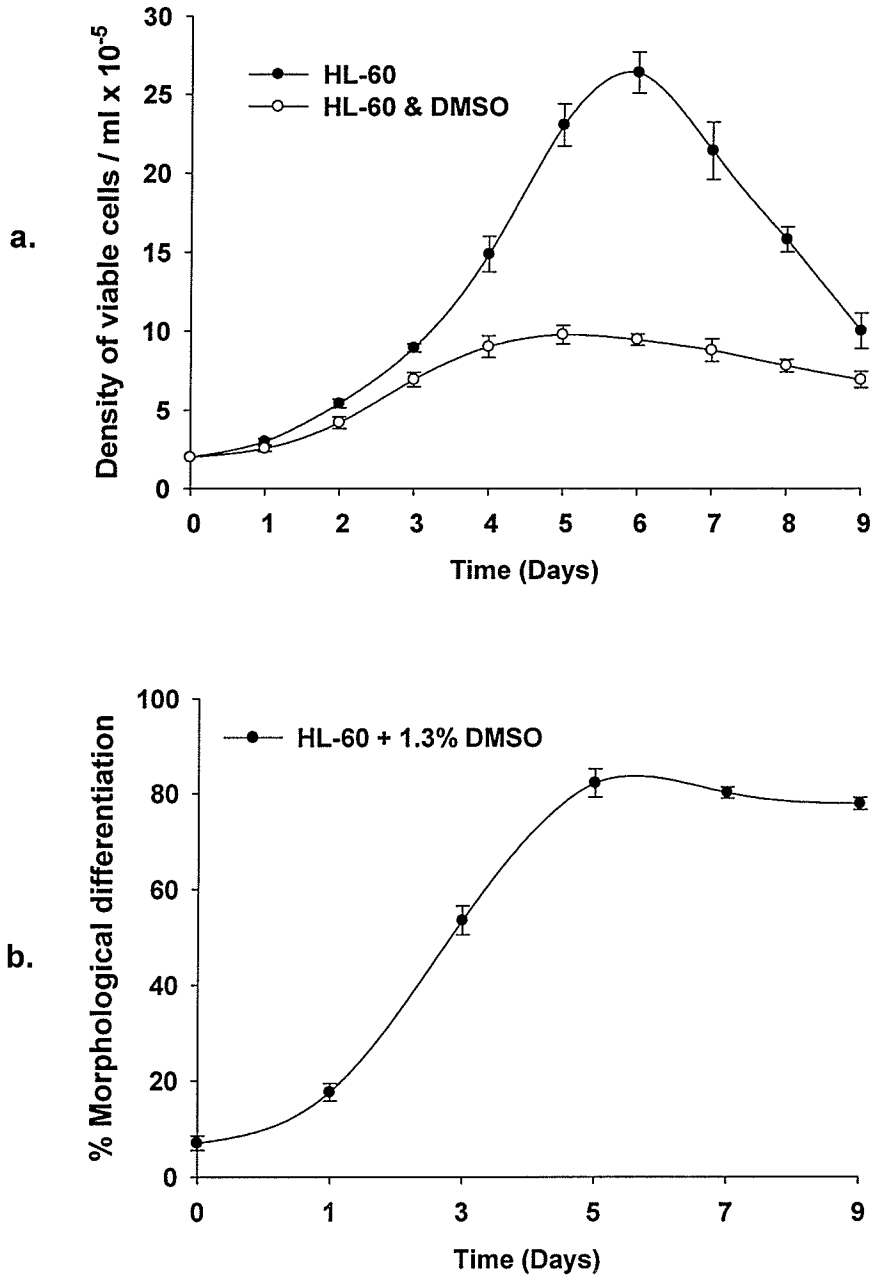

Figure 8: Proliferation (viable cells density, mean ± s.d.) and morphological maturation (percentage, mean ± s.d.) of promyelocytic or DMSO-induced HL-60 cells over nine days. (a). The maximum cell densities of promyelocytic and DMSO-treated HL-60 cells were reached around day 5. (b). The maximum neutrophilic differentiation occurred on day 5 after 1.3% (v/v) DMSO induction. Stages of neutrophil differentiation are provided in Table 4.

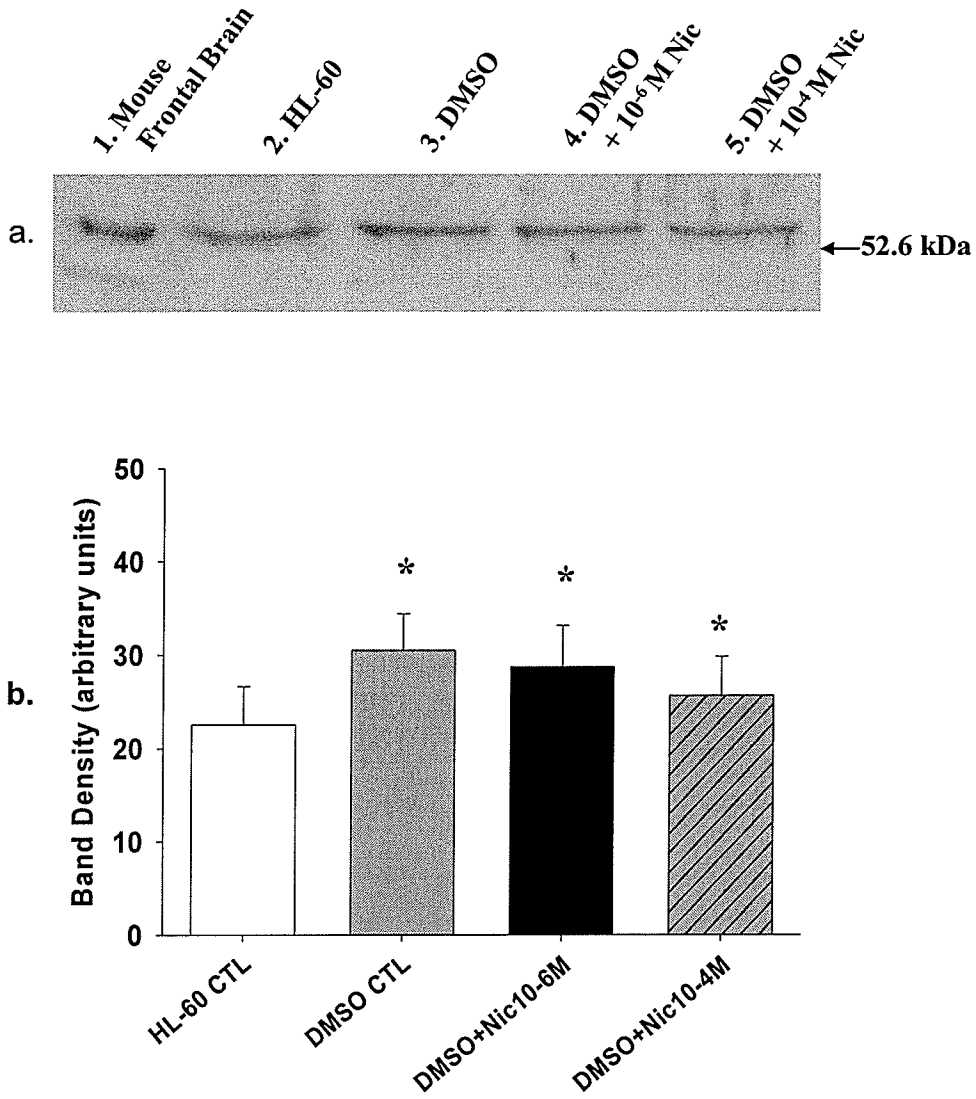

Figure 9: Immunoblot analysis of the α7 nAChR subunit protein expressed by HL-60 cells. (a): Lane 1. Mouse frontal brain extract (10 μg) was used as a positive control; lane 2. Lysate (40 μg) of undifferentiated HL-60 cells; from lane 3 to lane 5, lysate (40 μg) of five day, DMSO-induced HL-60 cells without nicotine, or with 10^{-6} M nicotine, or with 10^{-4} M nicotine treatment, respectively. An α7 nAChR-specific antibody (AChRsα7 [H-302]) bound to protein bands exhibiting a relative molecular mass of 55.0 KDa in all lanes. **(b):** Densitometric analysis (mean, s.d.) shows that band intensity is significantly increased in the DMSO-treated samples, irrespective of nicotine exposure, relative to undifferentiated cells (* $p<0.05$).

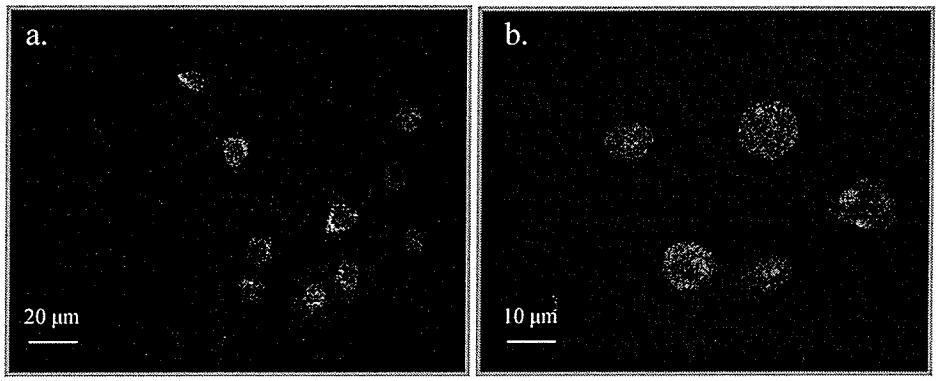

Figure 10: Immunofluorescence staining of the α7 nAChR subunit protein expressed by HL-60 cells. Fixed HL-60 cells were incubated with a polyclonal rabbit anti-α7 antibody (AChRsα7 [H-302]) before staining with a FITC-conjugated anti-rabbit antibody (Santa Cruz SC-2253). (**a**). Undifferentiated HL-60 cells, x400; (**b**). Five-day, DMSO-induced HL-60 cells, x1000. HL-60 cells and five-day, DMSO-differentiated HL-60 cells both stained positively (green fluorescence represents FITC excitation).

9.3. Proliferation and viability of HL-60 Cells

The proliferation and viability of DMSO-treated or -untreated HL-60 cells exposed to nicotine or not, as determined using 0.4% trypan blue exclusion coupled with cell counting on a hemocytometer, were observed over five days, as shown in Figures 11 and Figure 12, respectively.

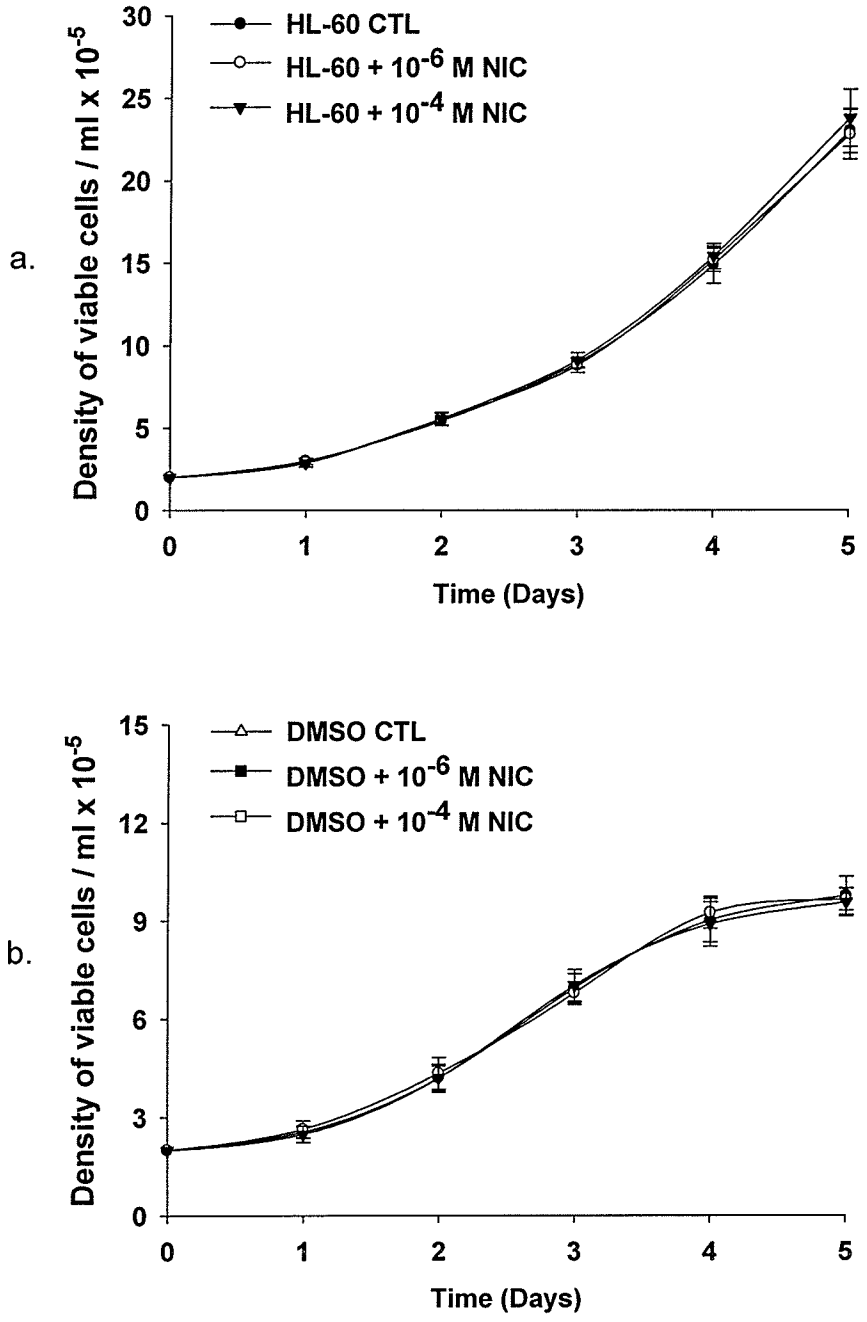

Figure 11: Nicotine effect on the proliferation of DMSO-differentiated or -undifferentiated HL-60 cells. Viable cell density (mean, s.d.) was counted using trypan blue exclusion on a hemacytometer. Nicotine (10^{-6} M or 10^{-4} M) exposure did not have a significant influence on the proliferation of promyelocytic (**a**) or DMSO-induced HL-60 cells (**b**) over five days of cell culture.

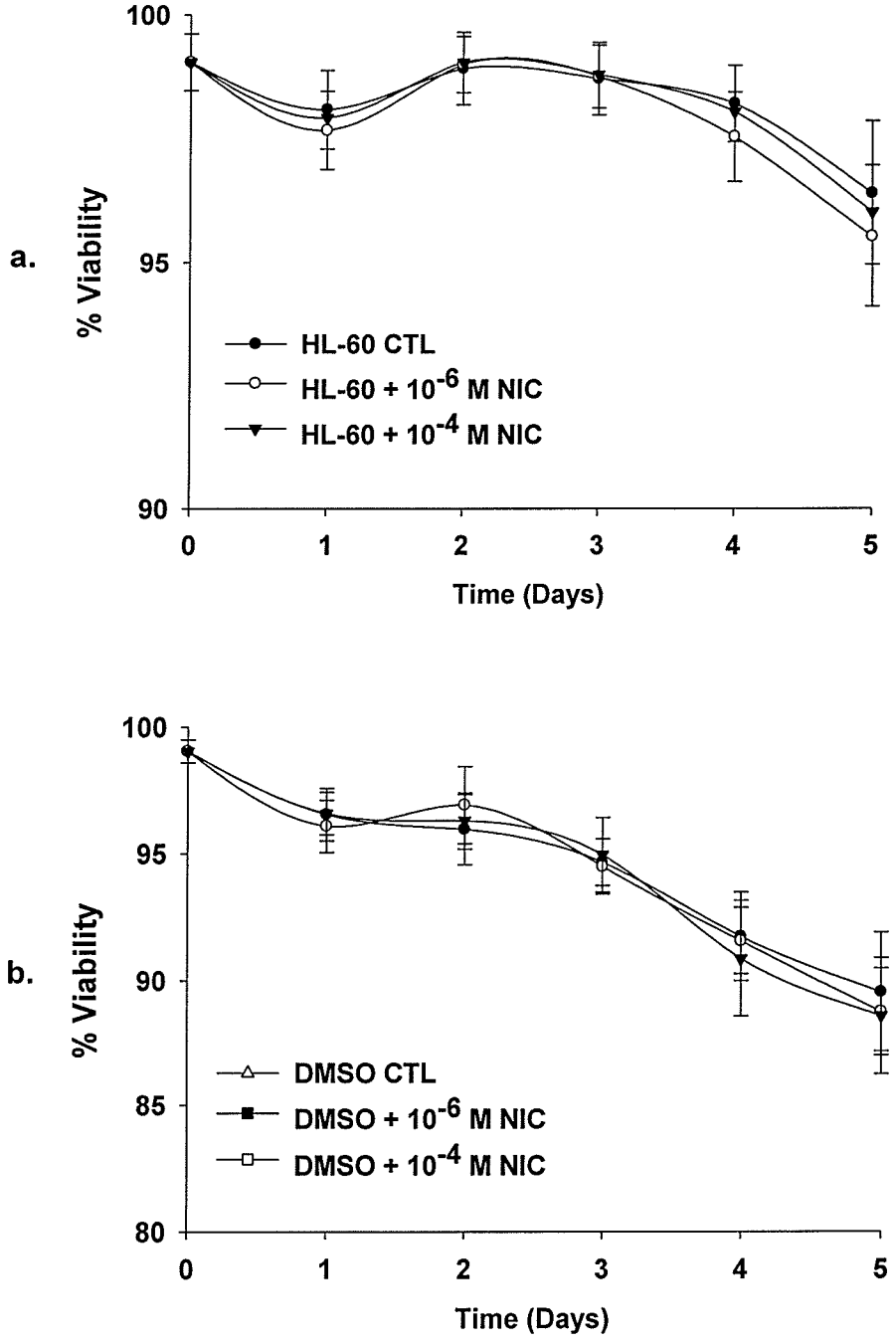

Figure 12: Viability (mean, s.d.) of promyelocytic or DMSO-induced HL-60 cells exposed to nicotine (10^{-6} M or 10^{-4} M) over five days. The viability of both (**a**) promyelocytic and (**b**) DMSO-differentiated HL-60 cells were not affected by nicotine exposure.

9.4. Morphology of HL-60 cells

The morphological features of promyelocytic and DMSO-differentiated HL-60 cells were examined by Hema 3 staining, as illustrated in Figure 13. In the absence of DMSO, HL-60 cells were predominantly typical promyelocytes with large round or oval nuclei, each containing 2-4 nucleoli and dispersed, fine nuclear chromatin. The cytoplasm was basophilic with prominent azurophilic granules. The ratio of nucleus/cytoplasm was relatively high (3:1 to 2:1, *see* Figure 13a). Compared with undifferentiated cells, DMSO-induced HL-60 cells exhibited the following morphological changes: smaller cell size, decreased ratio of nucleus/cytoplasm, fewer prominent cytoplasmic granules, marked reduction or complete disappearance of nucleoli, pyknotic changes in nuclear chromatin, and notable indentation, convolution, and segmentation of the nuclei (Figure 13b, c, d). Thus, the addition of DMSO induces striking morphological changes in HL-60 cells, which resemble terminally differentiated neutrophilic cells, and a cell population comprised of myelocytes, metamyelocytes, band- and segmented neutrophils. These morphologic data are fully consistent with pioneer studies on the HL-60 cell line by Collins *et al.* (1978).

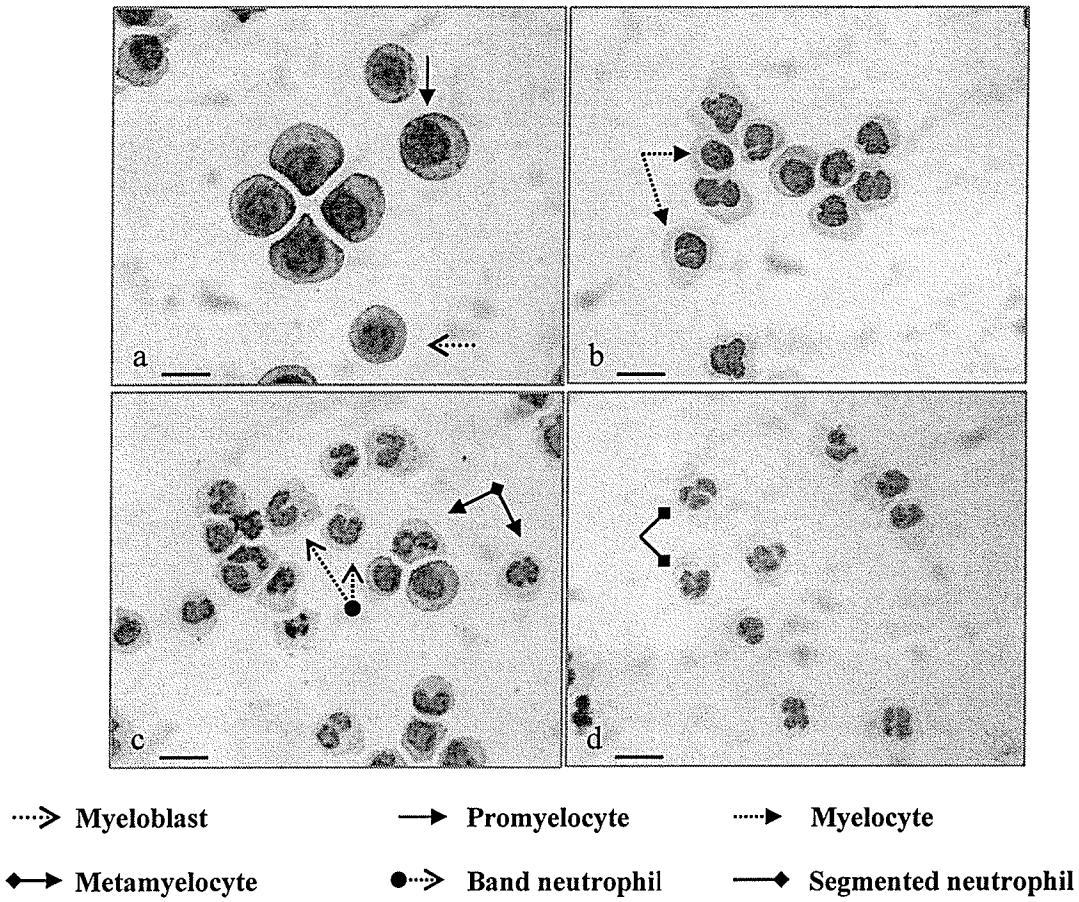

Figure 13: Morphological characteristics of differentiating HL-60 cells. (a). Undifferentiated HL-60 cells; **(b)** HL-60 cells were induced by 1.3% (v/v) DMSO over three days, or **(c)** five days, or **(d)** eight days. Compared to undifferentiated HL-60 cells, the morphological characteristics of differentiating cells include smaller cell size, decreased nucleus/cytoplasma ratio, disappearance of nucleoli, transformation of nuclei, and fewer cytoplasmic granules. Images were taken on an Olympus BX41 microscopy with a magnification of 400x using a SONY 4.1MP digital camera. The bar represents 10 um.

9.5. HL-60 ultrastructure

The ultrastructural characteristics of DMSO-induced HL-60 cells, following a five-day co-incubation with or without nicotine, were revealed by transmission electron microscopy, as illustrated in Figure 14. The most distinctive feature of undifferentiated HL-60 cells was the presence of large cytoplasmic vacuoles containing loosely packed floccular material. Some vacuoles contained a more densely packed core, resembling azurophilic granules. In addition, a large and round or oval nucleus with diffuse chromatin and prominent nucleoli was observed in HL-60 cells (Figure 14a). These ultrastructural observations are consistent with a previous electron microscopic study of HL-60 cells by Rovera *et al.* (1979). After a five-day induction with DMSO, the cells presented a wide variety of morphological changes. The nuclei showed prominent indentation and segmentation, accompanied by the disappearance of nucleoli with the heterochromatin clumped at the nuclear periphery and the euchromatin more centrally located. Typical granules containing material of variable density as well as membrane bound vacuoles partially packed with a dense substance, were readily observed in the differentiated cells (Figure 14b, c, d). DMSO-differentiated HL-60 cells containing fewer vacuoles with a multiple dense core, relative to promyelocytic HL-60 cells, might be in accordance with the decreased azurophilic granule content in DMSO-differentiated HL-60 cells observed by light microscopy (Figure 12). Exposure to nicotine (10^{-6} M or 10^{-4} M) did not induce qualitatively-observable changes in ultrastructure of cells in the late phases of differentiation.

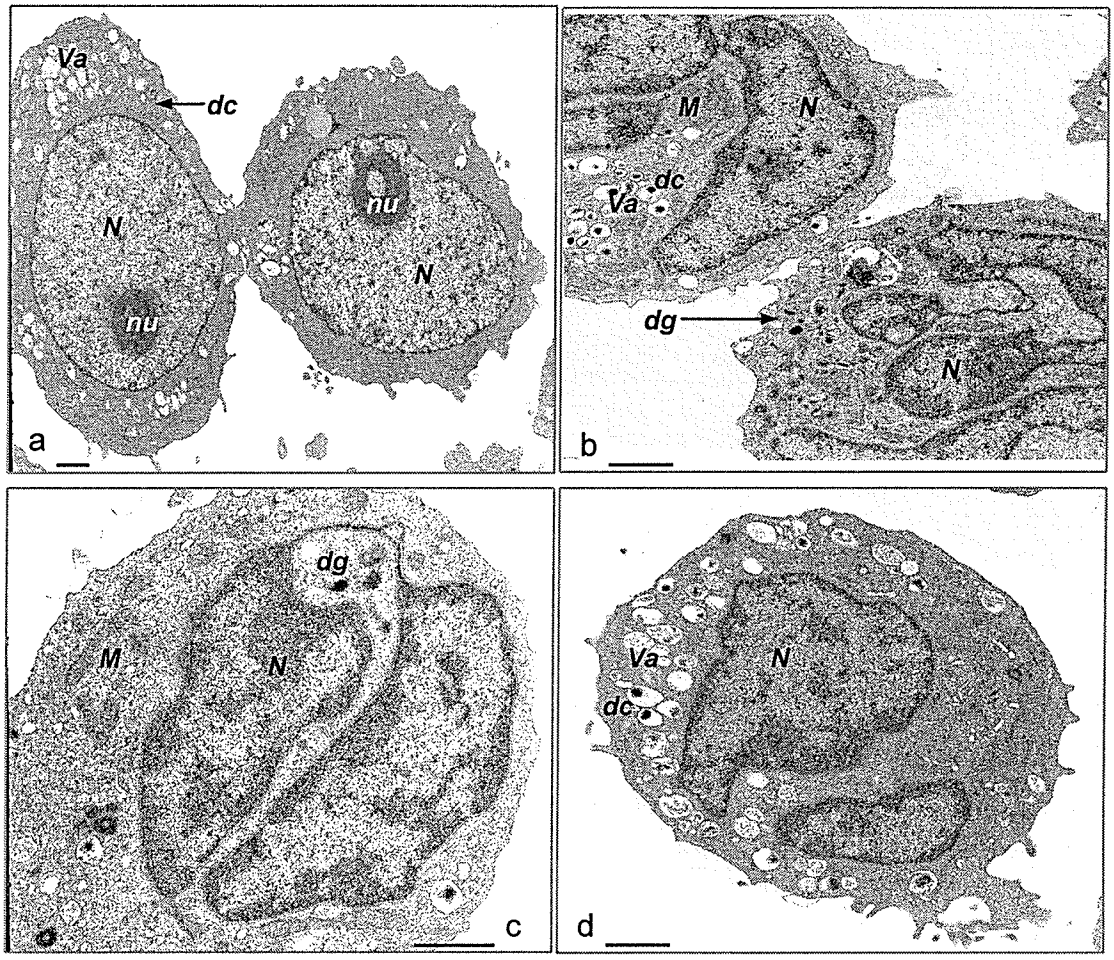

Figure 14: Ultrastructure of differentiating HL-60 cells. **(a).** HL-60 cells (x 1550). Cells contain a large, oval nucleus *(N)* with diffuse chromatin and a prominent nucleolus *(nu)*; a variable number of large cytoplasmic vacuoles containing variously dense core *(dc)* were easily observed in the cells. **(b).** DMSO-differentiated HL-60 cells over five days without nicotine treatment (x 2650), or **(c)** exposed to 10^{-6} M nicotine (x 4300), or **(d)** exposed to10^{-4} M nicotine (x 2650). These cells contain a markedly indented and segmented nucleus with pyknotic nuclear chromatin and the disappearance of nucleolus. Small uniformly dense granules *(dg)* as well as some large vacuoles with *dc* were found in the differentiating cells. *M*: mitochondria. Length of the bar represents 1 μm.

9.6. Morphological differentiation of HL-60 cells

Since DMSO-differentiated HL-60 cells exhibit unique morphological characteristics, as described above, we used Hema 3 staining to determine the stages of the differentiation of HL-60 cells with or without 1.3% (v/v) DMSO treatment over five days (Table 4). The spontaneous myeloid differentiation rate of DMSO-untreated HL-60 cells remained stable [7.1 ± 1.5%; (mean, s.d.), Table 4], regardless of nicotine exposure. Nicotine (10^{-6} M or 10^{-4} M) had no statistically significant influence on the percentage of morphologically mature cells at day 5 following DMSO-stimulation [81.9 ± 1.6% and 83.1 ± 2.4% (mean, s.d.), respectively], relative to DMSO control [80.8 ± 2.7%, (mean, s.d.)], as shown in Figure 15.

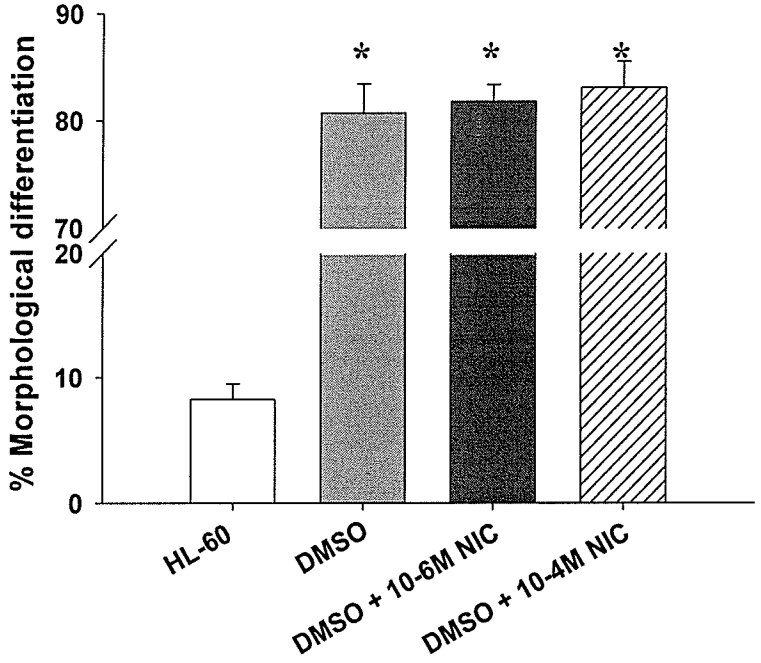

Figure 15: Morphological differentiation of DMSO-induced HL-60 cells treated with or without nicotine over five days. The percentage (mean, s.d.) of morphologically mature HL-60 cells significantly increased upon the addition of DMSO (* $p<0.05$), in contrast to undifferentiated HL-60 cells, irrespective of nicotine exposure. Morphologically differentiated cells refer to myelocytes, metamyelocytes, band cells and mature (segmented) neutrophils

Table 4: Percentage (mean, s.d.) differentiation stages of HL-60 cells stimulated with or without DMSO over five days. Five days nicotine exposure (10^{-6}M or 10^{-4}M) did not significantly affect the spontaneous neutrophilic differentiation of promyelocytic HL-60 cells. Morphologically differentiated cells refer to myelocytes, metamyelocytes, band cells and mature (segmented) neutrophils.

			Differentiated cells			
	Myeloblast	**Premyelocyte**	**Myelocyte**	**Metamyelocyte**	**Band cell**	**Neutrophil**
HL-60 CTL (day 0)	15.25 (2.13)	78.19 (1.11)	4.94 (1.20)	1.81 (0.24)	0.31 (0.24)	0
HL-60 CTL (day 5)	14.88 (1.11)	77.00 (1.47)	5.50 (0.91)	2.25 (0.65)	0.38 (0.25)	0
HL-60+Nic 10^{-6}M (day 5)	14.83 (2.29)	76.63 (2.90)	5.63 (1.03)	2.75 (0.65)	0.38 (0.48)	0
HL-60+Nic 10^{-4}M (day 5)	13.75 (2.22)	76.75 (2.10)	6.50 (0.71)	2.50 (0.41)	0.50 (0.41)	0
DMSO CTL (day 5)	0.38 (0.44)	18.88 (2.53)	27.63 (3.04)	30.56 (2.20)	21.38 (2.77)	1.44 (0.50)

9.7. Nitroblue Tetrazolium (NBT) Reduction Test for HL-60 Cells

Like mature primary neutrophils, differentiated HL-60 cells have a developed respiratory burst sufficient to reduce NBT. Reduced NBT forms intracellular purple or dark blue granular deposits that can be visualized by light microscopy. Indeed, the ability to reduce NBT can serve as a biochemical marker of neutrophilic maturation (Glasser and Fiederlein, 1987 and Hua *et al.*, 2000). After five days of culture, the percentages of cells reducing NBT (NBT positive cells) remained low [7.5 ± 1.8 %, (mean, s.d.)] in DMSO-untreated HL-60 cells (Figure 16a), in contrast to the dramatic increase [82.8 ± 3.1 %, (mean, s.d.)] in the DMSO-treated groups, irrespective of the presence of 10^{-6} M or 10^{-4} M nicotine (Figure 16b). A quantitative analysis of the respiratory burst is provided later.

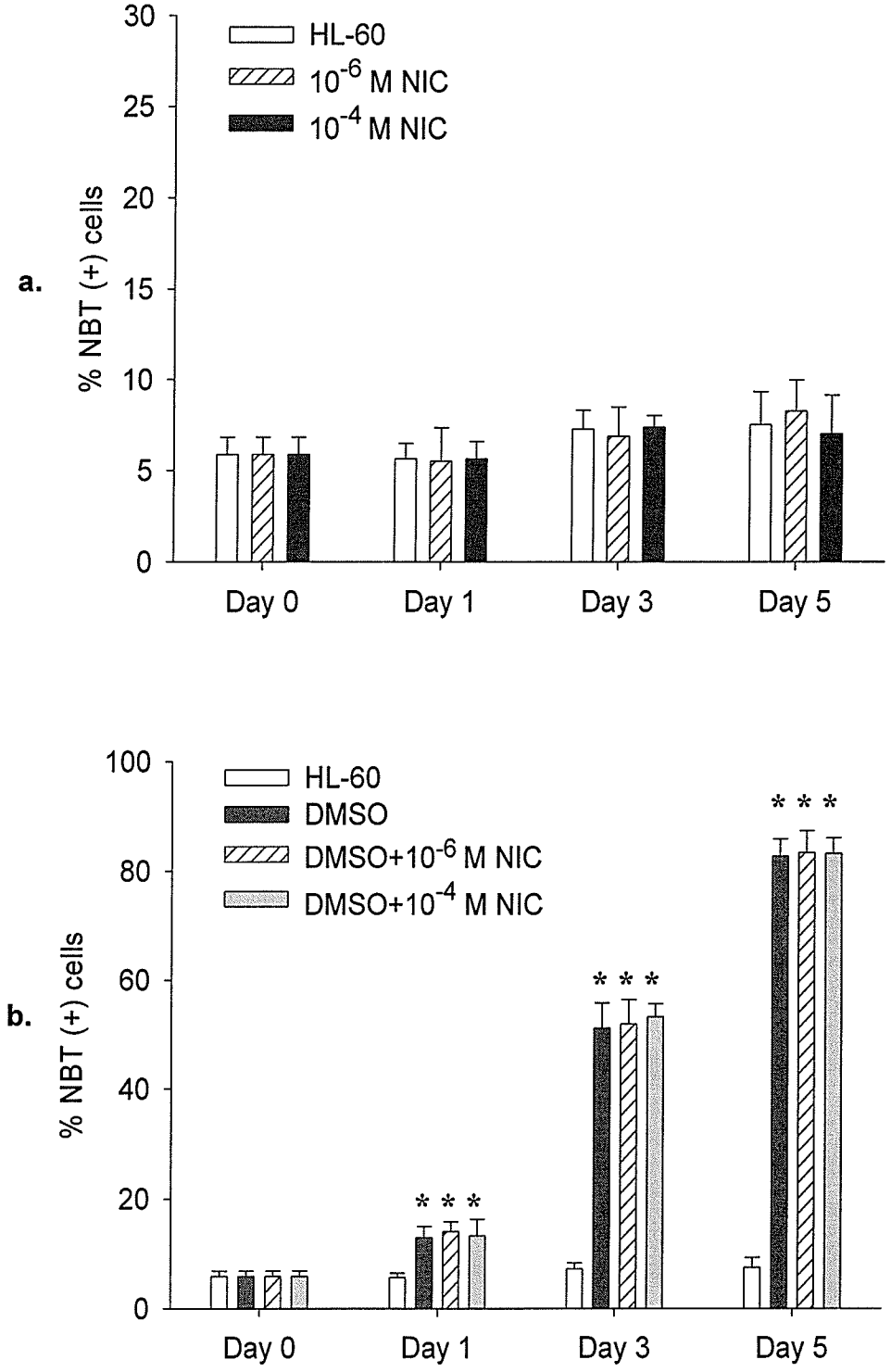

Figure 16: NBT reduction test of promyelocytic or DMSO-differentiated HL-60 cells in the presence of nicotine. The percentages (mean, s.d.) of NBT positive cells in five-day HL-60 cell culture **(a)**, and DMSO-induced HL-60 cell culture **(b)** exposed to 10^{-6} M or 10^{-4} M nicotine. DMSO exposure significantly increased the percentages of NBT positive cells (* $p<0.05$), relative to DMSO-untreated HL-60 cells, irrespective of the presence of nicotine.

9.8. Expression of CD11b (integrin κM) by HL-60 cells

In order to determine whether nicotine changes the expression of the terminal myeloid differentiation molecular marker-CD11b by DMSO-induced HL-60 cells, flow cytometry was used. The results are shown in the Figure 17. The percentages of HL-60 cells expressing spontaneously CD11b were as low, at 5 - 8%. However, CD11b expression levels increased dramatically after a five-day stimulation with DMSO (79.7 ± 7.4%), irrespective of the presence of nicotine (10^{-6} M or 10^{-4} M).

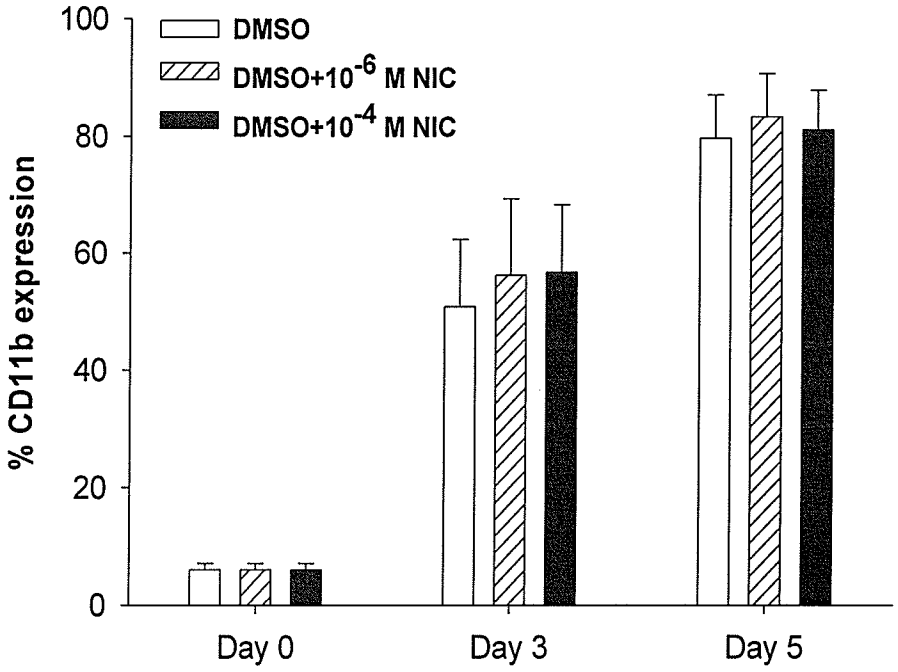

Figure 17: CD11b expression (%, mean, s.d.) by DMSO-induced HL-60 cells with or without nicotine treatment over five days. The CD11b expression of DMSO-differentiated HL-60 cells was significantly elevated with time, irrespective of nicotine exposure.

9.9. Cell Cycle of HL-60 cells

Cell cycle characteristics of promyelocytic or DMSO-stimulated HL-60 cells exposed to nicotine (10^{-6} M or 10^{-4} M) over five days, as determined by PI DNA staining using a flow cytometer, are shown in Figure 18 and Table 5. Cells sorting into the area before the Go/G1 peak were presumed to be apoptotic cells. Both nicotine concentrations did not have a significant effect on the apoptotic rate of promyelocytic HL-60 cells, whereas DMSO induction increased the apoptotic HL-60 cells significantly, compared to undifferentiated HL-60 cells, irrespective of nicotine treatment (Figure 18). DMSO also inhibited HL-60 cell proliferation and mitosis by blocking more cells in the static stage (G0/G1) of cell cycle, relative to DMSO-untreated cells, regardless of nicotine exposure (Table 5).

	AP (pro-G0/G1)	G0/G1	G2/M
HL-60 (day 0)	2.5 (0.8)	47.6 (3.2)	20.7 (2.5)
		Day 5	
HL-60	6.7 (0.4)	51.3 (1.5)	17.2 (1.0)
HL-60+10^{-6}M NIC	6.7 (0.6)	50.0 (1.8)	17.5 (0.6)
HL-60+10^{-4}M NIC	6.3 (0.4)	49.7 (1.3)	18.9 (0.3)
DMSO	13.4 (1.1)	79.6 (3.5)	3.5 (0.2)
DMSO+10^{-6}M NIC	14.3 (3.5)	78.4 (3.6)	3.6 (0.9)
DMSO+10^{-4}M NIC	15.8 (2.0)	78.1 (1.9)	2.7 (0.6)

Table 5: Cell cycle (PI DNA staining) of DMSO-treated and -untreated HL-60 cells exposed to nicotine over five days. The cell cycle stage percentages [mean (s.d.)] of DMSO-treated and -untreated HL-60 cells were not significantly altered upon nicotine (10^{-6} or 10^{-4}M) exposure over five days.

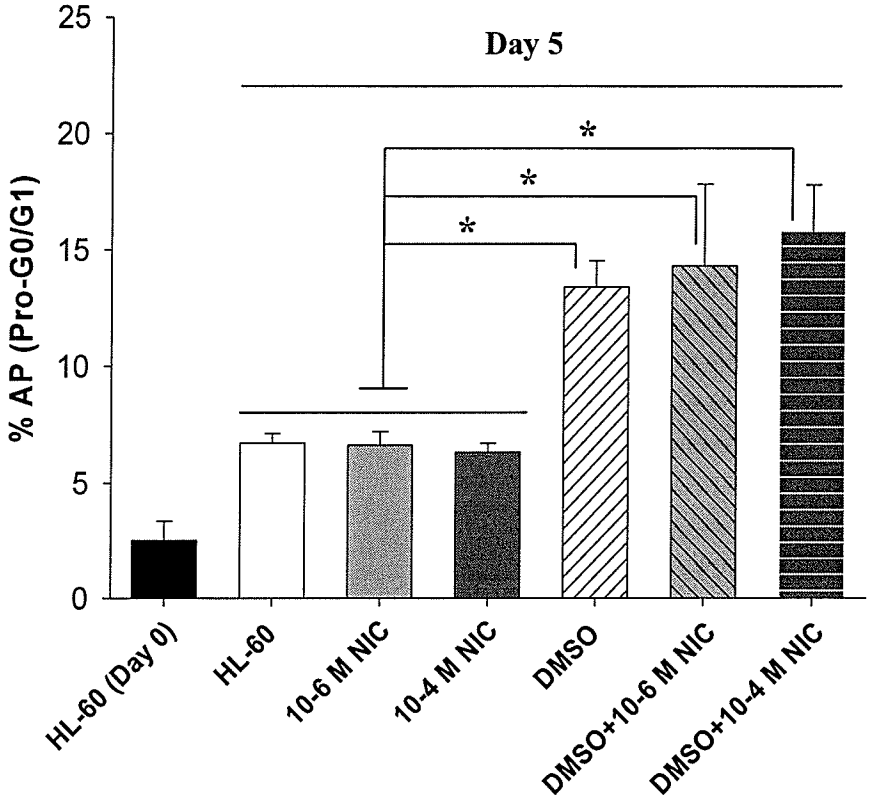

Figure 18: Apoptosis (percentage of pro-G0/G1) of five days, DMSO-treated and -untreated HL-60 cells with or without nicotine exposure. Normally maintained HL-60 cells were used as a standard control. The percentages (mean, s.d.) of apoptotic cells were increased by approximately 100% in DMSO-treated cell cultures (* $p<0.05$), compared to five days DMSO-untreated HL-60 cell group, regardless of the addition of nicotine (10^{-6}M or 10^{-4}M).

9.10. Conformational changes

FT-IR spectroscopy revealed alterations to the relative IR band intensities of lipid and DNA signals, and to the ratios of protein (amide I: amide II) and DNA : amide II noted in five-day cultures of undifferentiated HL-60 cells exposed to nicotine (Figure 19-II) and five-day, DMSO-differentiated HL-60 cell cultures (Figure 19-IV). These specific molecular and sub-molecular profile alterations are indicative of pro-apoptotic changes (Zhou *et al.*, 2002). Additionally, IR spectra of all cell samples did not show a shoulder

band centered at 1621 cm^{-1} (Figure 19-I and 19-III), a recommended band specific for HL-60 cell necrosis (Gasparri and Muzio, 2003).

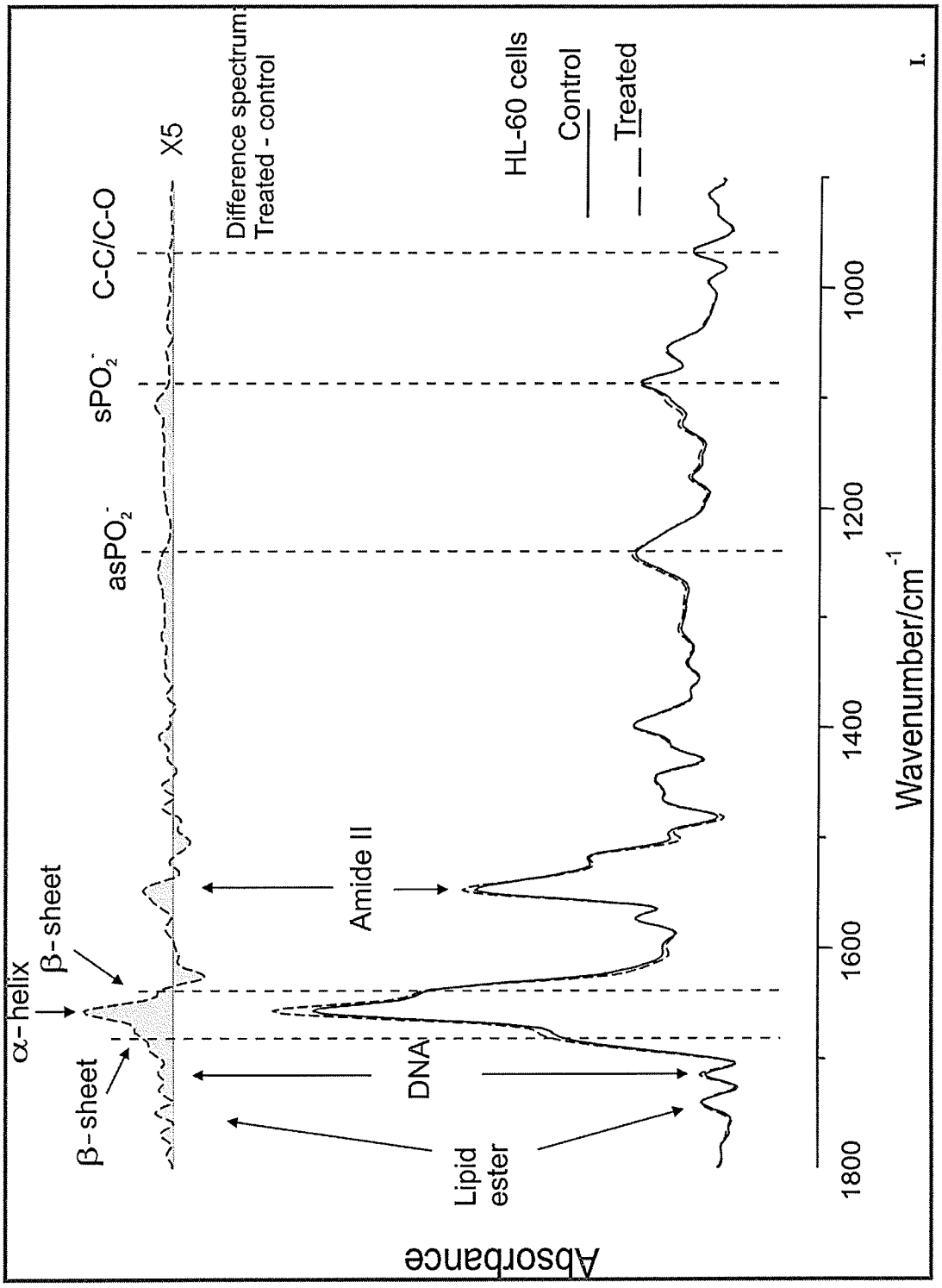

Figure 19 - I: Characteristic IR spectrum of HL-60 cells treated with or without 10^{-6} M nicotine over five days. The difference spectrum of nicotine-treated cells and control HL-60 cells is shown (top).

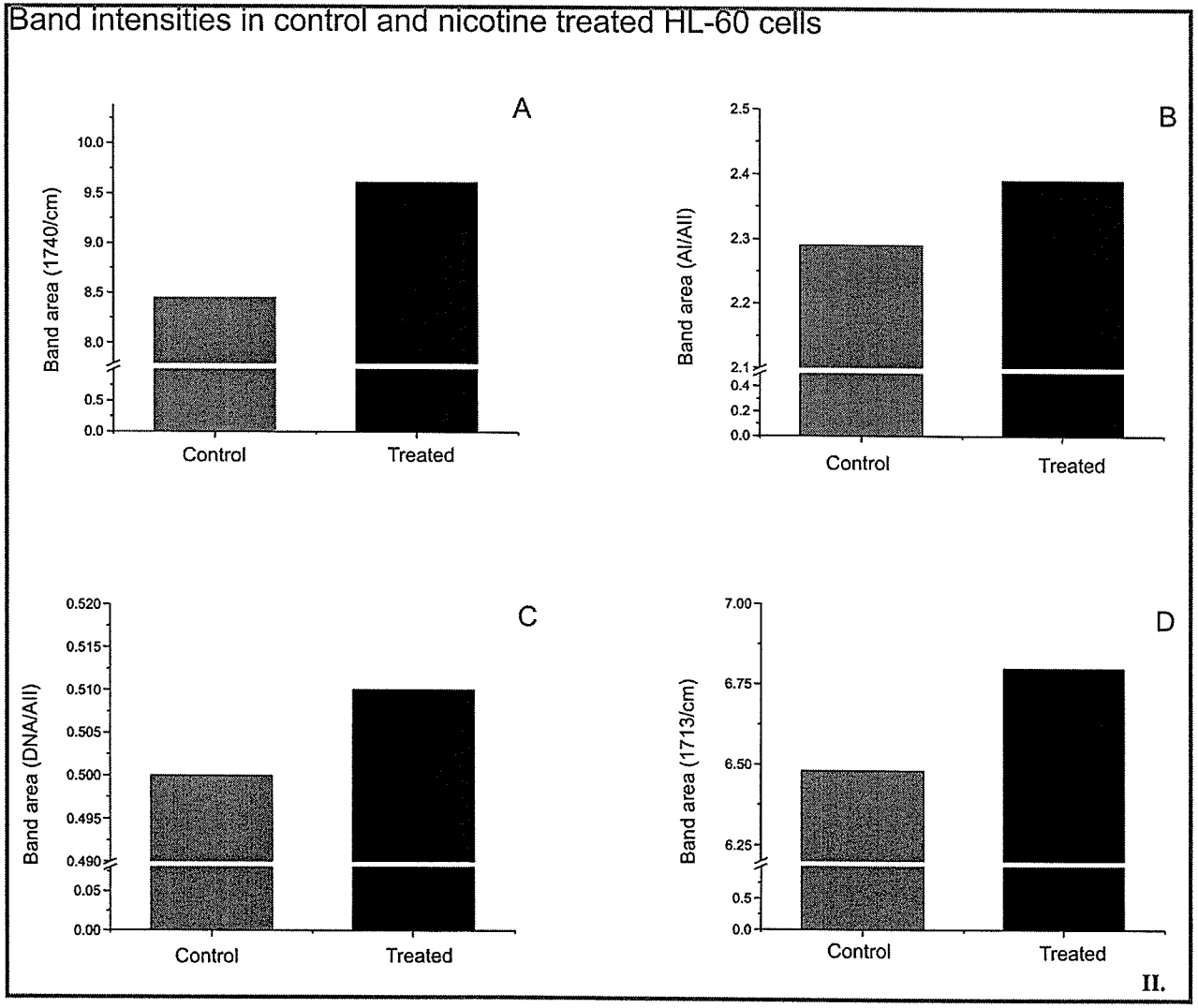

Figure 19 - II: Bar diagrams of specific integrated IR spectral area of HL-60 cells treated with or without 10^{-6} M nicotine over five days. Comparison of the major molecular components, represented by bar diagrams of the integrated areas of cellular (A) lipid; (B) protein (amide I / amide II); (C) DNA-protein ratio (1086 cm^{-1} / amide II); and (C) DNA content (1713 cm^{-1}).

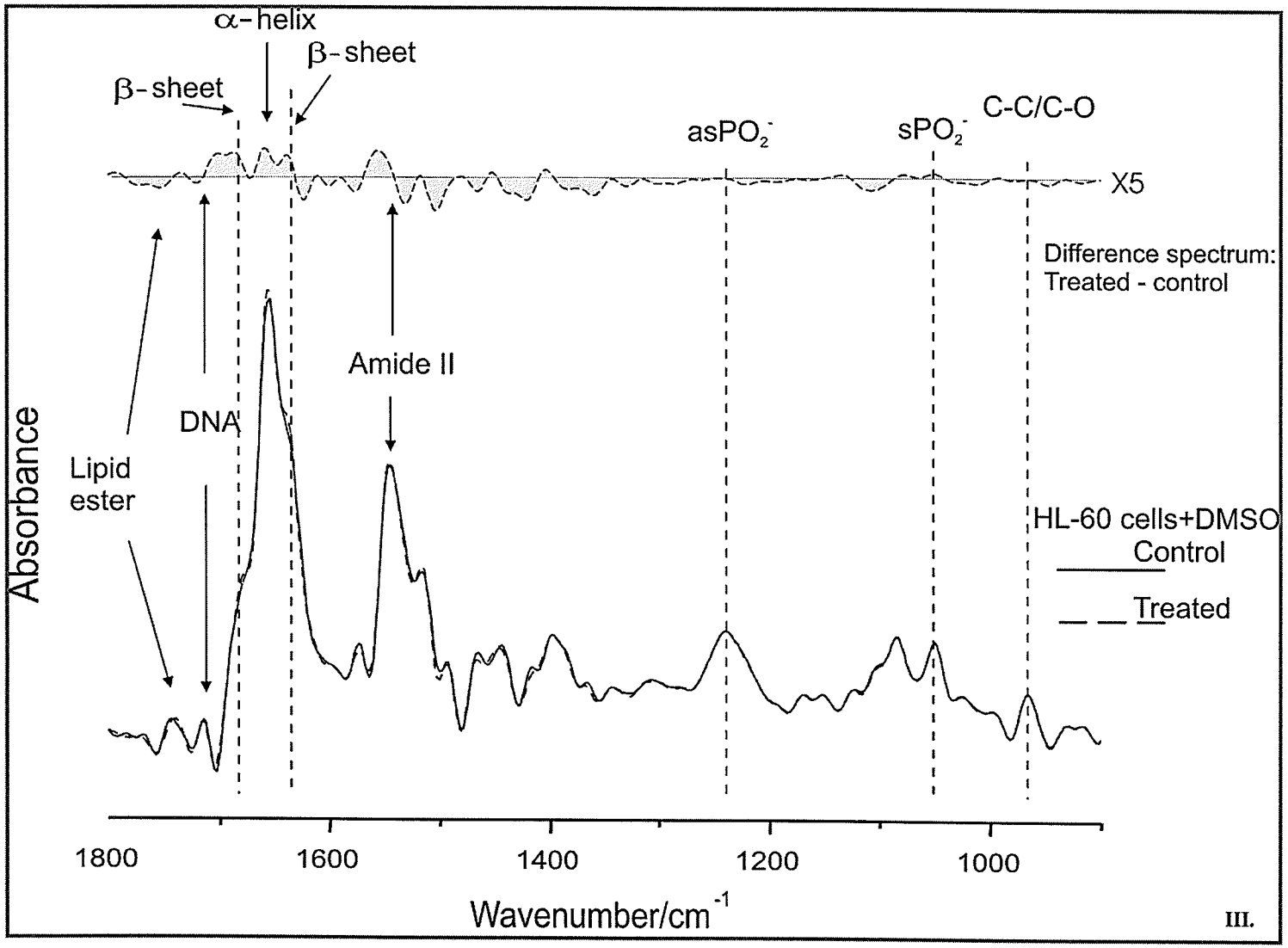

Figure 19 - III: Characteristic IR spectrum of DMSO-induced HL-60 cells treated with or without 10^{-6} M nicotine over five days. The difference spectrum of nicotine-treated cells and DMSO control cells is shown (top).

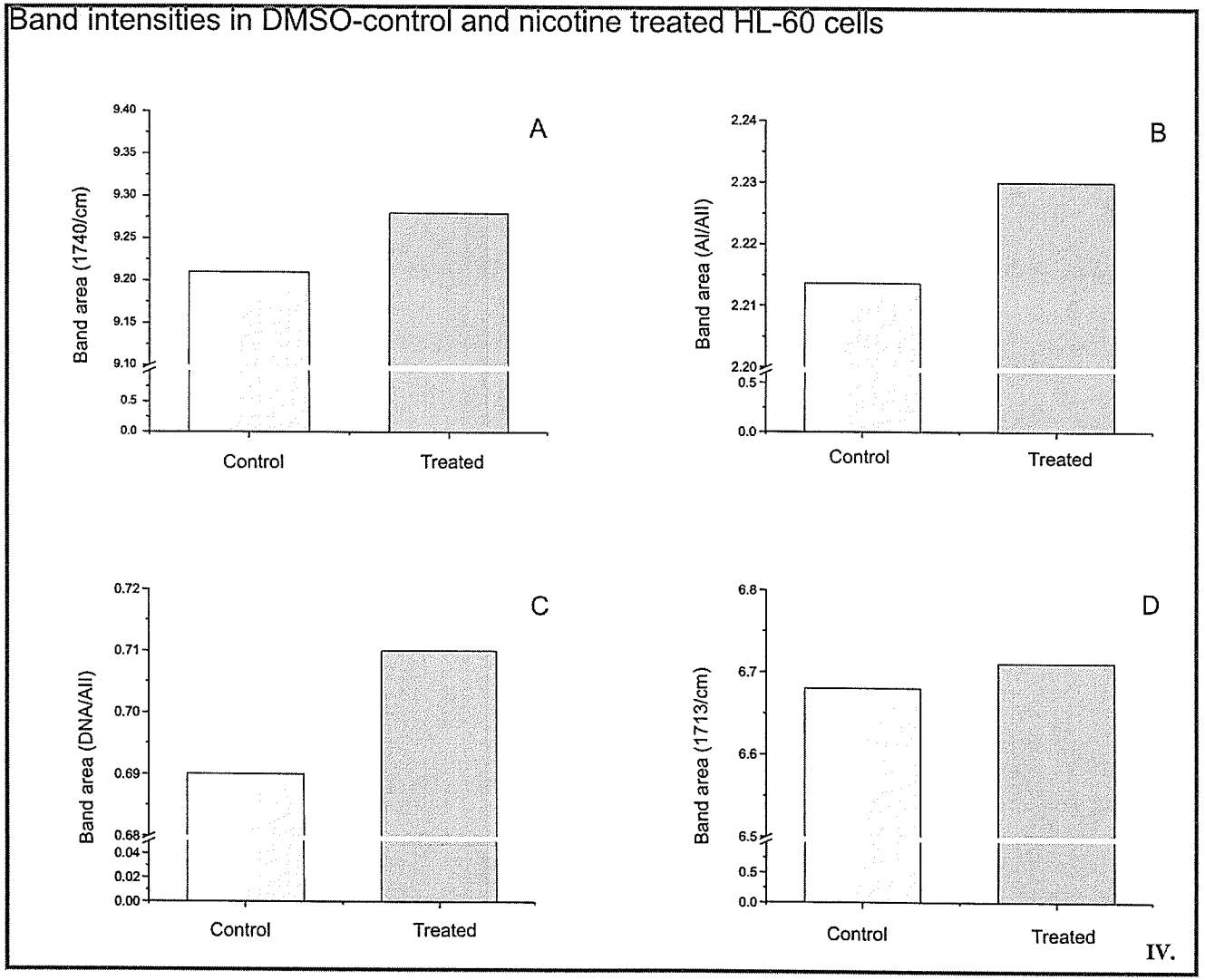

Figure 19 - IV: Bar diagrams of specific integrated IR spectral area of DMSO-induced HL-60 cells treated with or without 10^{-6} M nicotine over five days. Comparison of the major molecular components, represented by bar diagrams of the integrated areas of cellular (A) lipid; (B) protein (amide I / amide II); (C) DNA-protein ratio (1086 cm^{-1} / amide II); and (C) DNA content (1713 cm^{-1}).

9.11. Quantitative Analysis of the Respiratory Burst

To determine whether nicotine changes the respiratory burst profiles of five day, DMSO-differentiated HL-60 cells, we quantitatively measured the PMA-induced cell oxidative burst ability to reduce NBT, as shown in Figure 20. Nicotine (10^{-4} M or 10^{-6} M) impaired the capacity of DMSO-differentiated cells to reduce NBT in a dose-dependent manner, (approximately 50% and 30% respectively), relative to DMSO control.

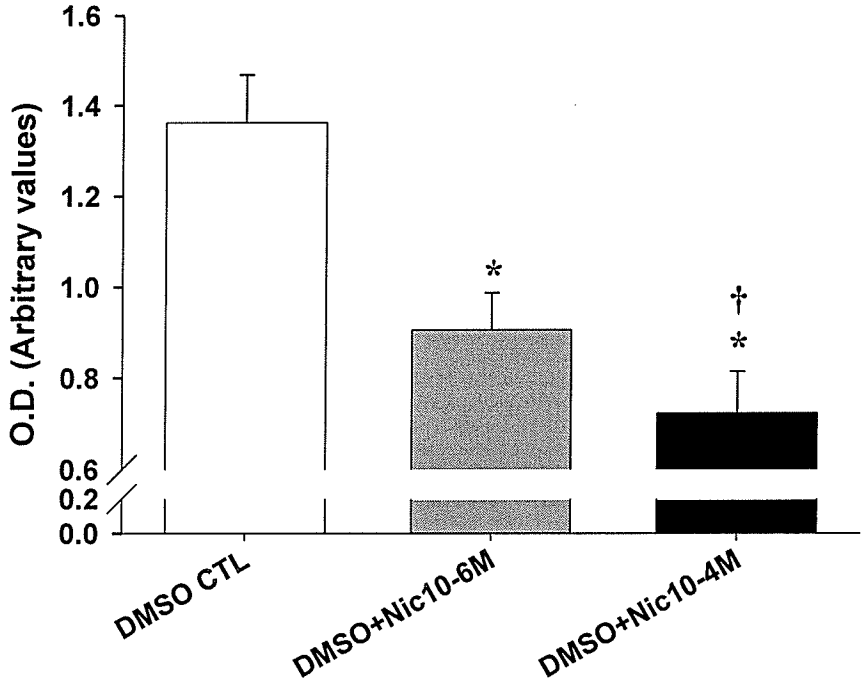

Figure 20: Oxidative burst of five day, DMSO-differentiated HL-60 cells treated with or without nicotine (10^{-6} M or 10^{-4} M) by quantitative NBT reduction test. The NBT formazan optical densities (mean, s.d.) of DMSO-differentiate HL-60 cells decreased significantly upon nicotine treatment. (* $p<0.05$, refer to DMSO control; † $p<0.05$, relative to nicotine 10^{-6} M treated-groups only)

9.12. MMP- 9 and MMP-2 Release by HL-60 cells

To examine whether nicotine alters MMP-2 and MMP-9 secretion of DMSO-differentiated HL-60 cells, gelatin zymography was performed using conditioned media collected from five-day DMSO-differentiated HL-60 cells with or without nicotine (10^{-6} M or 10^{-4} M) exposure, followed with LPS stimulation over 24 hour, as illustrated in Figure 21 and 22. Differentiated HL-60 cells secreted both MMP-9 (92kDa) and MMP-2 (72 kDa) zymogen, as indicated by the clear bands (digesting gelatin substrate) contrasted with the dark background (Figure 21a and Figure 22a). Nicotine exposure increased the MMP-9 released by DMSO-differentiated HL-60 cells upon one-hour LPS stimulation in a dose dependent manner, as well as 24-hour LPS stimulation (Figure 21b). MMP-2 profiles were not significantly changed by nicotine (Figure 22b). Pretreatment with the α7-nAChR inhibitor, α-bungarotoxin (200ng ml^{-1}, final concentration), prior to the addition of nicotine, abrogated the enhanced MMP-9 secretion by neutrophil-differentiated HL-60 cells, as demonstrated in Figure 23. In addition, small amounts of MMP-9 were released spontaneously by DMSO-differentiated HL-60 cells only exposed to 10^{-4} M nicotine over five days, but not DMSO control and lower nicotine dose groups, supporting that nicotine augments the MMP-9 secretion by differentiated HL-60 cells in a dose-dependent manner (data not shown).

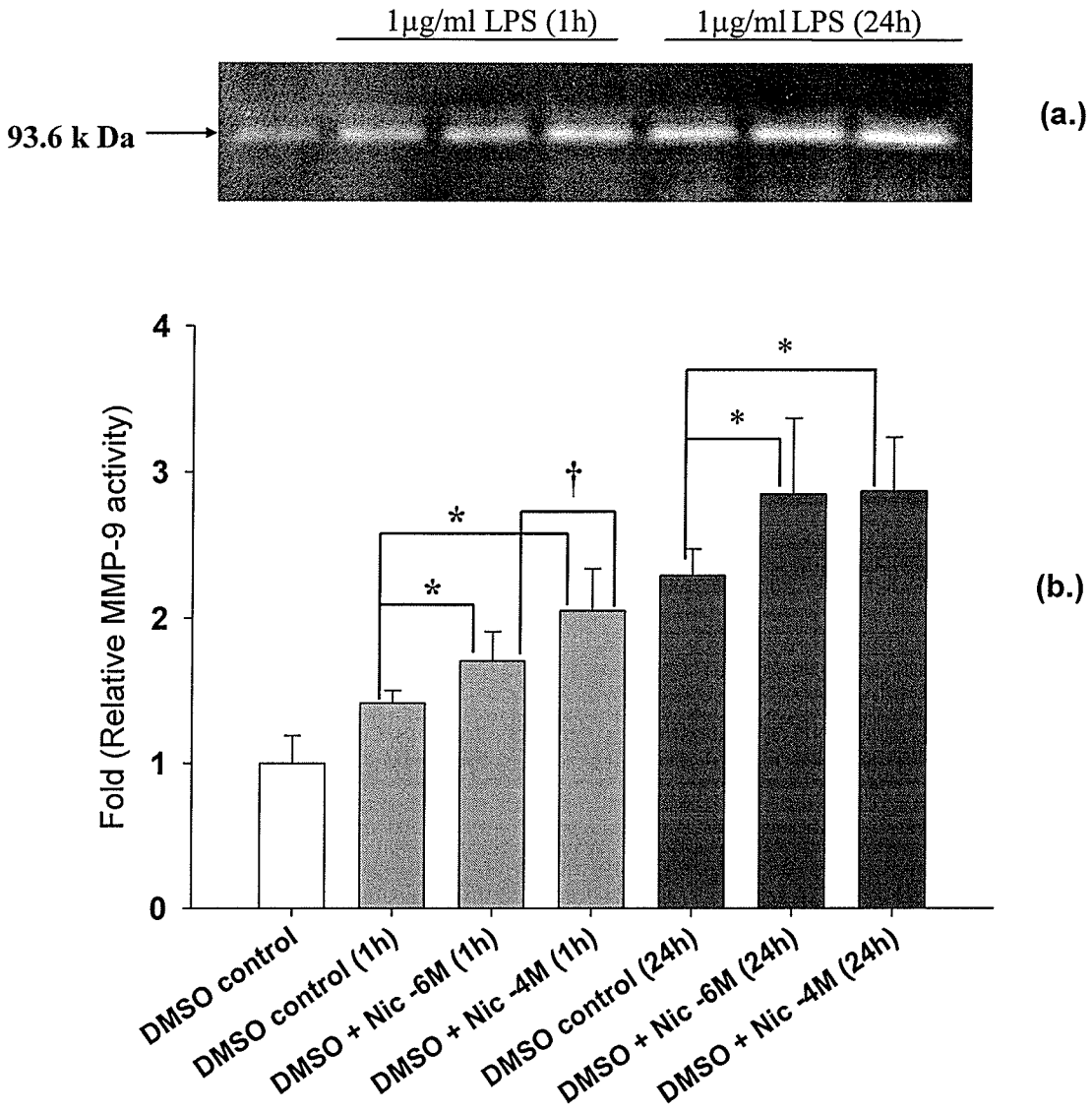

Figure 21: MMP-9 gelatin zymography of DMSO-differentiated HL-60 cells. **(a).** A representative gelatin zymographic gel image clearly indicated the MMP-9 band (92kDa), from the left to the right, conditioned media (corresponding to 10000 cells) of five-day DMSO-differentiated HL-60 cells (lane 1), or followed with a one-hour (lane 2) or 24-hour (lane 5) LPS stimulation, as well as five-day DMSO-differentiated HL-60 cells exposed to 10^{-6} M (lane 3) or 10^{-4} M nicotine (lane 4) after a one-hour (lane 6) or 24-hour (lane 7) LPS stimulation, was loaded and size-fractionized by 8.0% SDS-PAGE gels. **(b).** The relative fold (mean, s.d.) of MMP-9 proteolytic activity was quantified by densitometry. (* $p<0.05$, * $p<0.05$, † $p<0.05$)

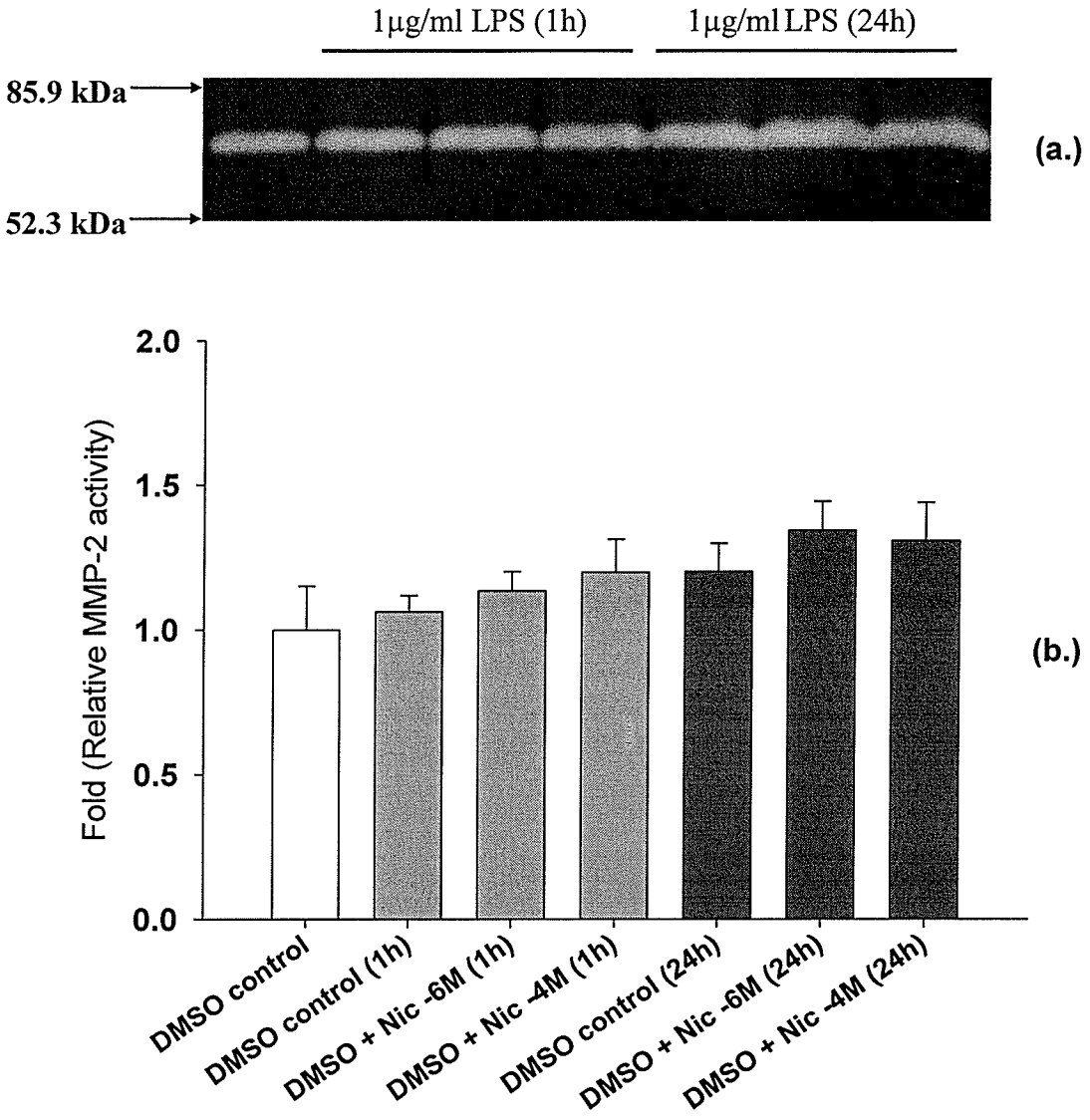

Figure 22: MMP-2 gelatin zymography of DMSO-differentiated HL-60 cells. **(a).** A representative gelatin zymographic gel image clearly indicated the MMP-2 band (70kDa), from the left to the right, conditioned media (corresponding to 10000 cells) of five-day DMSO-differentiated HL-60 cells (lane 1), or followed with a one-hour (lane 2) or 24-hour (lane 5) LPS stimulation, as well as five-day DMSO-differentiated HL-60 cells exposed to 10^{-6} M (lane 3) or 10^{-4} M nicotine (lane 4) after a one-hour (lane 6) or 24-hour (lane 7) LPS stimulation, was loaded and size-fractionized by 8.0% SDS-PAGE gels. **(b).** The relative fold (mean, s.d.) of MMP-2 proteolytic activity was quantified by densitometry.

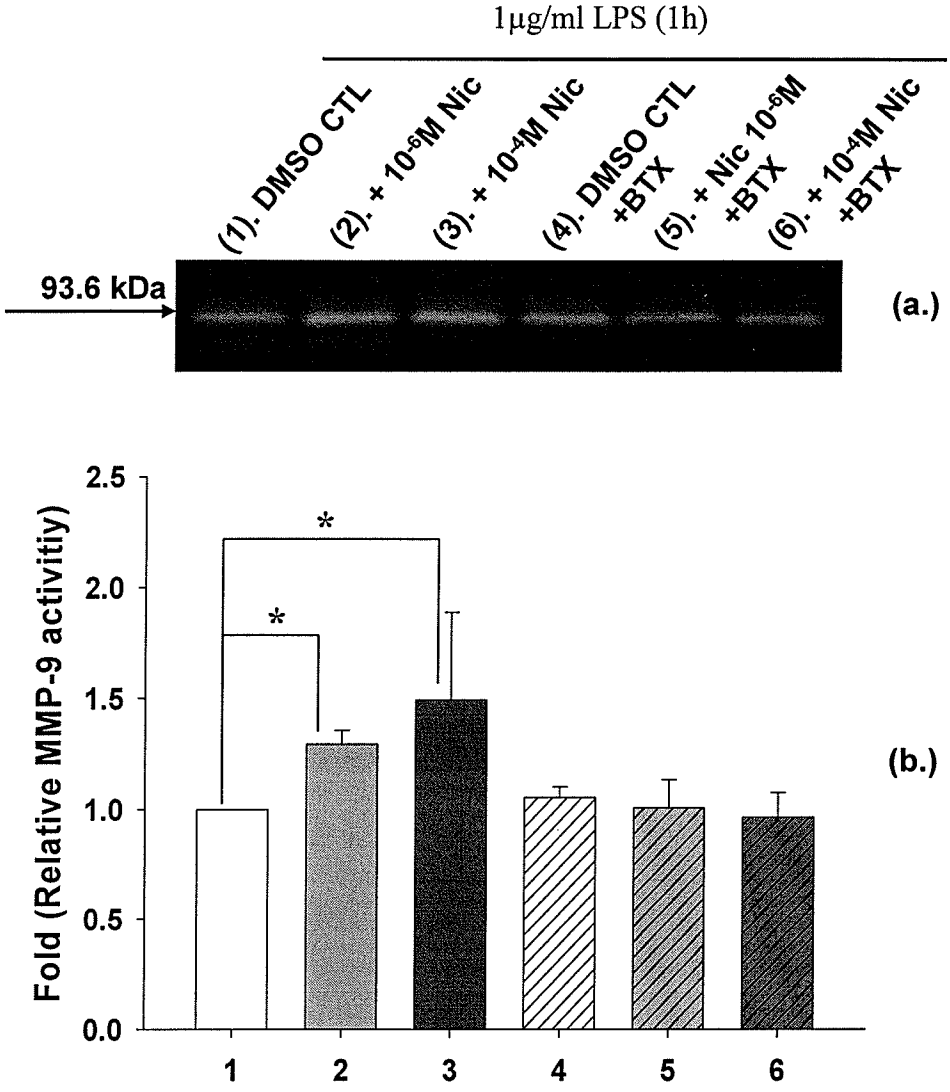

Figure 23: Pretreatment of α-bungarotoxin (BTX) abrogates nicotine-enhanced, LPS-induced MMP-9 release by DMSO-differentiated HL-60 cells. BTX (200ng/ml^{-1}, final concentration) was added to cell culture 30 minutes prior to the exposure to nicotine. (a). A representative gelatin zymographic gel image clearly indicated the MMP-9 band (92kDa) in conditioned media (corresponding to 10000 cells) of five-day DMSO-differentiated HL-60 cells without nicotine exposure (lane 1), and with 10^{-6} M (lane 2) or 10^{-4} M nicotine exposure (lane 3); the individually corresponding groups with the pre-treatment of BTX are lane 4, lane 5 and lane 6, respectively. All cell cultures were stimulated with 1μg/ml (final concentration) LPS for one hour before harvesting the cell free media for zymography. (b). The relative fold (mean, s.d.) of MMP-9 proteolytic activity was quantified by densitometry. (* $p<0.05$)

CHAPTER 10

DISCUSSION AND CONCLUSIONS

Leukocytosis, and particularly granulocytosis, reflects an active, systemic inflammatory response (Hubl *et al.*, 1997) which is thought to contribute to increased morbidity and mortality (Friedman *et al.*, 1974; Grimm *et al.*, 1985; Weiss *et al.* 1995; Furman *et al.*, 1996 and Suwa *et al.*, 2001). Leukocytosis, and particularly neutrophilia, has been well documented in smokers (Chan-Yeung *et al.*, 1981 and Vanuxem *et al.*, 1984). This leukocytosis is adversely correlated with lung function, contributing to higher mortality rates in smokers (Sparrow *et al.*, 1984; Bridges *et al.*, 1985 and 1986; Chan-Yeung *et al.*, 1988; Carel *et al.*, 1988 and Jensen *et al.*, 1998). Although potential mechanisms underlying neutrophilia in smokers have been proposed - such as tobacco-induced the alteration of neutrophil chemotactic profile or the dysregulation of neutrophil apoptosis (Aoshiba *et al.*, 1996; Mariggio *et al.*, 2001 and Iho *et al.* 2003) - the results obtained are conflicting. Several profound effects of tobacco smoke or smoke components on multiple aspects of neutrophil physiology, including the effects on cell adhesion, migration, chemotaxis, oxidative burst, proteinase release and apoptosis, have been widely reported, which may be relevant to the progression of several inflammatory diseases and conditions, including COPD (Shapiro *et al.* 2003; Churg and Wright *et al.*, 2005 and Drost *et al.*, 2005), atherosclerosis and other vascular diseases (Blann, 1997; Dunn *et al.*, 2005 and Kougias *et al.*, 2005), asthma (Cundall *et al.*, 2003 and Wenzel, 2003), and periodontitis (Deas *et al.*, 2003; Scott and Singer, 2004; Palmer and Scott *et al.*, 2001 and 2005). Nevertheless, the collective data are conflicting and controversial, as reviewed in

the Introduction. Hence, it is urgently necessary to clarify the essence of tobacco-related neutrophilia and associated alterations in the effector functions of neutrophils.

Neutrophils leave the bone marrow as terminally differentiated cells with low transcriptional activity that, without appropriate inflammatory stimuli, normally have a short life span of one to two days (Borregaard, 1997 and Witko-Sarsat *et al*, 2000). Indeed, many biologically active effectors of mature neutrophils, such as multiple granules containing various proteinases and bacteriostatic/bactericidal agents, are well or fully developed even before neutrophils are liberated to the peripheral blood from the bone marrow (Ross and Pawlina, 2006). Glasser and Fiederlein (1987) indicated that the sequence for the functional differentiation of the human neutrophil appeared to be the following: Fc receptors -- immune phagocytosis -- complement receptors -- oxygen-independent microbial killing -- oxygen-dependent microbial killing -- chemotaxis. Therefore, many important tobacco-induced alterations to neutrophil physiology may occur before neutrophils even reach the systemic circulation. In rabbits, *in vivo* cigarette smoke exposure has been associated with the increased numbers of both mature and band cell (immature) neutrophils liberated from the bone marrow into the circulation, resulting in preferential sequestration in the microvasculature (Terashima *et al.*, 1999). Considering the high particulate content of tobacco smoke, it may be relevant to note that the supernatant from alveolar macrophages exposed to air pollution particles smaller than 10 μm can promote cell differentiation in HL-60 cells, as measured by surface CD11b and CD14 expression (Suwa *et al.*, 2002).

Of the more than 4000 compounds in tobacco smoke complex, nicotine is primarily responsible for addiction and is the best studied. In smokers, the pharmacological concentrations of nicotine are detectable in multiple human tissues and organs, and various body fluids.

In the present study, we primarily examined the influence of nicotine on promyelocyte to mature neutrophil differentiation in an HL-60 cell model, which expresses functional nAChRs. We found that nicotine significantly affected several neutrophil functions, i.e. a reduced oxidative burst, increased MMP-9 release, and pro-apoptosis associated molecular modifications. However, cell proliferation and viability, cell cycle, cell ultrastructure, expression of the terminal differentiations markers - CD 11b, and the percentage of NBT positive cells in the neutrophil-differentiated cell model were not influenced by nicotine exposure.

Recently, considerable evidence has revealed that the expression of the α7 nAChR is not limited to neurons but is also expressed by multiple leukocytes (Villiger *et al.*, 2002 and Gimonet *et al.*, 2003). α7 nAChR-mediated nicotine signaling has been shown to regulate antibody production by, and the proliferation of, lymphocytes (Rosa *et al.*, 2004 and Skok *et al.*, 2005); to alter cytokine production profiles in macrophages (Wang *et al.* 2002; Scott and Martin, 2006); and to impact on apoptosis in HL-60 cells (Gimonet *et al.*, 2003). We herein confirm α7 nAChRs expression by HL-60 cells using western blot and immunoflourescence assays. To the best of our knowledge, the present study is the first to prove the positive expression of the α7 subunit (55kDa) in HL-60 cell

line using an immunoblot method. Our data showed that the 55kDa protein expression was up-regulated upon the addition of DMSO, irrespective of nicotine exposure. Previously, Cormier *et al.* (2004) reported that the basal expression levels of homomeric α7 nAChRs were not affected in leukocytes of both smokers and mice administered nicotine, which is consistent with an earlier study (Benhammou *et al.*, 2000) which showed increased nicotine binding in neutrophils from smokers along with an up-regulation of non-α7 nAChRs. Thus, our data agree with and extend the findings of Cormier *et al.* (2004), in that nicotine exposure did not affect the expression levels of α7 nAChRs by neutrophils, even when exposed throughout the differentiation process.

Armstrong *et al.* (1996) showed that five days exposure of undifferentiated HL-60 cells to 0.06 and 0.8 μM nicotine did not initiate HL-60 cell differentiation, a finding later confirmed by Yoshida *et al.* (1998) (2 mM nicotine did not induce HL-60 cell differentiation over a three-day observation period). Interestingly, Maturana *et al.* (1991) observed that a 4-day, 3mM nicotine exposure resulted in the partial monocyte/macrophage-like differentiation of HL-60 cells (20% ~ 25%). The same authors suggested that a transient down-regulation of c-MYC gene expression upon nicotine exposure explains the resultant HL-60 differentiation. It seems that high nicotine doses, which are not pharmacologically relevant, and likely cytotoxic (Yoshida *et al.* 1998 and Konno *et al.* 1986), may partially induce myeloid-like differentiation of HL-60 cells by unknown mechanisms. Our data agree with Yoshida *et al.* and Armstrong *et al.* in that the pharmacologically relevant concentrations of nicotine had no significant influence on the spontaneous differentiation rate of HL-60 cells.

We used 1.3% (v/v) DMSO to induce the neutrophilic differentiation of HL-60 cells over the optimal time period of five days (Figure 8 and Figure 13). In contrast to unstimulated HL-60 cells, the percentages of differentiated HL-60 cells were dramatically increased over five days of DMSO stimulation, irrespective of nicotine exposure, as demonstrated by the fully consistent results obtained from the Hema 3 staining (Figure 15), the NBT reduction test (Figure 16) and the CD11b differentiation marker expression (Figure 17). Thus, our data would imply that, within the limits of this *in vitro* model, the mechanisms responsible for neutrophilia in smokers do not include a nicotine-induced increase in cellular differentiation. Furthermore, the major markers of late or terminal differentiation remain unaffected by nicotine exposure during differentiation.

Nicotine (10^{-6} M or 10^{-4} M) did not significantly affect the viability or the proliferation of promyelocytic or DMSO-induced HL-60 cells. These findings are consistent with Yoshida *et al.* (1998) and Konno *et al.* (1986) who reported that only higher doses of nicotine (>2 ~ 4 mM) induced cytotoxic and cytostatic effects on HL-60 cells, and consistent with the prior study of Pabst *et al.* (1995) who observed that nicotine did not affect the viability of mature neutrophils *in vitro*, as determined by trypan blue exclusion. The nicotine doses used in our study did not induce any qualitative ultrastructural changes in DMSO-differentiated HL-60, as shown by the transmission electronic microscopy (Figure 14).

Neutrophils enter the circulation as terminally differentiated cells. Without appropriate inflammatory stimuli, the programmed cell death of peripheral neutrophils is

initiated rapidly. Thus, circulating neutrophils normally have a short life span of less than a day. However, their longevity can be significantly increased in tissues due to the suppression of apoptosis by pro-inflammatory cytokines and other mediators (Haslett, 1997; Condliffe *et al.*, 1998 and Murray *et al.*, 2003). Induction of apoptosis causes loss of effector function in neutrophils, including chemotaxis, degranulation, generation of the respiratory burst, and is, thus, a key step in the resolution of inflammation. Neutrophils that die by apoptosis are predominantly phagocytosed and destroyed without releasing their proteolytic arsenal (Aoshiba *et al.*, 1996 and Finkelstein *et al.*, 2001). However, neutrophils that die by necrosis release degradative proteases, including MMP-9 and elastase, and other factors that can contribute to tissue degradation (Aoshiba *et al.*, 1996; Haslett, 1997; Condliffe *et al.*, 1998; Finkelstein *et al.*, 2001 and Murray *et al.*, 2003). Consequently, inappropriate suppression of apoptosis would be expected to facilitate or potentiate neutrophil-mediated tissue injury through prolonging neutrophil functional lifespan and abrogating the phagocytotic clearance of dead neutrophils.

The available evidence for an effect of tobacco smoke on neutrophil longevity is controversial. Acrolein, a tobacco smoke component, has been shown to suppress pro-apoptotic signals in systemic neutrophils in an *in vitro* model (Finkelstein *et al.*, 2001). Yoshida *et al.* (1998) have reported that high nicotine doses induced DNA cleavage in HL-60 cells. Aoshiba *et al.* (1996) found that nicotine suppressed neutrophil apoptosis in a dose-dependent manner, suggesting a potential mechanism of neutrophilia in smokers. A more recent report concluded high nicotine doses did not influence apoptosis in freshly isolated peripheral neutrophils (Yoshida *et al.*, 1998). Mariggio *et al.* (2001) have

presented the alternate message, showing nicotine at high doses to be a potent inducer of apoptosis in systemic neutrophils. Therefore, an urgent need to clarify the influence of tobacco smoking on apoptosis in neutrophils has been identified.

We hypothesized that the neutrophilia noted in smokers may partly be due to suppression of apoptosis. Yet, contradicting this hypothesis, infrared spectroscopy analysis of the molecular profiles of nicotine-stimulated HL-60 cells revealed apoptosis-associated cellular alterations, including increases in the cellular lipid content and in the DNA-protein ratio (Figures 19). However, such pro-apoptotic influences of nicotine on HL-60 cells during differentiation did not result in committed apoptosis, as would have been confirmed by propidium iodide staining in the flow cytometry experiments (Figure 18 and Table 5). In addition, HL-60 cells can acquire the spontaneous apoptosis upon obtaining the biochemical and functional properties of mature neutrophils through the differentiation by certain inducers, including DMSO (Martin *et al.*, 1990; Santos-Beneit and Mollinedo, 2000), which may explain the DMSO-induced HL-60 cell apoptosis noticed in our flow cytometry assays (Figure 18).

It has been known for some time that cigarette smoking can induce leukocyte-endothelial adhesion, microvascular and macrovascular entrapment of leukocytes, and leukocyte aggregation in humans and animal models, and that key adhesion molecules including CD11b (a ß2-integrin chain) are central to these processes, as Scott *et al.* (2002) have recently reviewed. Scott *et al.* (2000a and 2000c) examined the influence of tobacco smoking on CD11b expression, and other adhesion molecules, on circulating

human neutrophils *in vivo*. They found that tobacco smoking did not acutely influence CD11b expression level in primary neutrophils. However, Maestrelli *et al.* (1996) have reported that the numbers of neutrophils expressing CD11b and CD18, but not CD11a or CD11c, are increased in chronic smoking subjects with airway obstruction, compared to smokers without airway obstruction, and hypothesized that CD11b/CD18 expression by sputum neutrophils may represent a marker for the development of chronic airway obstruction among smokers. Recently, Speer *et al.* (2002) have demonstrated the dose-dependent inhibition of the integrin (CD62L, CD11a, and CD11b) expression by nicotine in neutrophils, *in vitro*. In our study, CD11b expression was used as a marker of terminal neutrophilic differentiation. We noted a five-day nicotine exposure did not cause statistically significant differences in mean CD11b expression levels of DMSO-treated HL-60 (Table 4). This would imply that, within the limits of this *in vitro* model, any alterations in CD11b expression profiles in chronic smokers are more likely to be the result of either the exposure of differentiating neutrophils to components of tobacco other than nicotine, or that neutrophil CD11b expression profiles are influenced by smoke components in mature, circulating cells rather than during differentiation.

The reactive oxygen and nitrogen species synthesized during neutrophil respiratory burst are essential weapons neutrophils use to eliminate microbial insults. A compromised respiratory burst may impair neutrophil ability to destroy bacteria, leading to elevated risk for infections. Additionally, the leakage of these chemically active species from neutrophils can fuel inflammatory responses and cause potential damage to surrounding cells and tissues (Witko-Sarsat *et al.*, 2000). Tobacco-related dysregulation

of the respiratory burst of neutrophils is important in the pathogenesis of periodontal diseases (Palmer and Scott *et al.*, 1999, 2003 and 2005) and several other tobacco-related systemic diseases (Ryder *et al.*, 1998), including COPD (Sparrow *et al.*, 1984) and atherosclerosis (Ernst *et al.*, 1987). Nicotine exposure has a profound and complicated impact on the profiles of circulating, mature neutrophil reactive oxygen species generation, as we reviewed in the Introduction. In this study, DMSO-differentiated HL-60 cells developed a functional oxidative burst ability, as demonstrated by the dramatic increase in the percentage of NBT positive cells in DMSO-treated cell cultures. Our data showed that nicotine inhibited PMA-stimulated oxidative burst of differentiating HL-60 cells in a dose-dependent manner with 10^{-4} M nicotine causing a 50% inhibition, relative to cells without nicotine exposure. Thus, the suppressed respiratory burst in neutrophils in chronic smokers may occur due to nicotine exposure during differentiation and prior to systemic exposure.

Matrix metalloproteinases (MMPs) are a family of at least eighteen secreted and membrane-bound zinc-endopeptidases. Collectively, these enzymes can degrade most, if not all, the components of the extracellular matrix (Nagase and Woessner, 1999; Chakrabarti and Patel, 2005). Dysregulation of MMP-9 (gelatinase B) and/or MMP-2 (gelatinase A) has been closely associated with the pathologic processes of tobacco smoking-related diseases, such as vascular atherosclerosis (Nordshog *et al.*, 2003), unbalanced tissue remodeling in periodontitis (Scott *et al.*, 2001 and 2005) and COPD (Russell *et al.*, 2002; Selman *et al.*, 2003, Chakrabarti and Patel, 2005). As to human

leukocytes, MMP-2 is produced by macrophages, while the expression of MMP-9 is restricted to neutrophils and eosinophils.

It is has been reported that HL-60 cells produce and secrete both MMP-9 and MMP-2 (Richard *et al.*, 2002; Devy *et al.*, 2002 and Zhang *et al.*, 2003). Normally, MMP-9 and MMP-2 are secreted as the form of zymogen and finally activated extracellularly (Witko-Sarsat *et al.*, 2000 and Chakrabarti and Patel, 2005). LPS stimulation can induce the rapid release of pre-formed MMP-9 from neutrophils by degranulation, while continuous LPS exposure has been reported to stimulate *de novo* MMP-9 synthesis in neutrophils (Pugin *et al.*, 1999 and Kanai *et al.*, 2004). This study showed that nicotine augmented the rapid LPS-induced proMMP-9 (92 kDa) secretion by DMSO-differentiated HL-60 cells in a dose-dependent manner, and also enhanced LPS-induced *de novo* MMP-9 secretion. However, the proMMP-2 (72 kDa) profile of these cells was not significantly changed by nicotine treatment. These findings are in keeping with unpublished data by Scott that systemic levels of neutrophil-derived MMP-9, but not MMP-2, are significantly elevated in smokers (personal communication). Further, we observed that the increased MMP-9 secretion was abrogated by the pre-treatment of DMSO-differentiated HL-60 cells with α-bungarotoxin, a potent antagonist of human nAChR-α1, α7 and α10 (Scott and Martin, 2006). No evidence has revealed that human neutrophils including HL-60 cells express α-bungarotoxin-sensitive nAChRs other than α7 (Cormier *et al.*, 2004; Gimonet *et al.*, 2003 and Villiger *et al.*, 2002), this suggests the involvement of α7 nAChR mediated-nicotine signaling in the potentiated MMP-9 secretion by DMSO-differentiated HL-60 cells. Taken together, our results support that

a potential nicotine-induced enhancement of MMP-9 synthesis by neutrophils occurs in a nAChR dependent manner and implies that MMP-9 may be involved in the inflammatory processes leading to certain tobacco-induced diseases.

Finally, we used three different methods to evaluate the differentiation of promyelocytic and neutrophil-differentiated HL-60 cells, i.e. morphological examination, expression of CD11b myeloid differentiation marker, and the capacity to reduce NBT. Nicotine did not influence any of these markers in the promyelocytic or DMSO-induced HL-60 cells over five days.

In summary, physiologically relevant nicotine concentrations have pro-apoptotic potential during neutrophil differentiation in an HL-60 cell model expressing α7-nAChRs, but nicotine does not affect other major differentiation markers (growth kinetics, cell cycle, morphological maturation, NBT reduction, and CD11b expression). Certain phenotypic differences in differentiating neutrophils exposed to nicotine were addressed in this study, including reduced oxidative burst, augmented synthesis of MMP-9. Therefore, the contribution of neutrophils to tobacco-induced pathologies partially results from nicotinic effects on the phenotypic alteration of differentiating neutrophils rather than a direct influence on neutrophil gross differentiation. The impact of tobacco smoke constituents other than nicotine on neutrophil differentiation was not addressed. Future studies that address the potential effects of tobacco smoke and specific smoke components on functional aspects of neutrophil physiology, and particularly the

mechanisms by which nicotine enhances MMP-9 secretion and suppresses the respiratory burst, are warranted.

CHAPTER 11

FUTURE DIRECTIONS

The research presented here has examined the influence of nicotine, a primary tobacco smoke component, on HL-60 promyelocytic cell differentiation and associated effector function. The results showed that physiologically relevant doses of nicotine do not affect the gross markers of neutrophil differentiation or induce commitment to apoptosis in this cell model. However, exposure to nicotine during cellular differentiation altered effector function in neutrophils, i.e. suppression of respiratory burst and enhancement of MMP-9 release, which may partially explain the increase in susceptibility of tobacco smokers to bacterial infection.

The impact of tobacco smoke constituents other than nicotine on neutrophil differentiation was not addressed in this study. Also, the exact mechanisms underlying nicotine-induced certain phenotype changes of differentiating neutrophils, such as the suppression to oxidative burst and the dysregulation of MMP-9 release, are not fully understood. Increasing evidence has revealed the expression of multiple functional nAChRs by neutrophils, whereas the pathophysiologic roles of these receptors on neutrophils, particularly the neutrophils of smokers, have not been clarified yet. Therefore, future studies will address potential effects of tobacco smoke and other specific smoke components on functional aspects of neutrophil physiology, and particularly the mechanisms by which nicotine enhances MMP-9 secretion and suppresses the respiratory burst. Additionally, the influence of nicotine on other effector

functions of neutrophil during cell differentiation, including MMP-8 and elastase release, multiple cytokine secretion, has not been involved in current study, most possibly, which will be one of our future interests.

This study has confirmed the α7 nAChR expression by HL-60 cells and implied the potential of this receptor to regulate MMP-9 profiles and probably other phenotypes. Further investigation of fundamental relations between nAChR regulation and neutrophil phenotype changes will be another future interest. Using the experimental approaches of nAChR knockout animals and /or specific nAChR antisense oligonucleotide will essentially provide us better picture of the role of nAChRs in regulating neutrophil function. Overall, this work has addressed a potential nicotine-related mechanism involved in the pathogenesis of tobacco-induced diseases. More studies will be fulfilled to contribute to probe the molecular, biochemical, and pathological mechanisms of tobacco-associated diseases.

CHAPTER 12

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