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Modulation of monocyte-derived dendritic cell maturation and function by cigarette smoke condensate in a bronchial epithelial cell co-culture model

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Modulation of Monocyte-Derived Dendritic Cell Maturation and Function by
Cigarette Smoke Condensate in a Bronchial Epithelial Cell Co-Culture Model

by

Alison J. Montpetit

A dissertation submitted in partial fulfillment
of the requirements of the degree of
Doctor of Philosophy
College of Nursing
University of South Florida

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Dedication

This dissertation is dedicated to my family for their continued support and sacrifice during my PhD work and to our unborn baby, Weston Louis who unknowingly motivated me to persevere.

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List of Abbreviations

7-AAD: 7-amino-actinomycin

APC: allophycocyanin

BALF: bronchoalveolar lavage fluid

BDCA: blood dendritic cell antigen

CCL20: C-C motif ligand 20

CFSE: carboxyfluorescein succinimidyl ester

CSC: cigarette smoke condensate

CSCH: cigarette smoke condensate high dose, 50 µg/ml

CSCL: cigarette smoke condensate low dose, 10 µg/ml

CSE: cigarette smoke extract

CD: cluster of differentiation

COPD: chronic obstructive pulmonary disease

DC: dendritic cells

DEP: diesel exhaust particulate

DMSO: dimethyl sulfoxide:

EC: epithelial cells

η^2 : eta squared

ETS: environmental tobacco smoke

FITC: fluorescein isothiocyanate

GMCSF: granulocyte macrophage colony stimulating factor

HLA-DR: human leukocyte derived antigen:

ICAM1: intracellular adhesion molecule 1, also known as CD54

IFN γ : interferon gamma

IP10: interferon-inducing protein 10

IL: interleukin

LN: lymph nodes

Log: logarithm base 10

LPS: lipopolysaccharide

μ g: microgram

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

ml: milliliter

MLR: mixed lymphocyte reaction

mDC: myeloid dendritic cells

MDDC: monocyte-derived dendritic cells

NHBE: normal human bronchial epithelial cells

OS: oxidant stress:

PBMC: peripheral blood mononuclear cells

pDC: plasmacytoid dendritic cells

PE: phycoerythrin

PFA: paraformaldehyde

pg: picogram

PHA: phytohemmagglutinin

Poly I:C: polyinosinic-polycytidylic acid

SEM: standard error of the mean

siRNA: short interfering ribonucleic acid

TC: T cells

TCR: T cell receptor

TGF β : transforming growth factor beta

Th0: T helper type 0, T regulatory

Th1: T helper type 1

Th2: T helper type 2

TLR3: toll-like receptor 3

TNF α : tumor necrosis factor alpha

Treg: T regulatory, Th0

TSLP: thymic stromal lymphopoietin

Modulation of Monocyte-Derived Dendritic Cell Maturation and Function by Cigarette
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ABSTRACT

Lung airway epithelium is the first line of defense against inhaled particulates such as cigarette smoke. Subepithelial dendritic cells (DC) survey the airway epithelial lining and represent the link between innate and adaptive immune response. No study has investigated the effect of cigarette smoke on DC in the presence of epithelial cells (EC). The purpose of this 4x2 factorial design study was to co-culture normal human bronchial epithelial (NHBE) cells with monocyte-derived dendritic cells (MDDC) and examine the effect of cigarette smoke condensate (CSC) and poly I:C stimulation (TLR3 ligand that mimics viral infection) on MDDC homotypic clustering, phagocytosis ability, surface marker expression, DC cytokine response, T cell (TC) proliferation and TC cytokine response. Experiments were performed with MDDC and TC derived from four individual donors. Two planned comparisons (DMSO vehicle control compared to CSC high dose in both the no poly I:C and poly I:C stimulated groups) were analyzed. In MDDC stimulated with CSC, there was a significant increase in homotypic clustering, reduced phagocytosis, increased CD54, increased CD83 and CD86 maturation marker expression. Although no significant changes were observed in MDDC cytokine production, IL10 exhibited a trend to increase with CSC exposure but failed to reach statistical significance. Despite evidence that CSC exposed MDDC are maturing, there was no increase in TC proliferation; however in poly I:C stimulated co-cultures, CSC

exposure increased IL2, IL5, IL10 and IL13 expression while non-stimulated co-cultures exhibited an increase in IL10 following CSC exposure. This study indicates that cigarette smoke has the ability to decrease phagocytosis ability of DC and increases co-stimulatory maturation markers without inducing TC proliferation and potentiating a Th2 environment. These findings suggest that DC of smokers may be less likely to mount a normal immune response to invading pathogens thus providing evidence for smoker susceptibility to infection and how DC of smokers may respond to viral infection. In addition, these findings are particularly important in patients with allergic airway disease since increasing Th2 cytokines would increase the risk for disease exacerbation.

Chapter One

Introduction

Cigarette smoking has been identified as the most important cause of preventable morbidity and mortality in the United States. Over 438,000 Americans die annually from diseases directly related to smoking (American Lung Association, 2006). In the United States, conservative estimates of the economic burden due to smoking are \$167 billion in direct healthcare costs, \$92 billion in lost productivity and \$75 billion in excess medical expenditures (American Lung Association, 2006). Approximately 8.6 million Americans have at least one serious illness caused by smoking (American Lung Association, 2006).

For people suffering with lung disease, breathing is often a daily challenge. Symptom management of lung disease is a vital component of therapy. Relieving dyspnea and breathlessness by encouraging smoking cessation is one of the most effective strategies. Despite efforts of nurses and other health care professionals to educate smokers of the risk of pulmonary disease, many continue to smoke and approximately 15 billion cigarettes are smoked worldwide every day (American Lung Association, 2003). In addition, over 50% of teenagers aged 13-15 who claim to have never smoked are exposed to secondhand smoke in the home (Centers for Disease Control and Prevention, 2007). There is a need to conduct research that explores immune responses to cigarette smoke and potential use of immune modulating therapies and their effects on the pulmonary system in smokers and nonsmokers.

Lung airway epithelium is the first line of defense against inhaled particulates such as cigarette smoke and represents a component of the innate immune response. Dendritic cells (DC) in the respiratory tract are professional antigen presenting cells that reside within the epithelium, submucosa and lung parenchyma (Lambrecht & Hammad, 2003; Upham & Stick, 2006). One function of the DC is to survey the lung environment for antigens, microbes and dead or dying cells (Rossi & Young, 2005). DC also link the innate immune response with the adaptive immune response and play a key role in not only antigen presentation but also in determining Th1 and Th2 immune responses. Cigarette smoke has been shown to suppress DC function and lead to an induction of Th2 immune response thus potentiating the allergic response (Vassallo & Chen, 2004; Vassallo, Tamada, Lau, Kroening, & Chen, 2005). In addition, the crosstalk between epithelial cells (EC) and DC has been shown to be especially important in airway inflammation and asthma (Lambrecht & Hammad, 2003; Upham & Stick, 2006).

Several studies have shown that smokers and people exposed to secondhand smoke are at increased risk for infections (Carroll et al., 2007) and at high risk for the development of chronic obstructive pulmonary disease (COPD) and asthma (Goodwin, 2007; Halken, 2004; Teramoto, 2007). Asthmatics who smoke are less responsive to corticosteroid therapy than non-smoking asthmatics (Thomson, Shepherd, Spears, & Chaudhuri, 2006), making treatment of patients who smoke quite challenging for health care providers. In addition, patients with asthma and/or COPD who smoke or are exposed to secondhand smoke are more prone to disease progression and exacerbation (Alvarez, Schulzer, Jung, & Fitzgerald, 2005; Eisner & Iribarren, 2007; Eisner et al., 2005; Kanner, Anthonisen, & Connett, 2001; Koga, Oshita, Kamimura, Koga, & Aizawa, 2006; Wark & Gibson, 2006).

Lung disease exacerbation is linked with a decline in lung function, decreased quality of life and increased morbidity and mortality (Bai, Vonk, Postma, & Boezen, 2007; Cote, Dordelly, & Celli, 2007; Donaldson, Seemungal, Bhowmik, & Wedzicha, 2002; Rogers, Adelroth, Hattotuwa, Dewar, & Jeffery, 2007). Studies indicate that cigarette smoke has the ability to modulate the immune response most likely, dampening the Th1 type immune response and favoring a Th2 type immune response. Despite recent advances in research related to the immune response to cigarette smoke exposure, the exact mechanisms of smoker vulnerability remains unclear.

One possible explanation for the increased risk of infection, inflammation and exacerbation of pulmonary disease in smokers is that smokers have fewer numbers of DC in the bronchial mucosa. This finding was observed in bronchial biopsies of patients with COPD who smoke compared with non-smoking normal, asthmatic and COPD controls (Rogers et al., 2007). In addition, one study reported observing significantly less CD83+ mature DC in the airways of smoking asthmatics when compared to non-smoking asthmatics and healthy non-smoking controls (Tsoumakidou et al., 2007). This phenomenon has also been observed in cord blood from neonates of smoking mothers (Pachlopnik Schmid et al., 2007). This study reported a significant decrease in all leukocytes including CD11c+ myeloid and CD123+ lymphoid DC. By decreasing the number of DC in the airways, the innate immune system's ability to respond is hindered; however, the potential mechanism requires further inquiry as this observation may merely be explained by migration of DC to the lymph nodes, other tissues or down-regulation in surface marker expression.

Despite overwhelming evidence that smoking is harmful, it cannot be ignored that evidence suggests a protective effect of tobacco smoking against certain diseases including, but not limited to: Parkinsons' and Alzheimer's disease (Fratiglioni & Wang,

2000), sarcoid arthritis (Visser et al., 2002), and ulcerative colitis (Mahid, Minor, Soto, Hornung, & Galandiuk, 2006). Interestingly, these diseases have an inflammatory or autoimmune component and particulates in cigarette smoke and/or nicotine may suppress the immune response and potentially protect against these diseases. Overall, it is clear that cigarette smoking modulates the immune system by activation and suppression making research inquiry complex.

People who smoke often face challenges in the life-dependent act of breathing. Nursing care is guided toward symptom assessment and management as well as playing a critical role in educating patients and family regarding symptom management, smoking cessation, disease pathophysiology and progression, and prevention of exacerbations.

No study has investigated the effect of cigarette smoke in DC in the presence of epithelial cells (EC). By specifically studying the interaction of DC and EC exposed to cigarette smoke particulates, this study will provide focused insight into the immune effects of smoke and the link between innate and adaptive immunity. This will add to the body of knowledge regarding smoke immunomodulation enabling nurses and other health care providers to improve current methods of diagnosis, symptom management and monitoring in order to prevent disease exacerbation. In chapter two, a review of the literature will be presented in order to further develop the concepts to be investigated in this study.

Chapter Two

Review of Literature

Bronchial Epithelial Mucosa as First Line of Defense

Bronchial EC play a critical role in protecting submucosal tissue from inhalation injury by entrapment and removal of particulates by mucous secreting goblet cells, ciliated cells, and intact tight junctions. Although this is a key function of the bronchial lining, recent advances in immunology research have indicated that EC play an important role in linking the innate and adaptive immune systems. This is evident in the ability of EC to secrete cytokines and chemokines in response to inhaled allergens, pollutants and chemicals that lead to inflammatory cell recruitment, airway remodeling and development or potentiation of airway disease such as asthma, emphysema and chronic bronchitis (Gershwin, 2007; Kato & Schleimer, 2007; Lambrecht & Hammad, 2003; Schleimer, Kato, Kern, Kuperman, & Avila, 2007; Upham & Stick, 2006).

The immune system is composed of two major branches: innate and adaptive immunity. The innate immune response is the body's first line of defense against infection and hinges on the ability to recognize self from non-self. EC in the pulmonary system are heavily equipped with numerous systems that protect the body from microbial, viral, chemical and particulate invasion. Some of these mechanisms include but are not limited to the complement system, collectins, mucins, reactive oxygen species and toll-like receptors (Ezekowitz & Hoffman, 2003; Schleimer et al., 2007). In addition to the innate immune system, EC play a role in the adaptive immune system.

The adaptive immune system response is characterized by antigen processing/presentation and induction of a memory response to specific antigens and involves many cells including T and B cells. This review will focus primarily on one type of antigen presenting cell, DC.

Dendritic Cells: The Professional Antigen Presenting Cell

DC represent 1-2% of peripheral blood mononuclear cells and approximately 0.5% of cells found in bronchoalveolar lavage fluid (BALF) (Donnenberg & Donnenberg, 2003; Tsoumakidou, Tzanakis, Papadaki, Koutala, & Sifakas, 2006; van Haarst et al., 1994). In humans, there are two major types of DC, myeloid (mDC) and plasmacytoid (pDC). Myeloid DC express the surface marker CD11c and capture antigen by phagocytosis and macropinocytosis. Plasmacytoid DC are derived from a lymphoid lineage, do not express CD11c and are inefficient in phagocytosis (Schoorhuis, Fu, Ossendorp, & Melief, 2006). Both mDC and pDC have the ability to induce Th1 and Th2 type responses but the response is primarily determined by environmental stimuli such as pulmonary inflammation.

Both types of DC exist in the lungs; however, mDC are predominate and they are located in the conducting airway, interstitium and alveoli (de Heer, Hammad, Kool, & Lambrecht, 2005; Iwasaki, 2007). One study reported 0.06% of pDC in BALF and 0.47% mDC and demonstrated an increase in both pDC and mDC following a segmental allergen challenge (Bratke et al., 2007). In addition, one study investigated DC in lung digests and the presence of blood DC antigen (BDCA), a marker used to subtype blood DC. This study showed four subtypes of DC in the lungs: mDC1 (BDCA1+/HLA-DR+), mDC2 (BDCA3+/HLA-DR+), pDC (BDCA2+/CD123+) and CD1a+ DC in the conducting airway epithelium (Demedts, Brusselle, Vermaelen, & Pauwels, 2005). Another study

confirmed the presence of the three subtypes (mDC1, mDC2, pDC) in BALF (Tsoumakidou et al., 2006). It is apparent that with advances in flow cytometry techniques and identification of surface markers, more pulmonary DC subtypes will be elucidated.

DC are key in directing immune responses throughout the body, but for the purposes of this review, only pulmonary DC are examined. In the lungs, DC are located within the lung epithelium and interstitium and perfectly placed for sampling the bronchial airway for inhaled antigens and particulates (Grayson, 2006; Lambrecht, Prins, & Hoogsteden, 2001). Studies have shown that DC that reside underneath and within the epithelium are able to extend their dendrites through epithelial tight junctions in order to sample the airway. The DC then migrate to draining lymph nodes where they confer antigen-specific responses. Pulmonary inflammation plays a key role in the maturation process and trafficking of DC.

Immature DC phagocytose/endocytose bacteria, allergens, pollutants and cellular debris in the airway. They then process the antigen and carry immune signals from the periphery or site of capture to the secondary lymphoid tissue where, after maturation, they present antigen to TC (Cook & Bottomly, 2007). Pulmonary DC are the sentinels of the lung and they direct clonal expansion of TC by cross-presentation of allergens to CD4⁺ T helper cells, CD8⁺ cytotoxic TC and are the only cells capable of priming naïve TC (Cools, Ponsaerts, Van Tendeloo, & Berneman, 2007; Novak & Bieber, 2008).

The response of naive T helper cells after contact with pathogen-specific, mature DC is determined by three main signals: 1) antigen specific (by engagement of the TC receptor by MHC class II), 2) co-stimulation and 3) polarization. Signal 1 is characterized by antigen specificity. Signal 2 provides protective immunity by co-

stimulatory molecules and if signal 2 is not present, it leads to TC anergy and potentially tolerance. Signal 3 is important in determining the balance of cytokine production from primed TC. There is a functional difference in DC and TC depending on the micro-environment of the third signal. It is unclear where all of these third signals originate but the micro-environment, character of antigen, and the affected tissue all play a critical role. Since DC are in close proximity to EC, it is hypothesized that polarizing signals may be driven by EC (Kalinski, Hilkens, Wierenga, & Kapsenberg, 1999; Kapsenberg, 2003; Schuurhuis et al., 2006).

Naïve TC generally have four broad fates including: Th1, Th2, Th17 and Treg. For the purposes of this review, only CD4⁺ TC subsets will be discussed. Th1 is primarily characterized by IFN γ and TNF α release; Th2 is primarily characterized by IL4, IL5 and IL13 release, Th17 by IL17 and Treg by IL10 and TGF β . An imbalance towards one type has been implicated in many diseases for example, Th1 in autoimmune diseases, Th2 in allergic diseases, Th17 in chronic inflammatory and autoimmune disorders and Treg in cancer and immunosuppression (Hammad & Lambrecht, 2006; Romagnani, 2006).

Many antigens induce DC maturation following uptake resulting in TC activation; however, most antigens fail to induce maturation requiring secondary signals from the micro-environment (Kalinski et al., 1999; Kapsenberg, 2003). This is a critical balance since approximately 10,000 L of air circulate through the lungs every day exposing EC and DC to many particles (Cook & Bottomly, 2007). In fact, the outcome of inhalation of most harmless antigens results in tolerance. Under steady state conditions, DC remain in an immature or semi-mature state during which they are continue to phagocytize antigen and have high expression of receptors for pro-inflammatory chemokines and cytokines (Novak & Bieber, 2008). This active process is characterized by tolerogenic

DC presenting antigen to naïve TC which in turn acquire regulatory TC (Treg) properties including Foxp3+, production of IL10 and TGF β , the ability to suppress Th1 and Th2 processes. There is some debate about characteristics of tolerogenic DC but generally these DC are considered semi-mature, are inefficient at phagocytosis of antigen, produce IL10 and are less motile but may express maturation/co-stimulatory molecules (Cook & Bottomly, 2007; Morelli & Thomson, 2007; Reis e Sousa, 2006; Steinman, Hawiger, & Nussenzweig, 2003).

Epithelial and Dendritic Cell Interaction

EC are the first contact with inhaled particulates and they play a critical role in regulation of immune responses through interactions with DC, TC and B cells among others. This review will focus specifically on EC and DC interactions. EC may play a role in recruitment of DC into the airways via macrophage inflammatory protein 3 alpha also known as chemokine (C-C motif) ligand 20 (CCL20) and possess the ability to polarize DC to a Th1 or Th2 directing phenotype (Kao et al., 2005; Thorley, Goldstraw, Young, & Tetley, 2005). One example, EC-derived thymic stromal lymphopoietin (TSLP) secretion directs DC to activate naïve TC to Th2 cells that produce IL4, IL3, TNF but not IL10 or IFN γ (Kato, Favoreto, Avila, & Schleimer, 2007; Lee & Ziegler, 2007; Liu et al., 2007).

One example of the importance of EC-DC interaction is shown by the effect of diesel exhaust particulates (DEP) to induce DC maturation marker CD80, CD83, CD86 and TC proliferation when DC and EC are co-cultured and this effect is not observed when DC are cultured alone (Bleck, Tse, Jaspers, Curotto de Lafaille, & Reibman, 2006). In this study, supernatant from DEP-exposed EC induced DC maturation and was equivalent to DC cultures with DEP-exposed EC, suggesting that soluble mediators

rather than direct cell-to-cell contact are critical for DC maturation and function. In addition, when a blocking antibody (Ab) and short interfering RNA (siRNA) to the growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) was administered to DEP-exposed EC cultures, DC failed to induce TC proliferation (Bleck et al., 2006). This finding suggests the critical role of EC-derived GM-CSF in DC maturation and function. This study did not evaluate the cytokine production of TC from the MLR; therefore, it is unknown if DEP-exposed EC co-cultured with DC induce TC to produce Th1 or Th2 cytokines. Based upon this study, it is reasonable that cigarette smoke-exposed EC culture may modulate patterns of DC maturation and TC proliferation when cultured with DC since cigarette smoke and DEP are both pro-inflammatory and potent oxidant stressors.

Cigarette Smoke as an Oxidant Stressor

The pathogenesis of many lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma may be a function of free oxygen radicals or oxidant mediators which can act as immune adjuvants, enhancing the response to inhaled allergens or antigens (Nouri-Shirazi & Guinet, 2006; Siafakas & Tzortzaki, 2002; Sopori, 2002; Tsoumakidou et al., 2007). A number of environmental stimuli or oxidant mediators induce cellular oxidative stress (OS) that stimulates several biological effects such as, increased inflammation and mucous production, decreased cilia function, impaired surfactant activity, promotion of epithelial permeability, inhibition of glucocorticoid responses and many others (Repine, Bast, & Lankhorst, 1997). All these effects compromise the effectiveness of the innate immune system.

Conceptually, OS has been defined as a loss of balance in which oxidation exceeds the antioxidant systems in the body. One cause of OS in the lungs is cigarette

smoke. Cigarette smoke has approximately 10^{17} oxidants/free radicals and 4,800 chemical compounds which increases the oxidant burden in smokers and the risk of developing pulmonary disease through chronic inflammatory processes and increasing airspace permeability (American Lung Association, 2006; MacNee, 2000; Marwick et al., 2002; Moodie et al., 2004). The respiratory tract is extremely differentiated and its ability for gas exchange is dependent on thin epithelial cell membranes in the respiratory tract and are the first contact with air particulates, making them more susceptible to the environment and cigarette smoke than other tissues; thus, they are more vulnerable to reactive oxygen species that can cause cellular damage and alter in the immune response by increased susceptibility to inflammation and infection (Jyonouchi, 1999; Lambre et al., 1996).

Effects of Cigarette Smoke on Humans in vivo

Several studies have investigated the effect of cigarette smoke in humans. Smokers have dramatically fewer numbers of DC in the bronchial mucosa which was observed in bronchial biopsies of patients with COPD who smoke compared with non-smoking normal, asthmatic and COPD non-smoking controls (Rogers et al., 2007). In fact, COPD ex-smokers had similar numbers of DC as asthmatics; therefore, the reduction in DC is not a function of the disease process. The location of these DC were determined by transmission electron microscopy images which revealed the presence of DC primarily in the bronchial mucosa epithelium and subepithelium (Rogers, Adelroth, Hattotuwa, Dewar, & Jeffery, 2008).

One study reported significantly less CD83+ mature DC in the bronchial biopsies of smoking asthmatics compared to non-smoking asthmatics and healthy non-smoking controls (Tsoumakidou et al., 2007). However, another study did not find a difference in

CD83+ DC in bronchoalveolar lavage fluid (BALF) of smokers versus never smokers. They did observe a difference in CD80+ and CD86+ DC in smokers. In addition, they reported decreased expression of CCR7, a lymph node homing receptor that is important in migration, suggesting that the reduction in DC is not due to DC migration.

In a randomized, placebo-controlled trial, non-smoking allergic subjects were exposed to two hours of environmental tobacco smoke (ETS) or clean air then subjected to a nasal challenge with ragweed allergen or saline and nasal lavages were obtained. Twenty-four hours after ETS exposure with allergen challenge, there was a significant increase in ragweed-specific IgE, histamine, IL4, IL5 and IL13 and a significant reduction in IFN γ , when compared with clean air exposure with allergen challenge (Diaz-Sanchez, Rumold, & Gong, 2006). This study was the first to show evidence in humans that cigarette smoke can directly exacerbate allergic disease.

Effects of Cigarette Smoke on Murine Dendritic Cells in vivo

Several studies have investigated the effect of smoke in murine models. One study exposed mice to two cigarettes per day, five days a week for two months. They reported a significant decrease in the number of CD11c+/MHC-II+ in smoke exposed mice compared with controls. In addition, this trend preceded emphysematous lesions in the lungs and was lung specific as there was no reduction in DC in regional lymph nodes (Robbins et al., 2004). This provides evidence that smoke effects on lung DC are not merely a result of DC migration.

Another murine study where mice were exposed to 6 cigarettes a day, five days a week for one month confirmed this reduction in pulmonary CD11c+ DC and reported that antigen induced migration to draining lymph nodes was not affected by smoke exposure. In addition, they reported a reduction in MHC-II, CD80 and CD86 but no

effect on CD40 in DC within the lymph nodes. They also observed a reduction in TC proliferation and significantly less CD4+ TC IL2 production from smoke exposed mice when compared to control (Robbins, Franco, Mouded, Cernadas, & Shapiro, 2008).

Effects of Cigarette Smoke on Human Dendritic Cells in vitro

Clinical evidence described previously suggests that smokers are more prone to Th2 type response. Since DC have the ability to sample the bronchial airway, take up smoke particulates and are key mediators of immune response, it is reasoned that DC function and DC-induced TC response may be affected by cigarette smoke. Only two studies have evaluated the effects of cigarette smoke on human DC function.

The first study investigated the role of cigarette smoke extract (CSE), the particulate phase of smoke on DC co-stimulatory markers (CD40, CD80, CD83, CD86), TC proliferation (BrdU assay) and polarization of TC cytokine response (IL2, IL4, IL10, IFN γ). This study provided evidence that CSE suppresses maturation markers (CD40, CD80, CD86) and suppresses IL12 and enhances IL10 production. In addition, CSE-exposed DC inhibit TC proliferation and promote Th2 (IL4) cytokine release from TC. Together, this suggests that smoke suppresses DC functions and potentiates a Th2 response (Vassallo et al., 2005).

The second study investigated TC proliferation (CFSE assay) and cytokine response (IL12p70, IL4, IFN γ) in response to DC cultured with nicotine and matured under non-polarized or polarized Th1 and Th2 conditions (Nouri-Shirazi & Guinet, 2006). The study provided evidence that nicotine exposed DC are able to induce TC proliferation but not Th2 differentiation (IL4 secretion) unless under a Th2 polarizing condition described as exposure to IL1 β , TNF α and prostaglandin E2.

In these two studies, DC were cultured alone. As discussed previously, DEP-treated DC did not induce TC proliferation when DC were cultured alone. This is the same observation with cigarette smoke in the first study but not in the nicotine study. This discrepancy may be a function of particulates in smoke-exposed EC. It has been shown that smoke-exposed bronchial EC produce pro-inflammatory cytokines including, IL6 and IL8; however, it has also been shown that smoke induces downregulation of GM-CSF in lipopolysaccharide (LPS)-treated EC (Glader et al., 2006; Li, Xu, & Shen, 2007; Phillips, Kluss, Richter, & Massey, 2005). GM-CSF was shown to have significant effects on DC maturation in DEP-exposed co-cultures of DC and EC (Bleck et al., 2006).

By culturing DC with smoke-exposed EC, it will take into account the impact of the tissue micro-environment on environmental exposures. Therefore, the proposed study will provide insight into EC-DC interactions in smoke exposure and may provide evidence for disease and infection susceptibility in cigarette smokers.

Polyinosinic-Polycytidylic Acid as an Immunological Stimulant

A major signal for DC priming are toll-like receptors (TLR) which are highly conserved immune receptors responsible for recognition of pathogen-associated molecular patterns (PAMP) of bacteria and viruses thereby initiating the immune response (Duez, Gosset, & Tonnel, 2006; Guillot et al., 2005; Kapsenberg, 2003). Specifically, TLR3 recognizes double stranded RNA (dsRNA) of viruses such as influenza and rhinovirus and induces a potent antiviral response (Doyle et al., 2003; Guillot et al., 2005; Hewson, Jardine, Edwards, Laza-Stanca, & Johnston, 2005). In both epithelial and monocyte-derived DC, TLR3 is primarily located in the cytoplasm and endoplasmic vesicles (Ciencewicki, Brighton, Wu, Madden, & Jaspers, 2006; Duez et al., 2006).

Poly I:C (Figure 1) is a TLR3 ligand and a dsRNA that mimics the effects of viral infections (Marshall-Clarke et al., 2007). EC stimulated with poly I:C have been shown to up-regulate numerous pro-inflammatory cytokines (IL1 β , IL6), growth factor (GM-CSF), chemokines (IP10, IL8) and adhesion molecules such as ICAM-1 (Matsukura et al., 2006; Ritter, Mennerich, Weith, & Seither, 2005). DC stimulated with poly I:C exhibit stable maturation and produce high levels of IL12, low levels of IL10 and a strong TC proliferative response (Verdijk et al., 1999). In addition, poly I:C-stimulated DC retain the ability to produce IL12p70 even after the initial maturation stage, thus driving a Th1 response (Rouas et al., 2004; Verdijk et al., 1999).

One study revealed that diesel exhaust enhanced poly I:C-induced expression of TLR3, interferon beta and IL6 in lung EC providing mechanistic evidence that inhalation of particulates affect the immune response to viral infection (Ciencewicki et al., 2006). Dysregulation of the innate immune response could lead to enhancement of inflammatory cell recruitment, DC activation, adaptive immune response and potentially an auto-immune or allergic type reaction. No studies have been conducted investigating modulation of poly I:C effects by cigarette smoke; however, it is important to determine if smoke down-regulate cytokine production and push the Th2 response despite the Th1 poly I:C effects. This will mimic the clinical scenario of cigarette smokers exposed to viral infection.

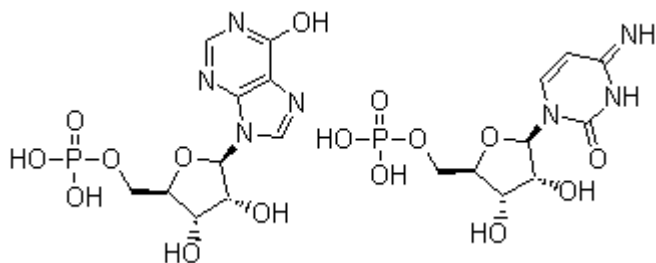


Figure 1. Structure of poly I:C. Reprinted from (Chemblink.com, 2007) with permission.

It is evident that smoke modulates the immune response and impacts disease development and progression. Unfortunately, the exact mechanism by which this occurs remains unclear. By culturing MDCC with smoke-exposed NHBE, it takes into account the impact of the tissue micro-environment on the response of MDCC. This study provides insight into EC-DC interactions in smoke exposure and may provide evidence for disease and infection susceptibility in cigarette smokers. In addition, by investigating the effect of smoke in poly I:C stimulated co-cultures, this study also provides insight into how a smoker may respond to viral infection. In chapter three, the purpose, models and methods of this study is described.

Chapter Three

Method

Purpose

The purpose of this preliminary 4x2 factorial design study was to examine the effect of cigarette smoke condensate (CSC) on monocyte derived DC (MDDC) clustering, cytokine response, maturation, endocytosis ability, TC proliferation and polarization. This study was guided by the conceptual model in Figure 2. This concept model shows the *in vitro* model model (NHBE, MDDC co-culture), four levels/doses (control/resting, DMSO/vehicle control, 10 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$) of cigarette smoke condensate (CSC) exposure, two levels/doses (0 and 25 $\mu\text{g/ml}$) of poly I:C stimulation. The dashed arrows and box, indicates the theoretical concept of DC maturation and function followed by the categories of dependent variables to be measured.

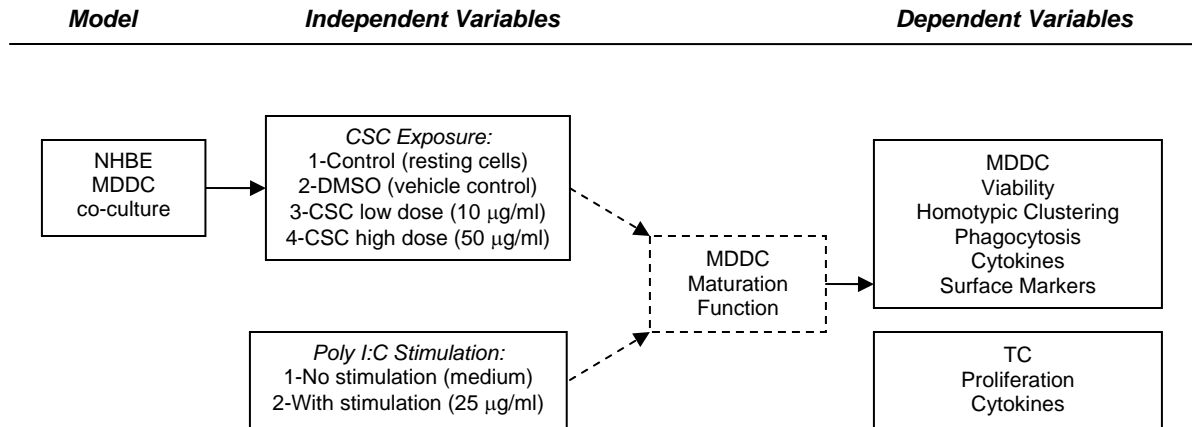


Figure 2. Conceptual model. *In vitro* study of CSC and poly I:C modulation of dendritic cell maturation and function in NHBE cell and MDDC co-culture model, DMSO = dimethyl sulfoxide.

Hypothesis

It was hypothesized that CSC will suppresses DC maturation and redirect DC-induced CD4+ T lymphocyte differentiation, leading to augmentation of T helper type 2 (Th2) cytokine secretion. The working model (Figure 3) depicts the innate and adaptive immune responses following exposure to CSC. EC up-regulate cytokine secretion and MDDC take up CSC particulates, modulating maturation and function. Stimulated MDDC undergo functional maturation and secrete T helper lymphocyte Th2 polarizing type cytokines. Theoretically, the DC then migrate to the lymph nodes (LN), promoting differentiation and proliferation of CD4+ TC into Th2 cells linking the innate immune response with the adaptive immune response.

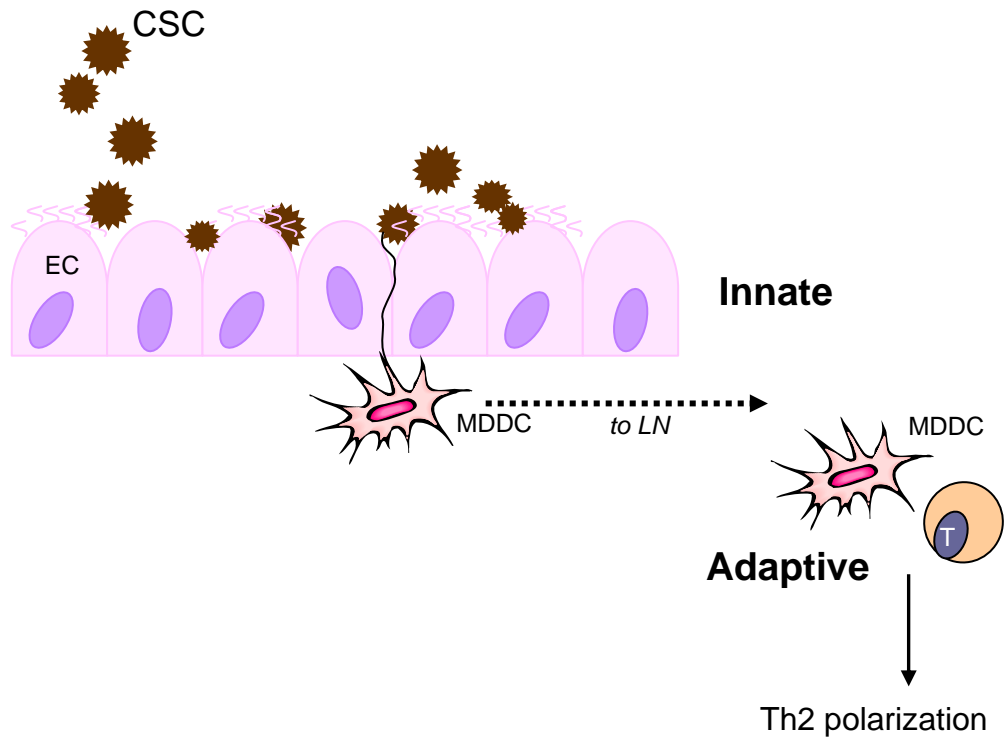


Figure 3. Working model. Depicts CSC exposed co-culture of EC and MDDC.

Specific Aims and Research Questions

This proposed study has two main aims. The first objective was to characterize the co-culture model and exposure systems and to determine the effect of cigarette smoke exposure and poly I:C stimulation on MDDC viability, homotypic clustering and cytokine secretion. The second objective was to determine the effect of cigarette smoke exposure and poly I:C stimulation on MDDC surface marker expression and phagocytosis. In addition, the effect of CSC exposed and poly I:C stimulated MDDC on TC proliferation and polarization was examined through mixed lymphocyte reaction and cytokine analysis.

Specific aim 1. Characterize and optimize CSC exposure and poly I:C stimulation of the NHBE cells, MDDC and a co-culture of NHBE cells and MDDC (Figure 4)

- Can NHBE cells, MDDC and the co-culture model be cultured effectively?
- Is there a difference in homotypic clustering of MDDC co-cultured with NHBE cells following exposure to CSC and poly I:C stimulation?
- What are the cytokine profiles of NHBE cells and MDDC co-cultured with NHBE cells following exposure to CSC and poly I:C stimulation?
- Does CSC exposure modulate poly I:C-stimulated cytokine/chemokine/growth factor expression of NHBE cells and MDDC co-cultured with NHBE cells?

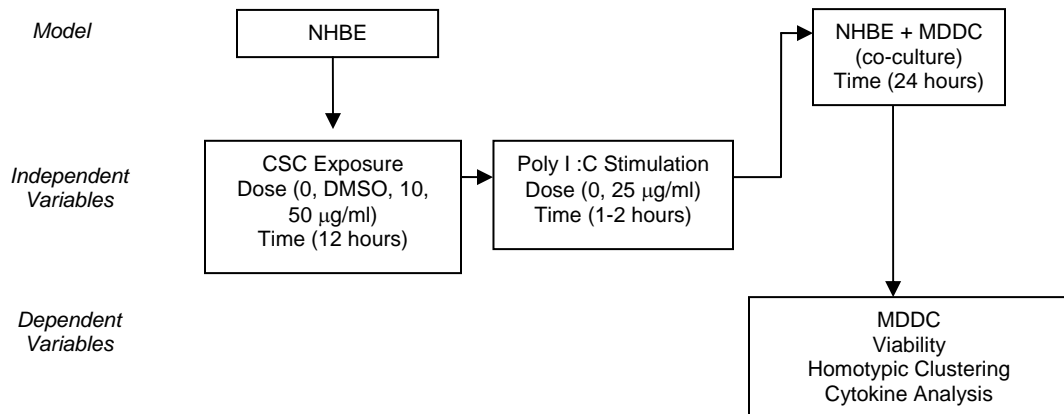


Figure 4. Aim 1 experimental schema. Depicts the experimental schema for aim 1 establishing and characterizing the model, µg/ml = microgram per milliliter.

Specific aim 2. Determine the effect of CSC and poly I:C stimulation on DC maturation, function and the ability of the exposed/stimulated DC to induce TC proliferation and priming. Compare the response of DC cultured alone or with bronchial EC (Figure 5).

- Does CSC exposure and poly I:C stimulation induce phenotypic DC maturation in MDDC co-cultured with NHBE cells as evidenced by maturation markers (CD80, CD83, CD86, and CD54) within the CD11c⁺ population?
- Does CSC exposure and poly I:C stimulation induce functional DC maturation in MDDC co-cultured with NHBE cells as evidenced by a lack of endocytic ability?
- Do CSC-exposed and poly I:C-stimulated MDDC co-cultured with NHBE cells induce CD4⁺ TC proliferation in MLR as evidenced by the CFSE proliferation assay?
- Do CSC-exposed and poly I:C-stimulated MDDC co-cultured with NHBE cells induce CD4⁺ cell proliferation towards a Th2 phenotype in the MLR as evidenced by a decrease in Th1 cytokines and an increase in Th2 cytokines?

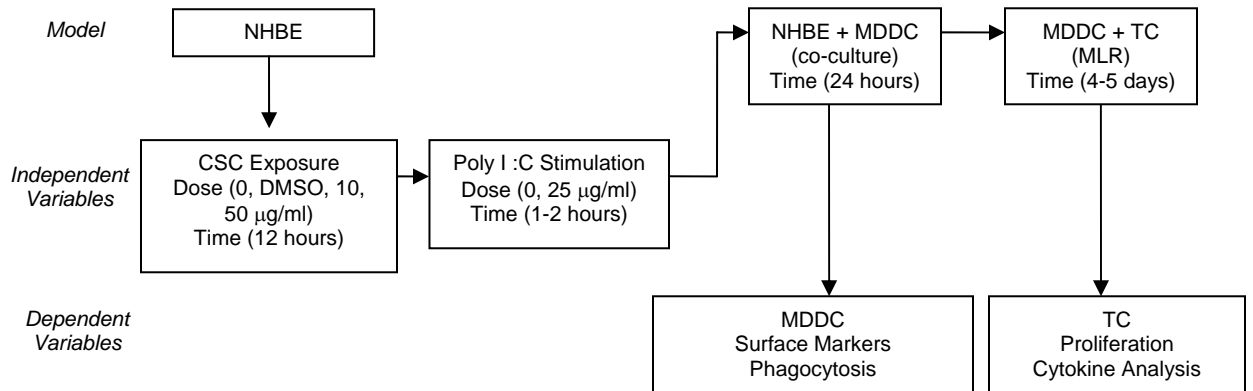


Figure 5. Aim 2 experimental schema. Depicts the experimental schema of aim 2 designed to test the effects of CSC and poly I:C exposure on MDDC surface marker expression, phagocytosis, TC proliferation and cytokine production.

Materials

Normal human bronchial epithelial cell culture. Primary NHBE cells were chosen for this study since they may more accurately mimic *in vivo* cell division characteristics as compared to transformed cell lines. NHBE cells were purchased from Clonetics/Cambrex (Lonza, Switzerland) which obtained the cells from healthy human volunteers after informed consent. Cells from one donor were obtained for replication of experiments. NHBE cells were grown according to the distributor guidelines. Briefly, primary NHBE cells (Passage 1) were grown to 75-80% confluency in an atmosphere of 5% CO² and 95% air at 37°C. Cell culture medium consists of bronchial epithelial growth medium supplemented with bovine pituitary extract, insulin, hydrocortisone,

human epithelial growth factor, epinephrine, transferrin, retinoic acid and triiodothyronine (Lonza, Switzerland). Cells were subcultured, aliquoted into freezing tubes (50,000 cells/vial), and stored in liquid nitrogen following passage 1. Cells used in these experiments were at a passage of 2 and 3. For NHBE only experiments, NHBE cells were seeded onto 12 well plates and grown to confluency. Confluent cultures will be exposed to CSC depending on experimental group (0, 10 or 50 $\mu\text{g/ml}$) for 12 hours. Poly I:C was then be added to CSC-conditioned medium depending on experimental group (0 or 50 $\mu\text{g/ml}$) for 1-2 hours. Following CSC exposure and poly I:C stimulation, DC were added to NHBE cultures. Supernatants were collected for subsequent cytokine analysis.

Monocyte-derived dendritic cells culture. Peripheral blood monocytes (PBMC) were isolated from buffy coats obtained from healthy volunteers (Florida Blood Services) after isolation by Ficoll-Paque (GE Healthcare/Amersham Biosciences, USA) density gradient centrifugation. CD14⁺ PBMC were isolated by positive selection using magnetically labeled CD14 antibodies and a magnetic separator (Miltenyi Biotec). PBMC can differentiate toward an immature, myeloid DC phenotype, CD11c⁺, HLA-DR^{lo}, E-cadherin⁻, langerin⁻ (de Heer et al., 2005) by an established method of culturing with IL4 and GM-CSF as described previously in the literature (Sallusto & Lanzavecchia, 1994). Briefly, CD14⁺ PBMC were cultured in RPMI 1640 medium supplemented with 2% L-Glutamin, 1% penicillin/streptomycin and 10% fetal bovine serum, 10 ng/ml IL-4, 20ng/ml GM-CSF (Peprotech), 1% nonessential amino acids and 1% sodium pyruvate (Sigma). Cultures were kept for 5-6 days in an atmosphere of 5% CO² and 95% air at 37°C. Immature MDDC (iMDDC) were harvested on day 5 or 6 and characterized by flow cytometry. One study compared the phenotype of DC using this method with lung

DC and reported similar phenotypic (low expression of CD40, CD80, CD83, CD86) and functional patterns (high capacity for the uptake of dextran and the ability to stimulate TC), making this an appropriate model for the *in vitro* study of pulmonary DC (Cochand, Isler, Songeon, & Nicod, 1999).

Co-culture of NHBE and MDDC cells. Methods for the co-culture of NHBE cells and MDDC are based on methods described in the literature (Bleck et al., 2006). Briefly, NHBE cells at passages 2 or 3 were seeded at a density of 2×10^4 cells/cm² on 12-well culture plates and maintained in an atmosphere of 5% CO² and 95% air at 37°C approximately 7 days or until 100% confluency is reached (Figure 6). Day 5 or 6 iMDDC were harvested and re-suspended in complete medium. MDDC (5×10^5 cells/well) to confluent NHBE cells in 12 well plates and exposed to CSC or poly I:C-conditioned medium depending on experimental group assigned. Following 24 hours of culture, light microscope images were taken with a 4x objective for homotypic clustering analysis and cells were harvested, washed and aliquoted for subsequent flow cytometry, endocytosis assay and mixed lymphocyte reactions. In addition, supernatants were collected for cytokine analysis.

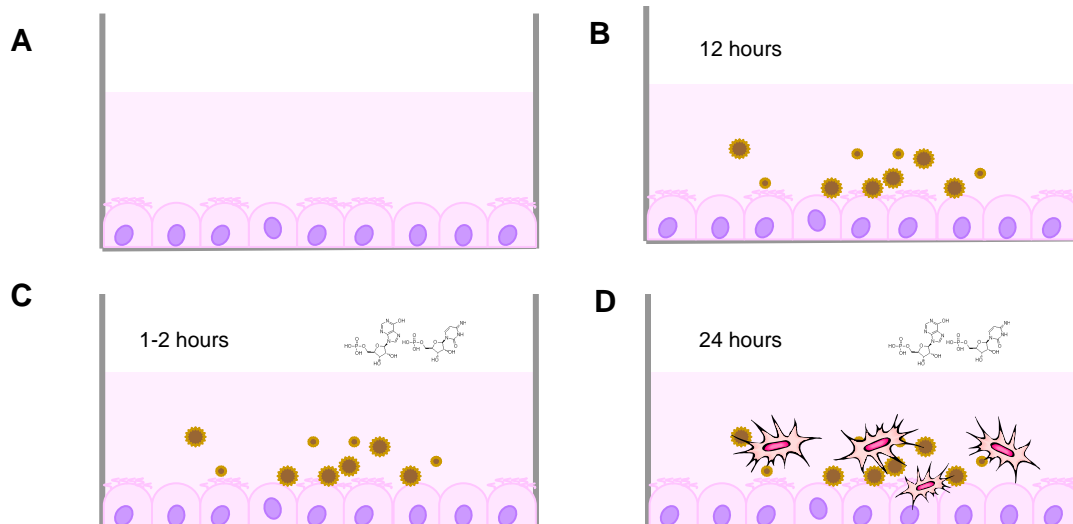


Figure 6. Generation of the co-culture model. A. culture of NHBE cells submerged in culture medium; B. exposure of confluent NHBE cells to CSC conditioned medium for 12 hours; C. stimulation of confluent NHBE cells with polyinosinic:polycytidylic acid (poly I:C) for 1-2 hours; D. addition of MDDC and co-cultured with confluent NHBE cells for 24 hours.

Treatment of cell cultures with CSC. Cigarette smoke consists of two components, the gas or vapor phase and smoke particulates. This study focused on the effects of smoke particulates by utilizing commercially available CSC. CSC purchased from Murty Pharmaceuticals (Lexington, KY, USA) was prepared by the manufacturer by using a Phipps-Bird 20 channel smoking machine. The particulate matter from Kentucky standard cigarettes (IR3F; University of Kentucky, KY, USA) was collected on Cambridge glass fiber filters and the amount obtained was determined by weight increase of the filter. CSC was prepared by dissolving the collected smoke particulates in dimethyl sulfoxide (DMSO) to yield a 4% solution (w/v). The average yield of CSC was 26.1 mg/cigarette. One ml of CSC is equivalent to 2 standard Kentucky IR3F cigarettes and each cigarette contains 1.3 mg of nicotine. CSC was added to culture medium in two doses (0 and 50 $\mu\text{g}/\text{ml}$) for 12 hours.

Treatment of cell cultures with poly I:C (Amersham Biosciences, Pittsburgh, PA, USA) was reconstituted in phosphate buffered saline (PBS) to 2 mg/ml by heating to 50°C to solubilize and allowing to slowly cool to room temperature in order to ensure re-annealing. Poly I:C was added to cell culture medium at two doses (0 or 50 µg/ml) for 1-2 hours. Poly I:C stimulated cultures were a positive control from MDDC maturation and mimic the effects of viral infection.

Homotypic clustering of DC. Homotypic and heterotypic clustering of DC, is critical for antigen presentation and correlates with DC maturation (Delemarre, Hoogeveen, De Haan-Meulman, Simons, & Drexhage, 2001; Lehner et al., 2003). In order to assess cluster formation following CSC exposure and poly I:C stimulation, an aggregation assay was performed prior to supernatant collection and harvesting of cells for flow cytometry analysis, endocytosis assay and mixed lymphocyte cultures. The homotypic cluster scoring system (Table 1) is a modification of methods reported previously (Lehner et al., 2003; Majdic et al., 1994). Figure 7 presents a light microscopy image of DC in various cluster states: single, small and large. Small and large clusters were discriminated by the density/darkness and size. In order to be considered large, over 75% of clusters had to be dark/dense and greater than 10 times the size of a single DC. Clusters were randomly counted by two blind investigators utilizing an inverted light microscope with a 4X objective and average scores were reported. The degree of aggregation or clustering was scored on a scale from 0 to 8 where a score of 0 indicates less than 10% of cells were in small clusters, a score of 1 indicates 10-50% of the MDDC were clustered in small clusters, a score of 2 indicates 10-50% of the MDDC were clustered in large clusters, a score of 3 indicates 51-75% of the MDDC were in small clusters, a score of 4 indicates 51-75% of the MDDC were in

large clusters, a score of 5 indicates 76-99% of the MDDC were clustered in small clusters, a score of 6 indicates 76-99% of the MDDC were clustered in large clusters, a score of 7 indicates 100% of the MDDC were clustered in small clusters, and a score of 8 indicates 100% of the MDDC were clustered in large clusters.

Table 1

Homotypic cluster scoring system

Score	Percent of Clusters	Size of Clusters
0	< 10 %	small
1	10-50%	small
2	10-50%	large
3	51-75%	small
4	51-75%	large
5	76-99%	small
6	76-99%	large
7	100%	small
8	100%	large

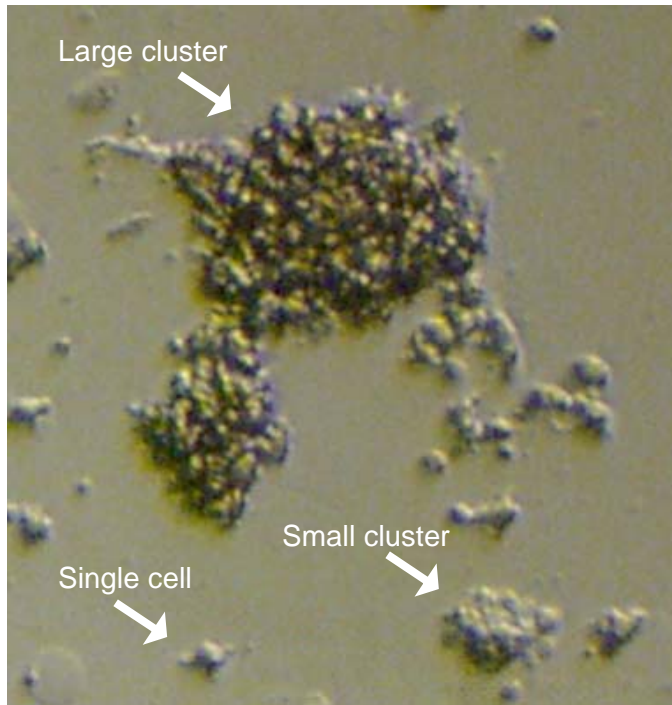


Figure 7. Homotypic cluster scoring. Light microscopy images depicting single cells, small and large clusters.

Quantification of cytokines by multi-plex bead assay. Cell culture supernatants from all experiments were collected, centrifuged and stored at -20°C until cytokine analysis was completed. Supernatant samples were analyzed using human LINCOPlex kits (Millipore, Billerica, MA, USA) multi-plex bead technology, which allows the analysis of multiple cytokines from one sample. The assay is based on characterization beads with a distinct emitting fluorescence pattern that are coated with capture antibodies specific for individual cytokines. A total of 7 cytokines were measured in NHBE and MDDC supernatants; they include: interleukin 6 (IL6), IL8, IL10, IL12p70, IL13, IFN γ -inducible protein 10 (IP10) and tumor necrosis factor alpha (TNF α). A total of 6 cytokines were measured in mixed lymphocyte reaction (MLR) supernatants; they

include: IL2, IL4, IL5, IL10, IL13, and IFN γ . Table 2 depicts each cytokine and the experiment in which it was collected along with the basic functional classification.

Cytokines were measured according to the manufacturer's instructions. Briefly, 25 μ L aliquots of medium samples were incubated with antibody coated capture beads for 1 hour, washed and incubated with biotin-labeled anti-human cytokine antibodies for 2 hours at room temperature followed by incubation with streptavidin-phycoerythrin for 30 minutes. Samples were analyzed using Luminex 100 IS System and IS 2.3 software (Luminex, Austin, TX, USA). Complete culture medium was used as the blank and added to all standards and quality controls. Each sample was assayed in duplicate and data were presented as the average. Standard curves of known concentrations of recombinant human cytokines (provided by the manufacturer) were used to convert mean fluorescence intensities to cytokine concentration in pg/mL by using 4 or 5 parameter logistic regression.

Table 2

Cytokine and chemokine analysis

Name	Th1/Th2	Experiment
Interleukin 2 (IL2)	Th1	MLR
Interleukin 4 (IL4)	Th2	MLR
Interleukin 5 (IL5)	Th2	MLR
Interleukin 6 (IL6)	Pro-inflammatory	NHBE+MDDC-EC
Interleukin 8 (IL8)	chemokine	NHBE+MDDC-EC
Interleukin 10 (IL10)	Th2	NHBE+MDDC-EC MLR
Interleukin 12p70 (IL12p70)	Th1	NHBE+MDDC-EC
Interleukin 13	Th2	MLR
Interferon gamma (IFN γ)	Th1	MLR
IFN γ -inducible protein-10 (IP10) or CXCR3	Th1 Chemokine	NHBE+MDDC-EC
Tumor necrosis factor alpha (TNF α)	Th1 or Th2	NHBE+MDDC-EC

Determination of viability and phenotypic maturation of MDDC by immunofluorescence labeling and flow cytometry. For phenotypic characterization of MDDC and NHBE cells following co-culture, MDDC were discriminated from EC by morphology (forward and side scatter). Flow cytometry was used to determine viability and the expression of cell surface maturation markers in MDDC and co-cultures of MDDC and NHBE cells exposed to CSC or poly I:C. Viability was determined by the addition of 7-Amino-Actinomycin D, 7-AAD (BD Biosciences Pharmingen) stain to identify non-viable cells during immunostaining. In order to phenotype MDDC, cells were stained with fluorochrome conjugated cell surface antibodies including: fluorescein isothiocyanate (FITC)- conjugated CD80 and CD83, phycoerythrin (PE)-conjugated

CD11c and CD86, allophycocyanin (APC)-conjugated HLA-DR and CD54 (BD Biosciences Pharmingen; Milteynyi Biotec). Table 3 depicts surface markers used to phenotype mature or activated MDDC.

Harvested MDDC were aliquoted into two tubes at approximately 2×10^5 cells per tube and washed. Each tube was stained with three conjugated Abs at the concentration recommended by the manufacturer. After surface staining, MDDC were washed with and fixed in PBS containing 4% paraformaldehyde (PFA), resuspended in stain buffer, protected from light and refrigerated at 4°C until flow analysis was performed. Flow cytometry was performed on a Becton Dickinson Canto II and LSR II Immunocytometry System. Expression of cell surface markers was determined by average mean fluorescence intensity (MFI). Poly I:C treatment (50 µg/ml for 24 hours) served as a positive control for MDDC maturation. Unstained cells and single stained cells were used to determine nonspecific binding and background auto-fluorescence.

Determination of functional maturation of MDDC by endocytosis assay. Since mature DC exhibit a loss of endocytic activity, an endocytosis assay was performed to confirm MDDC maturation. MDDC (5×10^5 /mL) cultured alone or in co-culture with NHBE cells were exposed for 24 hours to CSC, poly I:C, CSC and poly I:C or untreated. Following exposure, MDDC were harvested by pipetting, washed, re-suspended in complete RPMI medium and incubated on ice for 30 minutes. FITC-dextran (molecular weight 70,000, Molecular Probes) was added at a final concentration of 1mg/mL and exposed MDDC were incubated for 1 hour at 37°C or on ice (background staining). Following incubation, cells were immediately put on ice, washed and fixed with 4% paraformaldehyde, re-suspended in staining buffer and analyzed by flow cytometry (BD

FACS Canto). Immature MDDC will endocytose the FITC-dextran and mature MDDC will not. Data were expressed as MFI after subtracting for background staining.

Table 3

Dendritic cell surface marker analysis. Biological markers used for dendritic cell characterization

Marker	Immature	Mature	Function	Class
CD11c	+++	+++	dendritic cell marker	phenotypic
HLA-DR	++	+++	T cell receptor ligand	phenotypic
CD80	+	+++	co-stimulatory molecule	phenotypic
CD83	+	++	co-stimulatory molecule	phenotypic
CD86	+	+++	co-stimulatory molecule	phenotypic
CD54 (ICAM1)	++	+++	adhesion molecule	phenotypic
Phagocytosis	+++	--	antigen uptake	functional
MLR	--	+++	T cell proliferation	functional

Note. CD = cluster of differentiation; HLA-DR = human leukocyte antigen; + = minimal expression; ++ = moderate expression; +++ = high expression; -- = no expression.

Proliferation and polarization of allogeneic CD4+ TC in response to CSC

exposed and/or poly I:C stimulated MDDC. To determine the effect of CSC and poly I:C on DC-induced TC proliferation, MLR were performed with allogeneic (different donors) responder CD4+ TC confirmed by flow cytometry (FACS Canto II). Untouched human T helper cells were isolated from human blood buffy coats (Florida Blood Services)

following isolation by Ficoll-Paque and density gradient centrifugation and MACS cell suspension kit, CD4+ T cell isolation kit II (Miltenyi Biotec). The PBMCs were depleted of non-CD4+ TC including, CD8+ TC, gamma delta (γ/δ) TC, B cells, natural killer cells, DC, monocytes, granulocytes, and erythroid cells. In order to deplete these cell types, PBMCs were indirectly magnetically labeled using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ , and CD235a (glycophorinA) according to the manufacturer's instructions. The negative fraction was confirmed as the CD4+ population by double positive staining for CD3 and CD4 by flow cytometry analysis.

Immature MDDC (5×10^5) were exposed alone or in co-culture with NHBE cells for various culture times and doses of CSC and Poly I:C in complete medium supplemented with IL4 and GMCSF. Exposed MDDC were washed in fresh medium and treated with mitomycinC ($20 \mu\text{g}/\text{mL}$, 45 min at 37°C) to prevent proliferation. TC were treated with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen) in order to determine proliferation. The protocol for CFSE staining is based on methods described previously (Quah, Warren, & Parish, 2007).

In order to determine optimal MDDC to TC ratios, 3 different ratios of MDDC in $100 \mu\text{L}$ of medium were analyzed while the number of TC remains constant (2×10^5). Ratios were assayed in triplicate, including 1:8, 1:16, and 1:32. MDDC were co-cultured with responder allogenic TC ($100 \mu\text{L}$ of 1×10^5 cells/ mL) for 4-5 days in 96-well round bottom microplates.

MLR cell culture supernatants were collected for cytokine analysis (as described previously) in order to determine TC polarization as Th1 (IL2, $\text{IFN}\gamma$, $\text{TNF}\alpha$) or Th2 (IL4, IL5, IL10) as described in Table 1. TC were harvested and analyzed by flow cytometry.

Controls for this assay included: unstained (no CFSE) treated TC, TC cultured alone, TC-treated with mitomycin C. The positive control was MDDC cultured with TC and stimulated with a mitogen, phytohemmagglutinin (PHA) at a concentration of 5 μ g/mL.

The assay works by CFSE passively diffusing into the TC. The CFSE undergoes esterase cleavage of the acetate groups and diffuses throughout the cytoplasm where it reacts with amines forming highly fluorescent conjugates that are well retained. As the cells divide, the CFSE is split equally between the daughter cells resulting in diminished CFSE signal detection that occurs with each subsequent cell division allowing for a quantitative determination of the number of cell divisions.

Data Analysis

In this 4x2 factorial design study, experiments were performed with MDDC and TC derived from at least four individual donors. Data were reported as mean and standard error of the mean. Each of the dependent variables were transformed by base 10 logarithm except for DC viability (percent live), homotypic clustering (cluster score 0-8) and TC proliferation (percent proliferation); in these three cases, raw data was used. Log transformation was performed in order to normalize data. Each donor exhibited similar trends in data but a variation in magnitude between donors was observed.

Paired *t*-tests were conducted for two planned comparisons (see Figure 8), including DMSO vehicle control versus CSC high dose (CSCH) in non-stimulated and poly I:C stimulated co-cultures. In order to determine level of significance and reduce the likelihood of a Type I error, critical alpha values were determined using a modified Bonferroni correction (Holland & Copenhaver, 1988). Briefly, each test of significance was ranked. The smallest observed *p* value was compared against a critical alpha value of $p = 0.025$ (alpha 0.05 divided by the number of planned comparisons = 2). The

second test was compared against a critical alpha value of $p = 0.05$. In addition, eta squared ($\eta^2 / \eta^2 + \text{degrees of freedom}$) values are reported to display the effect size for each dependent variable. All statistics were performed with the Statistical Package for Social Sciences (SPSS version 15) and figures constructed with GraphPad Prism (Version 3.03). In chapter four is a summary of results obtained in this study is presented.

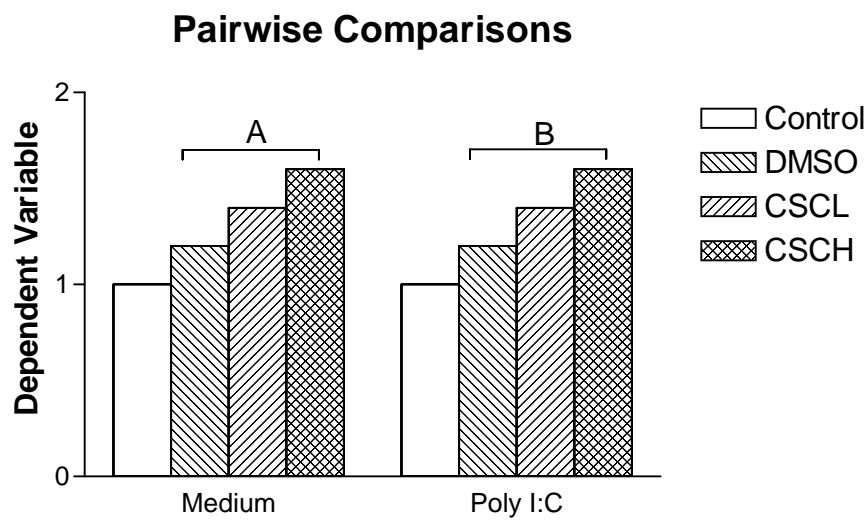


Figure 8. Pairwise comparisons for dependent variables.

Chapter Four

Results

Characteristics of the Sample

 Buffy coats from a total of four individual donors were used in this study to isolate MDDC and TC. Identifying data about each donor was de-identified by Florida Blood Services and only age and gender were given. Two of the four donors were female and the average age was 38 and ranged from 21 to 60 years (21, 21, 50 and 60 years). Identifying data about the NHBE donor was de-identified by Lonza (Walkersville, Inc.) and only age and gender were given. The NHBE donor was a 2 year old male. Overall descriptive statistics of the raw data for each dependent variable are displayed in Table 4.

Table 4

Descriptive statistics of all dependent variables

	Unit of Measure	N	Minimum	Maximum	Mean	SEM
Medium						
Viability	% Live	16	86.20	98.90	97.05	0.78
Cluster Score	0-8	32	0.00	5.00	2.78	0.22
Phagocytosis	MFI	16	17223	54312	30055	2912
CD11c	MFI	16	40783	67670	54132	2026
HLA-DR	MFI	16	995	2573	1640	137
CD80	MFI	16	10893	39214	19305	2016
CD83	MFI	16	6535	20412	11142	969
CD86	MFI	16	13740	43733	25712	2301
CD54	MFI	16	28699	115052	69096	6603
Interleukin 6*	pg/ml	16	0.00	16.13	6.76	1.22
Interleukin 8*	pg/ml	16	0.00	1287	123	86.13
Interleukin 10*	pg/ml	16	0.00	178	52.77	12.79
Interleukin 12p70*	pg/ml	16	0.00	6.68	0.79	0.54
Tumor Necrosis Factor α *	pg/ml	16	0.00	7.70	2.18	0.84
IFN-Inducing Protein 10*	pg/ml	16	0.00	17.43	2.23	1.20
T Cell Proliferation	% Proliferation	16	23.47	55.82	37.82	2.94
Interleukin 2	pg/ml	16	11.88	89.02	39.78	5.99
Interferon γ	pg/ml	16	35.27	3841	591	239
Interleukin 4	pg/ml	16	32.80	725	173	43.60
Interleukin 5	pg/ml	16	11.27	286	88.79	20.69
Interleukin 10	pg/ml	16	140	3613	986	233
Interleukin 13	pg/ml	16	56.63	2329	860	205
Poly I:C						
Viability	% Live	16	73.25	96.77	90.70	1.69
Cluster Score	0-8	32	3.00	6.00	4.81	0.18
Phagocytosis	MFI	16	457	7952	2531	509
CD11c	MFI	16	44360	72549	62684	2399
HLA-DR	MFI	16	1092	2457	1741	109
CD80	MFI	16	29630	92959	51408	5295
CD83	MFI	16	4458	39609	30029	224
CD86	MFI	16	11005	109085	81333	5939
CD54	MFI	16	74106	131152	100183	4475
Interleukin 6*	pg/ml	16	529	3131	1837	211
Interleukin 8*	pg/ml	16	0.00	5178	1891	363
Interleukin 10*	pg/ml	16	15.57	3176	1009	262
Interleukin 12p70*	pg/ml	16	50.14	5935	1199	450
Tumor Necrosis Factor α *	pg/ml	16	86.75	1957	492	137
IFN-Inducing Protein 10*	pg/ml	16	0.00	8797	2329	716
T Cell Proliferation	% Proliferation	16	33.44	64.92	49.02	2.60
Interleukin 2	pg/ml	16	21.31	286	100	22.01
Interferon γ	pg/ml	16	130	5022	1432	395
Interleukin 4	pg/ml	16	30.5	611	187	43.35
Interleukin 5	pg/ml	16	8.85	392	119	25.00
Interleukin 10	pg/ml	16	261	3984	1424	283
Interleukin 13	pg/ml	16	63.89	3667	1128	253

Note. * = Co-culture values minus EC only culture values; SEM = standard error of the mean.

Experimental Conditions Do Not Affect DC Viability

In order to determine that CSC effects were not related to a loss in DC viability experiments were conducted using 7AAD staining, analyzed by flow cytometry and percent viable/live was calculated (Figure 9). Descriptively, it appears there is no difference in DMSO vehicle versus CSCH in both non-stimulated and poly I:C stimulated co-cultures (Table 5).

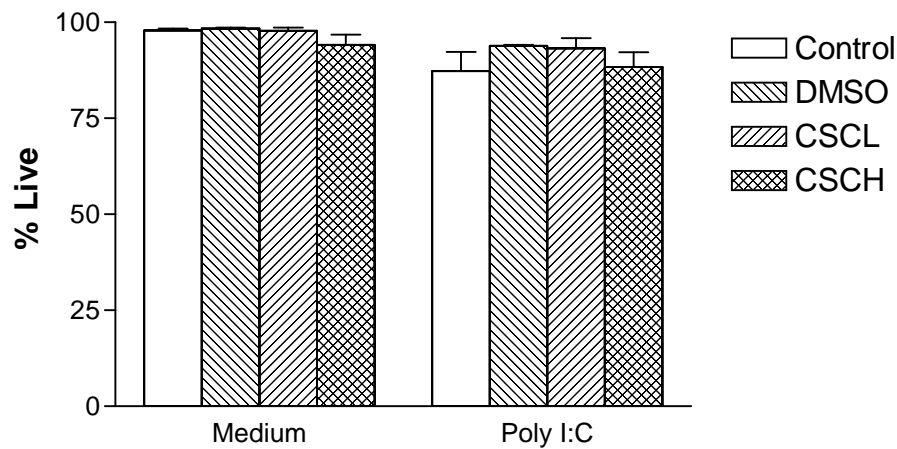


Figure 9. Dendritic cell viability. Data presented as mean \pm SEM. (7AAD staining)

Table 5

Dendritic cell viability

<i>Comparison (DMSO vs CSCH)</i>	<i>Mean 1 (DMSO)</i>	<i>Mean 2 (CSCH)</i>
Medium	98.4 \pm 0.27	94.2 \pm 2.7
Poly I:C	93.9 \pm 0.28	88.4 \pm 3.8

Note. Data are presented as mean percent live \pm SEM.

CSC Induces Homotypic Clustering in Non-Stimulated Co-Cultures

None of the previous human *in vitro* studies described DC homotypic clustering following smoke exposure. Homotypic clustering is the ability of DC to up-regulate adhesion molecules and cluster together and is an indicator of DC maturation (Delemarre et al., 2001). In order to determine if smoke exposure induced homotypic clustering, light microscopy images (4x) were obtained following smoke and poly I:C exposures and scored as previously described. A representative donor was chosen and typical clustering can be seen in Figure 10B. A total of 8 individual donors were used to calculate homotypic clustering and two blinded investigators scored each condition based on a scoring system (Table 1) and scores were averaged. Co-cultures that were not stimulated with poly I:C had a significant increase in cluster score secondary to CSC exposure (2.31 ± 0.47 versus 3.62 ± 0.26), $t(3) = -3.19$, $p < 0.025$ (two-tailed) (Figure 10C and Table 6). In poly I:C stimulated co-cultures, clusters were larger and accounted for a higher percentage of DC compared to no poly I:C stimulation; however, there was no effect of CSC exposure (4.88 ± 0.44 versus 4.75 ± 0.37), $t(3) = 0.73$, $p > 0.050$ (two-tailed), as depicted in Figure 10C and Table 6. A two-tailed test was performed because it was unknown if CSC or poly I:C would reduce or increase clustering.

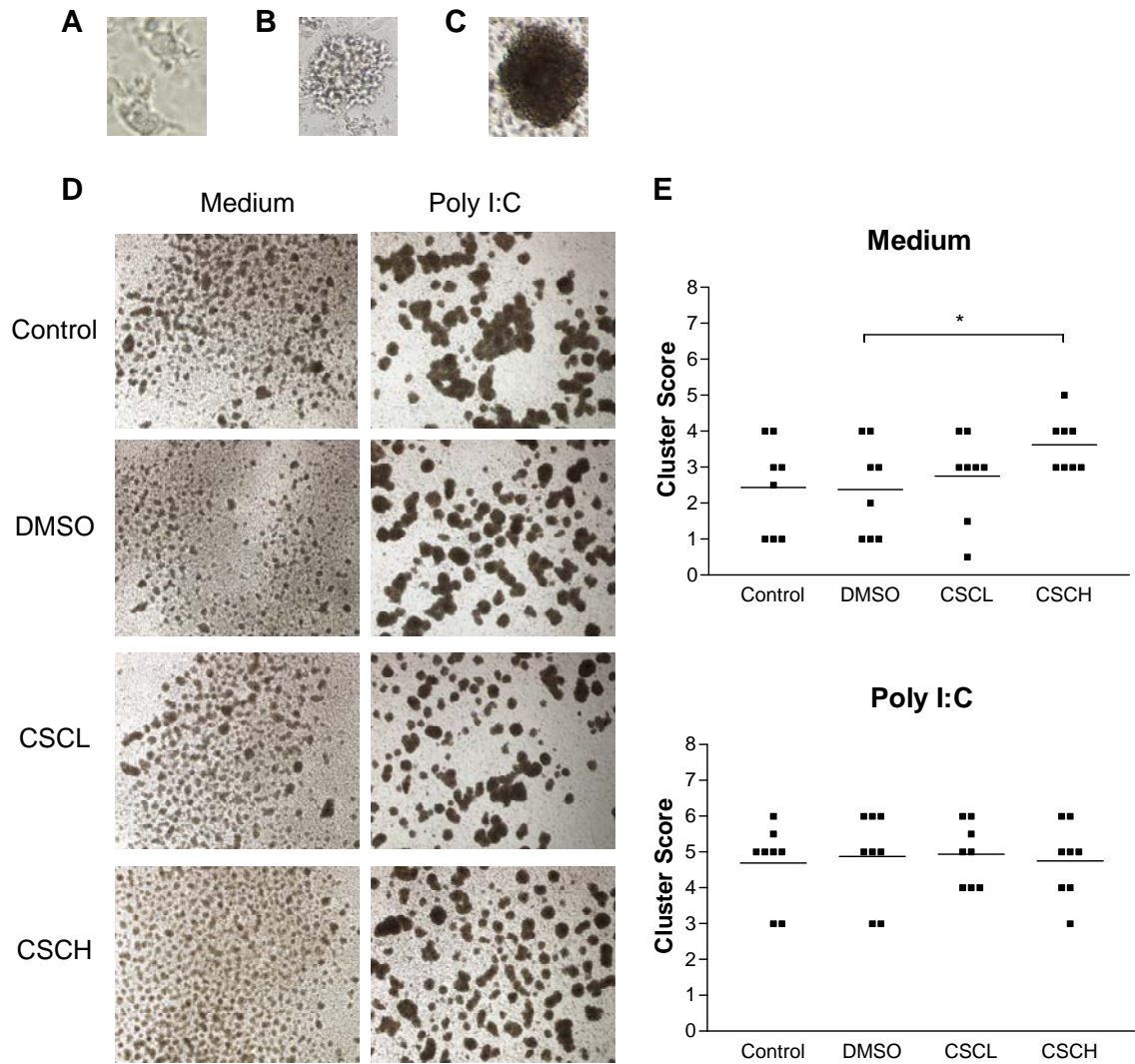


Figure 10. Dendritic cell homotypic clustering. Light microscopy images: A. Single dendritic cell (40x); B. small cluster (20x); C. large cluster (10x); D. Light microscopy images (4X) of all experimental conditions from a representative donor; E. Cluster score, horizontal bar represents mean, * = p value < critical alpha, 0.025 (two-tailed), n = 8.

Table 6

Dendritic cell homotypic clustering

<i>Comparison (DMSO vs CSCH)</i>	<i>Mean 1 (DMSO)</i>	<i>Mean 2 (CSCH)</i>	<i>eta²</i>	<i>t</i>	<i>Critical p</i>	<i>Observed p</i>
Medium	2.31 ± 0.47	3.62 ± 0.26	0.59	-3.19	0.025	0.015*
Poly I:C	4.88 ± 0.44	4.75 ± 0.37	0.02	0.73	0.050	0.732

Note. Data presented as mean cluster score ± SEM. * = p value < critical alpha 0.025, (two-tailed), n = 8.

CSC Reduces Phagocytosis in Non-Stimulated Co-Cultures

None of the previous human *in vitro* studies described phagocytosis of DC following smoke exposure. Phagocytosis or the ability for DC to take up antigen is another indicator of DC maturation and function. When a DC has taken up antigen and is mature, it will no longer phagocytize antigen or particulates; therefore, mature or maturing DC will not take up the FITC-dextran and will have lower mean fluorescence intensity (MFI). In order to determine if smoke exposure affected phagocytosis, FITC-dextran uptake was analyzed following CSC and poly I:C exposure. The evaluation of FITC-dextran phagocytosis demonstrated an inhibition following CSC exposure in non-stimulated co-cultures (4.50 ± 0.08 versus 4.34 ± 0.08), $t(3) = 6.13$, $p < 0.025$ (two-tailed) (Figure 11 and Table 7). Poly I:C stimulated co-cultures served as a positive control for MDDC maturation and exhibited very low MFI compared to non-stimulated co-cultures and there was no significant effect of CSC in poly I:C stimulated co-cultures (3.25 ± 0.12 versus 3.41 ± 0.22), $t(3) = -1.38$, $p < 0.025$ (two-tailed). A two-tailed test was performed because it was unknown if CSC or poly I:C would reduce or increase phagocytosis.

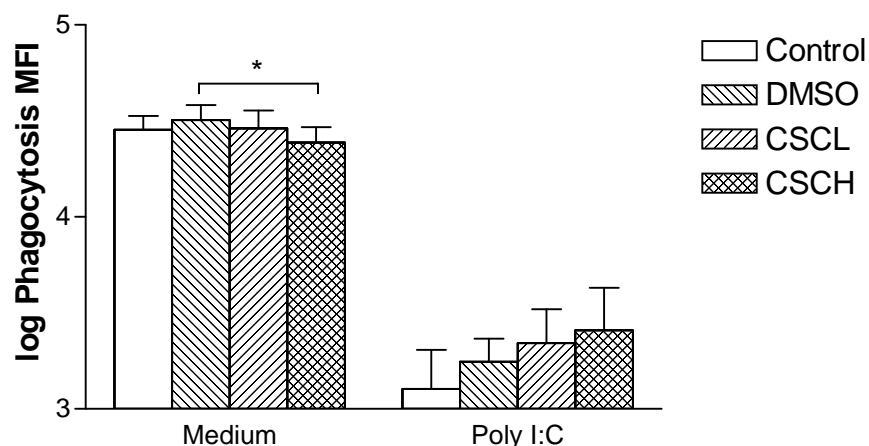


Figure 11. Dendritic cell phagocytosis. Data were transformed by base 10 logarithm and presented as mean \pm SEM. * = p value < critical alpha (two-tailed), n = 4.

Table 7

Dendritic cell phagocytosis

Comparison (DMSO vs CSCH)	Mean 1 (DMSO)	Mean 2 (CSCH)	η^2	t	Critical p	Observed p
Medium	4.50 \pm 0.08	4.34 \pm 0.08	0.93	6.13	0.025	0.009*
Poly I:C	3.25 \pm 0.12	3.41 \pm 0.22	0.39	-1.38	0.050	0.261

Note. Data were transformed by base 10 logarithm and presented as mean \pm SEM. * = p value < critical alpha (two-tailed), n = 4.

CSC Induces Maturation-Associated Co-Stimulatory and Adhesion Molecules

Surface markers or co-stimulatory markers are essential in inducing a TC response and are often used as an indicator of DC maturation. Only one study has investigated the effects of smoke on DC surface expression markers *in vitro* and in that study, DC were cultured alone (Vassallo et al., 2005). This study explored the effect of smoke on surface expression markers in MDDC and NHBE co-cultures. To address this

question, co-cultures were exposed to two doses of smoke, stimulated with poly I:C and surface marker expression was observed within the CD11c+ MDDC population. The percent of CD11c+ MDDC ranged from 98.94% to 99.88% in all experimental conditions and all donors (n = 4). As shown in Figure 12 and Table 8, smoke did not have an effect on CD11c or HLA-DR expression in either stimulated or non-stimulated cultures.

Although the previous study found a reduction in surface maturation markers when DC were exposed to smoke, another study investigating DEP reported an increase in CD80, CD83 and CD86 when DC were co-cultured with EC (Bleck et al., 2006). Therefore, it was speculated that maturation markers may be increased in co-culture conditions. As shown in Figure 13, smoke exposure significantly increased markers CD83 (3.96 ± 0.02 versus 4.34 ± 0.03), $t(3) = -11.86$, $p < 0.025$ (two-tailed) in non-stimulated co-cultures. CD80 and CD86 followed a similar pattern but failed to reach statistical significance (Table 8). This upregulation of surface maturation markers was confirmed in MDDC derived from four different donors.

No study has examined the effect of smoke on DC expression of CD54 *in vitro*, but previous reports demonstrated a reduction in CD54 expression in alveolar macrophages and circulating monocytes of smokers (Lensmar, Elmberger, Skold, & Eklund, 1998; Luppi, Lain, Jeyabalan, & DeLoia, 2007; Skold, Lundahl, Hallden, Hallgren, & Eklund, 1996). Therefore, it was speculated that smoke exposure may have an effect on DC CD54 expression. As shown in Figure 12 and Table 8, smoke exposure significantly increased CD54 expression in non-stimulated co-cultures (4.68 ± 0.08 versus 4.98 ± 0.05), $t(3) = -9.15$, $p < 0.025$ (two-tailed). In all poly I:C stimulated co-cultures, smoke did not induce significant changes of any DC surface markers (Table 8). A one-tailed test was performed because it was unknown if CSC or poly I:C would reduce or increase clustering.

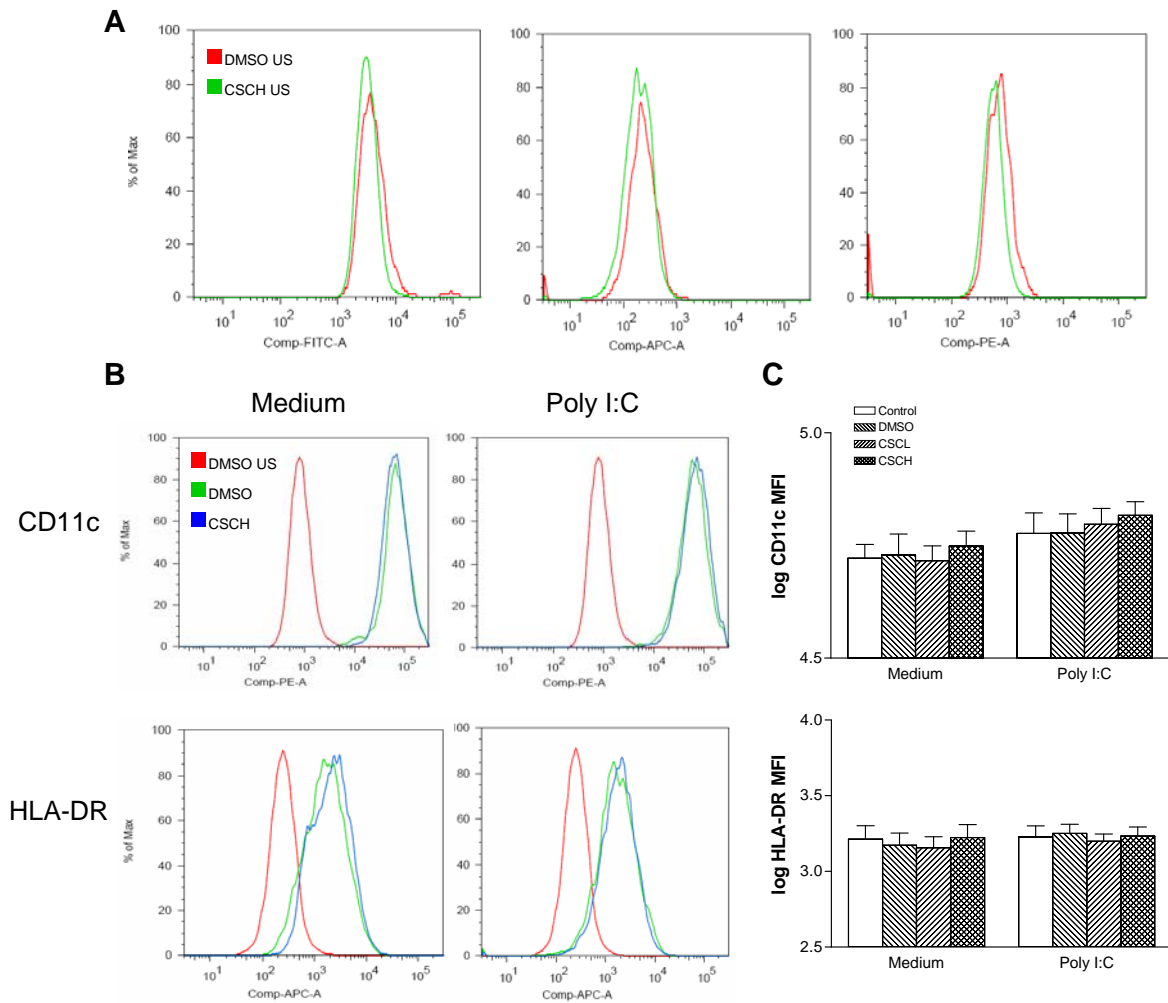


Figure 12. Dendritic cell surface markers: Controls, CD11c and HLA-DR. A. Unstained controls for each fluorochrome (FITC, APC, PE), B. Flow cytometry histograms from a representative donor; C. MFI data were transformed by base 10 logarithm and presented as mean \pm SEM, n=4.

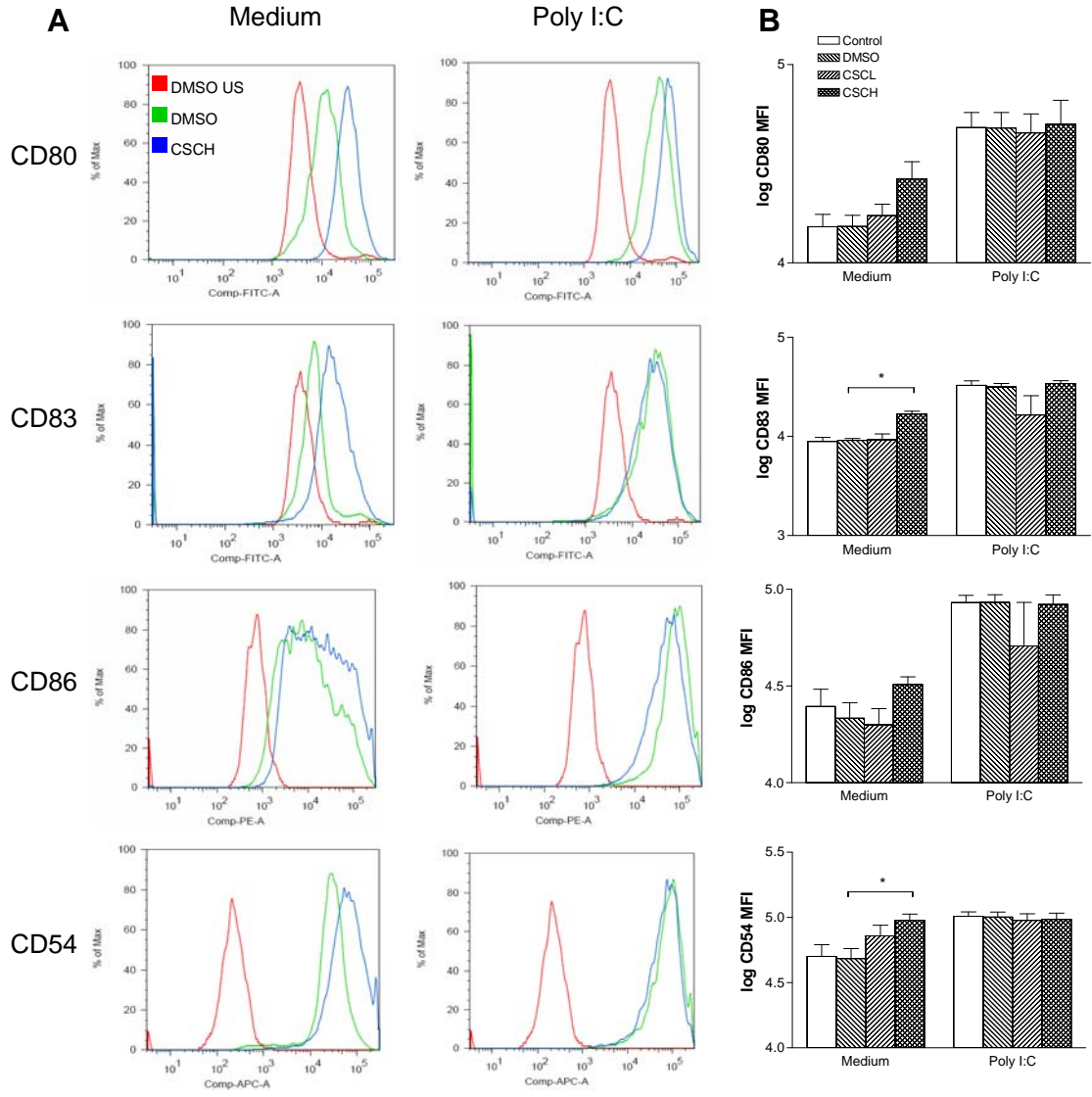


Figure 13. Dendritic cell surface markers: CD80, CD83, CD86 and CD54. A. Flow cytometry histograms from a representative donor; B. MFI data were transformed by base 10 logarithm and presented as mean \pm SEM, * = p value < critical alpha (two-tailed), n=4.

Table 8

Dendritic cell surface markers

<i>Comparison (DMSO vs CSCH)</i>	<i>Mean 1 (DMSO)</i>	<i>Mean 2 (CSCH)</i>	<i>eta²</i>	<i>t</i>	<i>Critical p</i>	<i>Observed p</i>
Medium						
CD11c	4.73 ± 0.05	4.78 ± 0.03	0.38	-1.36	0.050	0.265
HLA-DR	3.17 ± 0.04	3.22 ± 0.03	0.63	-2.27	0.025	0.108
CD80	4.18 ± 0.06	4.42 ± 0.09	0.72	-2.78	0.025	0.069
CD83	3.96 ± 0.02	4.23 ± 0.03	0.98	-11.86	0.025	0.001*
CD86	4.33 ± 0.08	4.51 ± 0.04	0.78	-3.24	0.025	0.048
CD54	4.68 ± 0.08	4.98 ± 0.05	0.97	-9.15	0.025	0.003*
Poly I:C						
CD11c	4.78 ± 0.09	4.82 ± 0.06	0.63	-2.25	0.025	0.110
HLA-DR	3.25 ± 0.06	3.23 ± 0.06	0.27	1.05	0.050	0.372
CD80	4.68 ± 0.08	4.70 ± 0.12	0.02	-0.25	0.050	0.819
CD83	4.50 ± 0.04	4.53 ± 0.05	0.10	-0.60	0.050	0.595
CD86	4.93 ± 0.04	4.92 ± 0.05	0.02	0.26	0.050	0.815
CD54	5.00 ± 0.04	4.99 ± 0.05	0.34	1.26	0.050	0.298

Note. MFI data were transformed by base 10 logarithm and presented as mean ± SEM. * = p value < critical alpha (two-tailed), n = 4.

CSC Failed to Significantly Affect Dendritic Cell Cytokines

Poly I:C stimulated co-cultures produced substantial amounts of all cytokines and chemokines assayed as compared to non-stimulated cultures. Although none of the DC cytokines were statistically different, two cytokines approached statistical significance, IL10 in both poly I:C and non-stimulated co-cultures and interferon inducing protein 10 (IP10) in poly I:C stimulated co-cultures (Figure 14 and Table 9). Smoke exposure increased IL10 suggesting that smoke induces DC Treg polarizing signal that could contribute to immunosuppressive effects. IP10 was also increased in poly I:C stimulated cultures which is an important chemoattractant of Th1 lymphocytes and is an appropriate response to a viral stimulus.

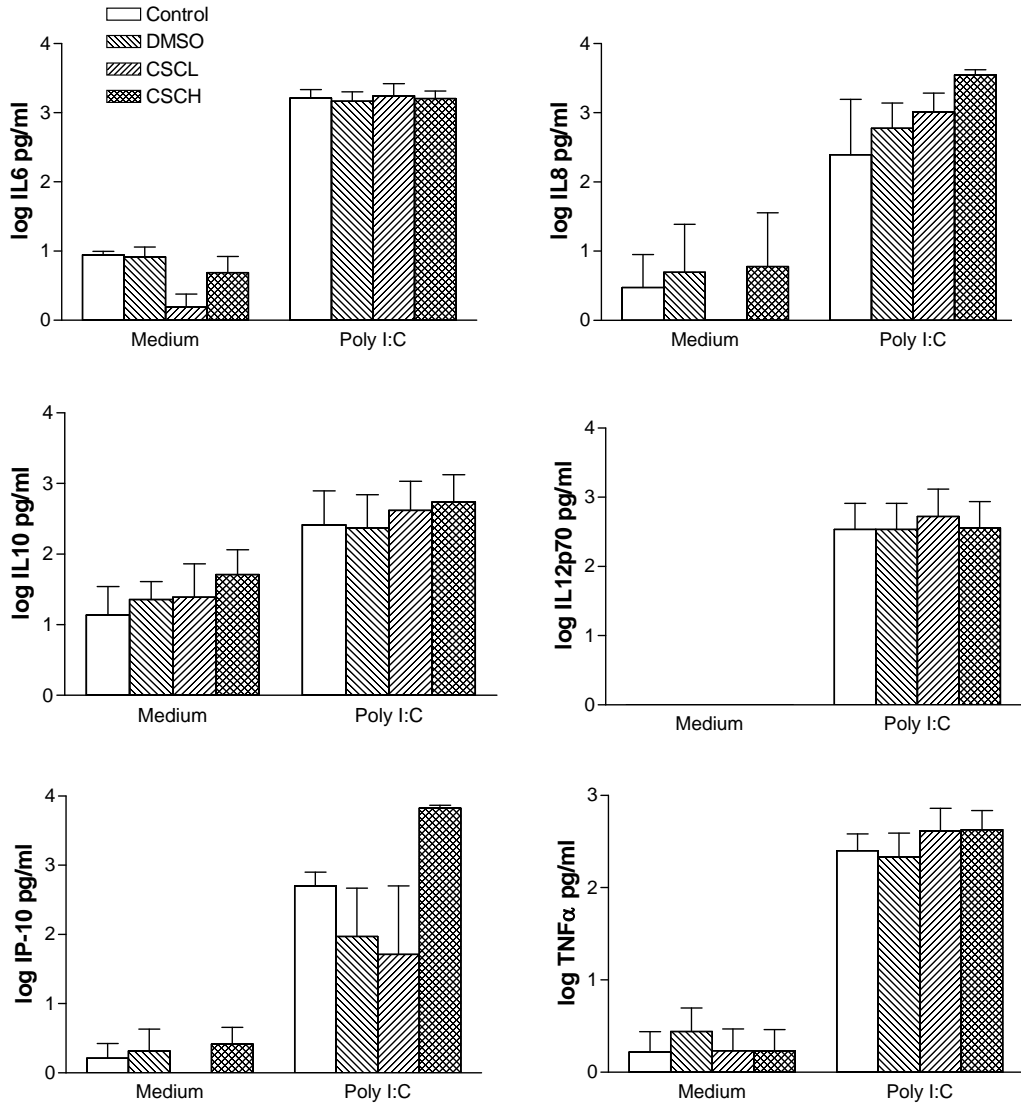


Figure 14. Dendritic cell cytokines and chemokines. Data were transformed by base 10 logarithm and presented as mean \pm SEM, n=4.

Table 9

Dendritic cell cytokines and chemokines

<i>Comparison (DMSO vs CSCH)</i>	<i>Mean 1 (DMSO)</i>	<i>Mean 2 (CSCH)</i>	<i>eta²</i>	<i>t</i>	<i>Critical p</i>	<i>Observed p</i>
Medium						
Interleukin 6	0.91 ± 0.14	0.98 ± 0.09	0.23	-0.95	0.025	0.207
Interleukin 8	0.69 ± 0.69	0.78 ± 0.78	0.00	-0.07	0.050	0.475
Interleukin 10	1.36 ± 0.25	1.71 ± 0.35	0.69	-2.61	0.050	0.040
Interleukin 12p70	0.00 ± 0.00	0.00 ± 0.00	--	--	--	--
Tumor Necrosis Factor α	0.44 ± 0.26	0.23 ± 0.23	0.23	0.94	0.050	0.208
Interferon-Inducible Protein 10	0.32 ± 0.32	0.41 ± 0.25	0.07	-0.46	0.050	0.338
Poly I:C						
Interleukin 6	3.17 ± 0.14	3.21 ± 0.11	0.05	-0.43	0.050	0.349
Interleukin 8	2.78 ± 0.37	3.54 ± 0.08	0.54	-1.88	0.025	0.079
Interleukin 10	2.37 ± 0.47	2.74 ± 0.39	0.75	-2.97	0.025	0.029
Interleukin 12p70	2.54 ± 0.38	2.56 ± 0.38	0.03	-0.31	0.025	0.389
Tumor Necrosis Factor α	2.33 ± 0.26	2.63 ± 0.21	0.37	-1.33	0.025	0.138
Interferon-Inducible Protein 10	1.97 ± 0.70	3.82 ± 0.04	0.70	-2.62	0.025	0.040

Note. -- = unable to calculate; Data were transformed by base 10 logarithm and presented as mean ± SEM; critical p value (two-tailed).

CSC Failed to Induce TC Proliferation but Enhances Th2 Cytokine Production

TC proliferation is an indicator of DC ability to induce an immune response to invading pathogens. Smoke exposed and poly I:C stimulated MDDC were co-cultured with allogeneic, CFSE stained CD4+ TC in mixed lymphocyte reactions (DC:TC ratios of 1:8, 1:16, 1:32) for 4-5 days. Figure 15A shows stained, unstained, non-proliferated and proliferated (PHA stimulated) controls and Figure 15B displays a representative donor of TC proliferation (1:16 ratio). This study showed that despite evidence that CSC exposed DC are maturing (increased clustering, decreased phagocytosis, increased maturation markers), they do not significantly increase TC proliferation (Figure16). Figure 16 and Table 10 represent pooled data from four experiments at a ratio of 1:16 (1:8, 1:32 data

not shown). There was no effect of smoke on TC proliferation in non-stimulated or poly I:C stimulated co-cultures and no trend was observed at any DC:TC ratio.

Multi-plex bead assays were used to assess TC polarization by examining the profile (T regulatory, Th1 or Th2 type) of cytokines that are secreted by the TC co-cultured with exposed DC. This study showed no significant effect of CSC on the major Th1 cytokine, IFN γ , but did show a statistically significant increase in the proliferative cytokine, IL2 (1.86 ± 0.20 versus 1.94 ± 0.29), $t(3) = -5.04$, $p < 0.025$ (two-tailed) in poly I:C stimulated co-cultures. In addition, several major Th2 cytokines were upregulated secondary to CSC exposure in poly I:C stimulated co-cultures, including: IL5 (1.83 ± 0.28 versus 2.13 ± 0.25), $t(3) = -5.97$, $p < 0.025$ (two-tailed), and IL13 (2.75 ± 0.28 versus 3.07 ± 0.28), $t(3) = -5.61$, $p < 0.025$ (two-tailed) in poly I:C stimulated cultures. A similar trend was observed in IL4 but it failed to reach statistical significance. One of the primary Treg cytokines, IL10, was upregulated in poly I:C stimulated co-cultures (2.89 ± 0.22 versus 3.14 ± 0.23), $t(3) = -3.30$, $p < 0.050$ (two-tailed) and IL10 approached statistical significance in non-stimulated co-cultures (Figure 17 and Table 11). Th1:Th2 ratios were also evaluated and only two significant ratios were observed and only in poly I:C stimulated cultures including, IL2:IL5 and IL2:IL13 (Table 12). CSC treatment significantly increased Th2 type cytokines (IL5 and IL13) despite the Th1 type poly I:C stimulus. In addition, IL10 was increased in both poly I:C stimulated and non-stimulated cultures suggesting smoke exposure induces a Treg and immunosuppressive response. In chapter five, a discussion of the results obtained in this study is presented. In addition, limitations, future directions and the significance to nursing is also discussed.

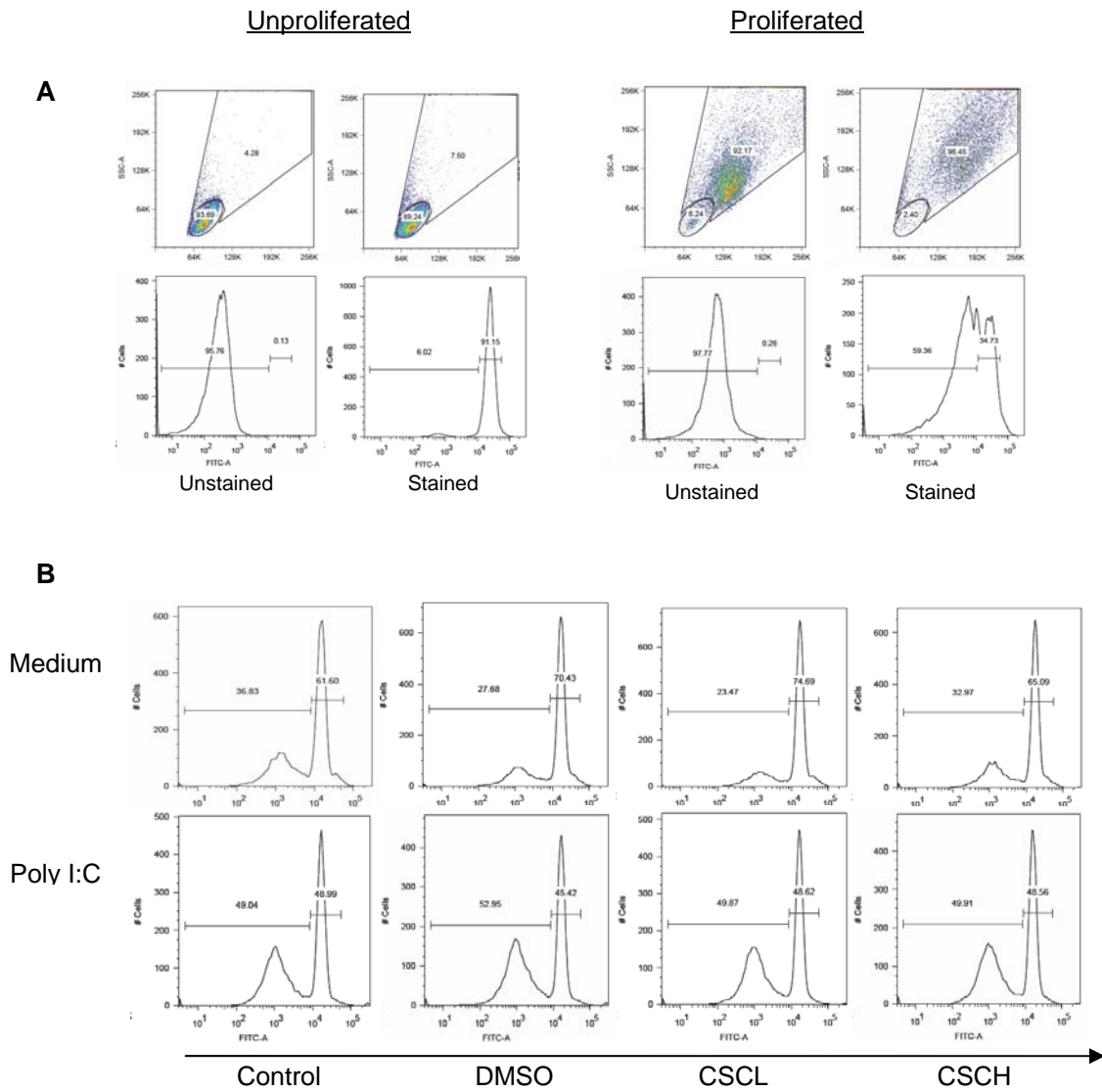


Figure 15. T cell proliferation: Flow cytometry. A. Proliferated and unproliferated, stained and unstained controls, B. flow cytometry images from a representative donor.

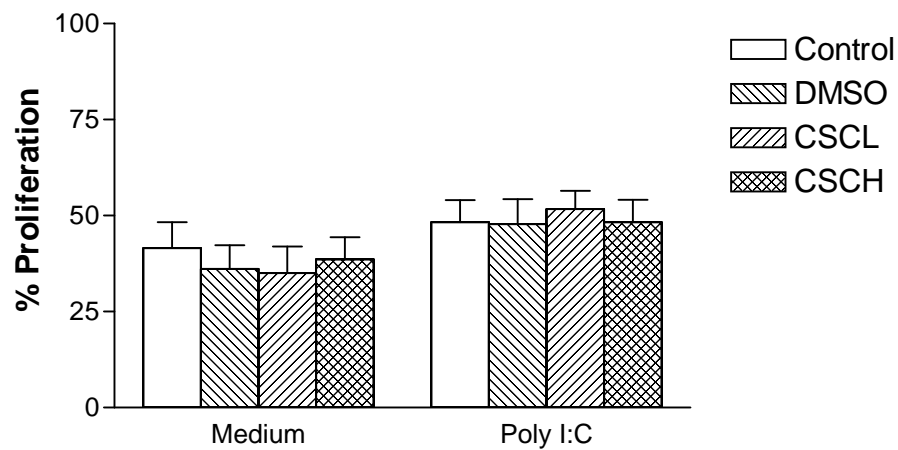


Figure 16. T cell proliferation: Percent proliferation. Data are presented as percent proliferation \pm SEM.

Table 10

T cell proliferation: Percent proliferation

Comparison (DMSO vs CSCH)	Mean 1 (DMSO)	Mean 2 (CSCH)	η^2	t	Critical p	Observed p
Medium	36.1 \pm 6.2	38.6 \pm 5.8	0.58	-2.05	0.025	0.066
Poly I:C	47.8 \pm 6.5	48.3 \pm 5.9	0.01	-0.14	0.050	0.899

Note. Data are presented as percent proliferated and presented as mean \pm SEM; critical p value (two-tailed).

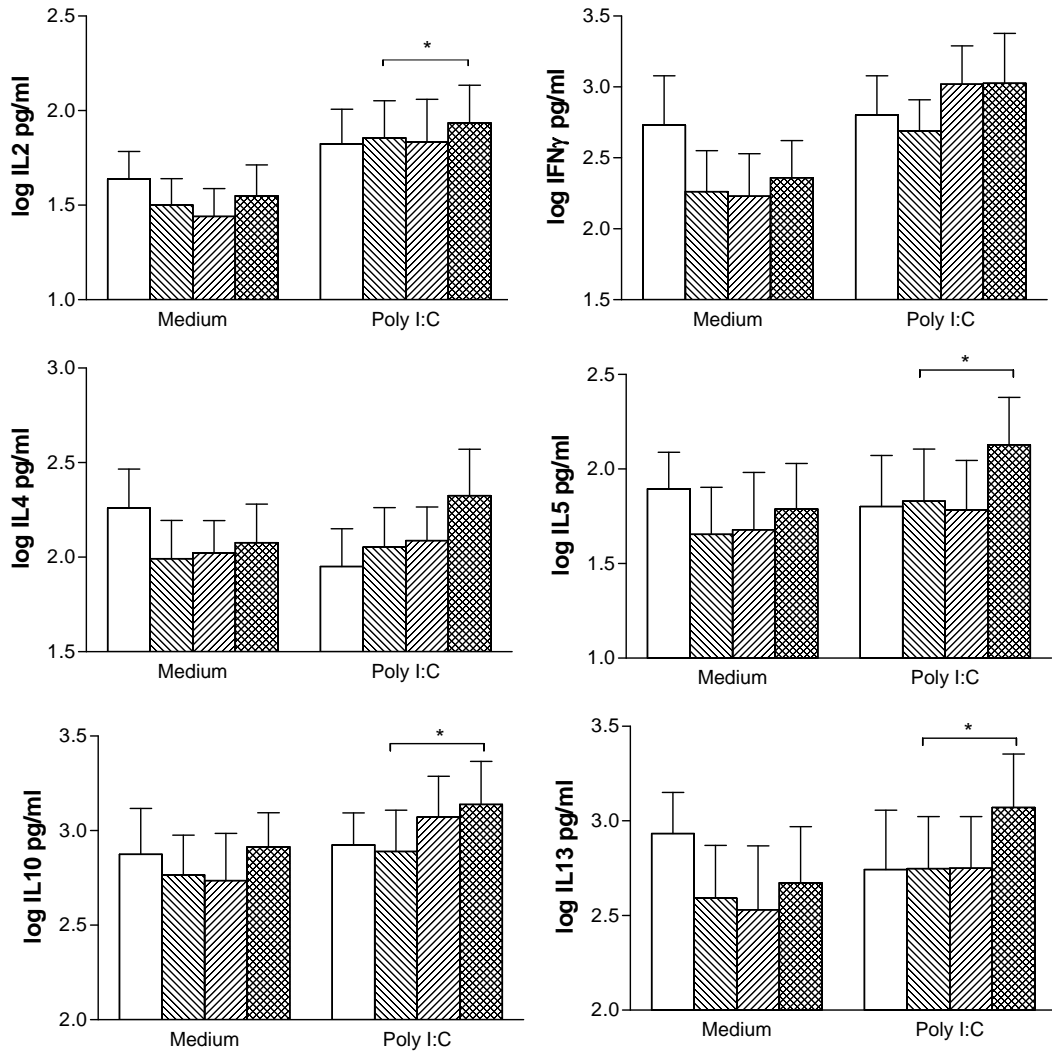


Figure 17. T cell cytokines. Analysis of Th1/Th2 cytokine profiles from mixed lymphocyte reactions. Data were transformed by base 10 logarithm and presented as mean \pm SEM; * = p value < critical alpha (two-tailed), n=4.

Table 11

T cell cytokines: Comparison of T cell cytokine profiles

<i>Comparison (DMSO vs CSCH)</i>	<i>Mean 1 (DMSO)</i>	<i>Mean 2 (CSCH)</i>	<i>eta²</i>	<i>t</i>	<i>Critical p</i>	<i>Observed p</i>
Medium						
Interleukin 2	1.50 ± 0.14	1.55 ± 0.16	0.24	-0.98	0.050	0.400
Interferon γ	2.27 ± 0.29	2.36 ± 0.26	0.69	-2.61	0.025	0.080
Interleukin 4	1.99 ± 0.20	2.08 ± 0.20	0.26	-1.03	0.050	0.380
Interleukin 5	1.65 ± 0.25	1.79 ± 0.24	0.54	-1.88	0.050	0.157
Interleukin 10	2.77 ± 0.21	2.91 ± 0.18	0.80	-3.46	0.025	0.041
Interleukin 13	2.59 ± 0.28	2.67 ± 0.30	0.15	-0.72	0.050	0.261
Poly I:C						
Interleukin 2	1.86 ± 0.20	1.94 ± 0.29	0.89	-5.04	0.025	0.015*
Interferon γ	2.69 ± 0.22	3.03 ± 0.35	0.51	-1.76	0.050	0.177
Interleukin 4	2.05 ± 0.21	2.33 ± 0.25	0.74	-2.89	0.025	0.063
Interleukin 5	1.83 ± 0.28	2.13 ± 0.25	0.92	-5.97	0.025	0.009*
Interleukin 10	2.89 ± 0.22	3.14 ± 0.23	0.78	-3.30	0.050	0.046*
Interleukin 13	2.75 ± 0.28	3.07 ± 0.28	0.91	-5.61	0.025	0.011*

Note. Data were transformed by base 10 logarithm and presented as mean ± SEM. * = p value

< critical alpha (two-tailed), n = 4.

Table 12

Th1/Th2 cytokine ratios

	<i>DMSO</i> (<i>n</i> =4)	<i>C5CH</i> (<i>n</i> =4)	<i>Contrast</i> <i>p</i>
IL2 : IL5			
Medium	-0.15 ± 0.24	-0.24 ± 0.22	0.503
Poly I:C	0.03 ± 0.27	-0.19 ± 0.21	0.012*
IL2 : IL13			
Medium	-1.10 ± 0.31	-1.14 ± 0.32	0.884
Poly I:C	-0.90 ± 0.23	-1.14 ± 0.27	0.180*
IFN γ : IL5			
Medium	0.62 ± 0.42	0.58 ± 0.24	0.608
Poly I:C	0.87 ± 0.27	0.90 ± 0.25	0.901
IFN γ : IL13			
Medium	-0.33 ± 0.51	-0.31 ± 0.28	0.894
Poly I:C	-0.06 ± 0.22	-0.04 ± 0.18	0.946

Note. Raw data ratios were transformed by base 10 logarithm and presented as mean ± SEM,

* = *p* value < critical alpha 0.025, (two-tailed), *n* = 4.

Chapter Five

Discussion

Summary of Findings

This is the first study to investigate the effects of cigarette smoke in a dendritic/bronchial epithelial cell co-culture model. Since DC possess the ability to sample the bronchial airway, take up smoke particulates and are key mediators of immune response, it was reasoned that DC function and DC-induced TC response may be affected by cigarette smoke. Only two studies have evaluated the effects of cigarette smoke on human DC function. In these two studies, DC were cultured alone. By culturing DC with smoke-exposed EC, it takes into account the impact of the tissue micro-environment. Therefore, this study provides insight into EC-DC interactions in smoke exposure and may provide evidence for disease and infection susceptibility in cigarette smokers; including how a smoker exposed to viral infection may respond.

In the context of smoke exposure only, this study indicates that cigarette smoke has the ability to decrease phagocytosis ability of DC and increases co-stimulatory maturation markers without inducing TC proliferation and inducing a potential Treg phenotype (Figure 18A). IL8, a potent neutrophil chemokine was predominantly expressed by EC and was significantly increased in smoke exposed, non-stimulated co-cultures. This is an important epithelial injury signal that recruits other inflammatory cells into the airway and critical *in vivo*.

DC maturation was significantly affected by CSC through a decrease in phagocytosis ability. No other studies have reported an effect of smoke on phagocytosis ability of DC. However, this reduction in phagocytosis was also observed in another antigen presenting cells, alveolar macrophages of healthy smoking subjects compared with non-smoking controls subjects. In addition, phagocytosis ability was significantly increased in COPD subjects who had quit smoking when compared with COPD current smokers (Hodge et al., 2007). DC maturation was also affected following CSC exposure by an increase in surface co-stimulatory markers, CD83 and CD86. This was not observed in a previous study of the effects of smoke on MDDC (Vassallo et al., 2005). However, this difference may be attributed to the presence of EC in the co-culture as DEP did not effect maturation markers unless MDDC were co-cultured with EC (Bleck et al., 2006). Together this data suggests that smoke may induce a semi-mature DC phenotype and reduce the ability for DC to sample the airway for antigens.

CD54 is another DC surface marker that is a vital component of cell-cell interaction and is also a receptor for rhinovirus. This is the first report that CSC significantly increased CD54 in MDDC. Other studies have investigated the effect of smoke on alveolar macrophages. However, they observed a reduction in CD54 in smokers in BALF and sputum (Lensmar et al., 1998; Skold et al., 1996). It is speculated that CD54 may play a different role in DC versus macrophages.

Although CSC did not significantly affect TC proliferation rates, low levels of IL2 may account for the lower TC proliferation observed in non-stimulated versus poly I:C stimulated co-cultures. The lack of TC proliferation and secretion of IL10 by MLR TC suggests a Treg phenotype in non-stimulated, CSC exposed co-cultures. This may be attributed to low IL6, TNF α and no IL12p70 expression levels observed in DC cytokine

supernatants. Together, these conditions push naïve TC to a Treg phenotype (Hammad & Lambrecht, 2006).

One possible explanation for the effects seen in CSC treated MDDC is the concept of tolerance or an alternatively activated DC, which is “a dendritic cell that is activated...resulting in a stable, semi-mature DC that can induce T-cell hyporesponsiveness *in vitro*...” (Morelli & Thomson, 2007, p. 612). This would require further investigation of other characteristics of tolerogenic DC such as, expression of programmed cell death ligand 1 (PDL1), production of indoleamine 2,3-dioxygenase (IDO), resistance to innate NK-cell mediated killing and the production of IL10/TGF β by TC stimulated by smoke exposed DC (Morelli & Thomson, 2007; Reis e Sousa, 2006).

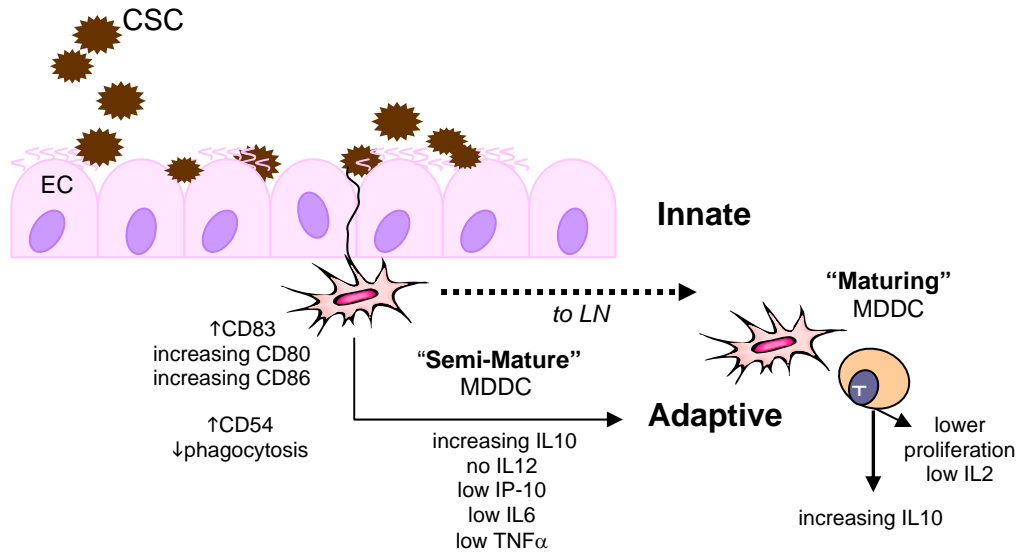
In the context of smoke exposure and poly I:C stimulation, high levels of EC derived IL8 were observed along with high expression of DC maturation markers (CD80, CD83, CD86) adhesion molecule, CD54 and low phagocytosis. All indicating an injured epithelium and activated/mature MDDC capable of producing high levels of IL6, TNF α , stable IL12p70 and high IFN γ (Figure 18B). These conditions would normally lead to a Th1 dominant phenotype in MLR TC cytokine expression; however, an overall mixed T helper response was observed. The specific effects of CSC were observed in increased DC IL10 (Treg polarizing) and increased IP-10 (Th1 chemokine). The profile of TC MLR cytokines revealed a mixed picture of high IFN γ (Th1) and a CSC-induced increase in IL10 (Treg) and Th2 cytokines (IL5 and IL13). Poly I:C is a Th1 stimulus (high IP-10, IL6, TNF α and stable IL12p70 expression) capable of inducing IFN γ production; however, CSC exposure may modulate T helper cytokine expression to increase both Treg cytokines (IL10) and Th2 cytokines (IL5 and IL13). Although CSC did not significantly affect TC proliferation rates, higher levels of IL2 may account for the overall

higher TC proliferation rate observed in poly I:C stimulated versus non-stimulated co-cultures.

The mixed presentation in T helper cytokines that was observed in MLR supernatants may be a function of DC to TC ratios. One study reported that high DC:TC ratios (1:4), like the ratio used in this study (1:16), resulted in a mixed Th1/Th2 response while low DC:TC ratios (1:300) resulted in a Th2 phenotype (Tanaka, Demeure, Rubio, Delespesse, & Sarfati, 2000). In addition, MLR reactions were carried out with CD4+ TC, not naïve therefore a true picture of TC polarization may not be clear due to the potential presence of all subtypes of T helper cells.

Overall, these findings suggest that DC of smokers may be less likely to mount a normal immune response to invading pathogens thus providing evidence for smoker susceptibility to infection. It may also provide insight into how DC of smokers may respond to viral infection. In addition, these findings are particularly important in patients with allergic airway disease since increasing Th2 cytokines would increase the risk for disease exacerbation. Because DC play a critical role in modulating immune response to environmental agents, they have become a primary target for drug development for diseases that are largely mediated by environmental triggers, such as asthma and COPD (Kool & Lambrecht, 2007). This study provides basic information that may be used in the development of therapeutic agents.

A. Cigarette smoke exposure



B. Cigarette smoke exposure and poly I:C stimulation

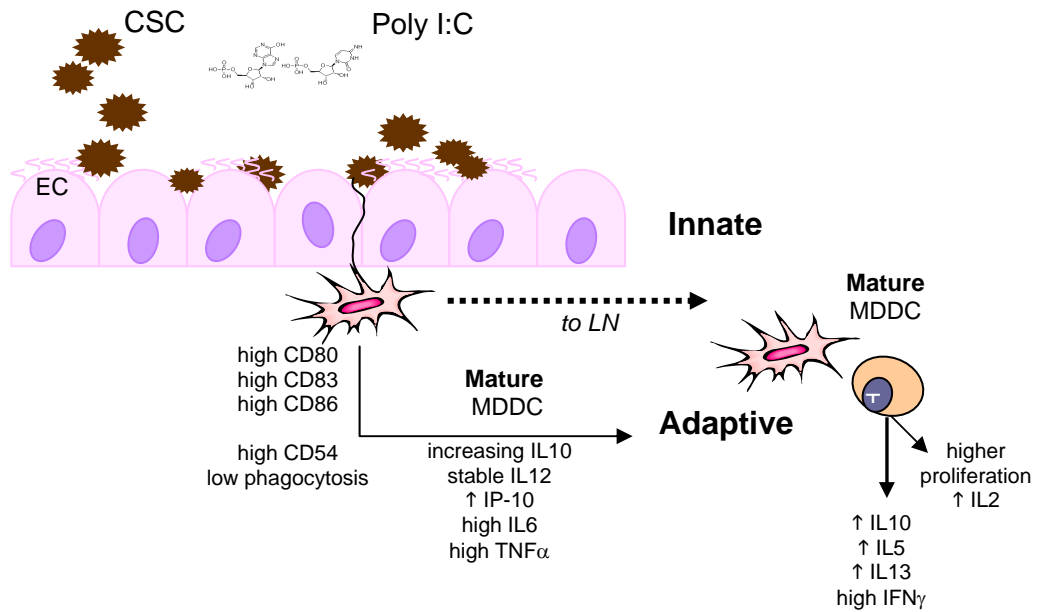


Figure 18. Summary of findings. A. The effect of cigarette smoke exposure dependent variables; B. The effect of cigarette smoke exposure and poly I:C stimulation on dependent variables; arrows indicate statistically significant increase (↑) or decrease (↓).

Limitations

A major strength of this study was the use of cells obtained from human subjects; however this is also a major limitation due to human subject variability a repeated measures analysis approach was used in an effort to control for this variability. One of the perplexing questions regarding COPD is why some smokers develop disease while others do not. In addition, there are mixed phenotypes including chronic bronchitis, emphysema and mixed presentations with asthma. Therefore it is not surprising that some subjects would immunologically respond to smoke while others would not. Also, all donor information was de-identified and there was no information regarding health history, medication use or smoking history and due to the limited sample size ($n = 4$) it is difficult to make general assumptions regarding the effects of smoke. Gender and age of the subjects may have also played a role in the immune responses investigated and the heterogeneous sample may have contributed to the mixed results. However, the small sample size did not permit investigation of the variation due to these demographic variables. Another limitation is that the donor of the NHBE cells is different than the donor of MDDC and TC. This may present an issue of self versus non-self recognition and result in an artificial immune response.

Another drawback of this study was the dose and time of CSC exposure. This study used a single acute exposure and NHBE cells were exposed for approximately 36 hours while MDDC were only exposed for 24 hours. Other studies of DEP exposure in EC-DC co-cultures used a 48 hour time course. Since this study used 24 hours, it may have been too early to detect late phase changes. In addition, length of poly I:C exposure may have limited the amount of $IFN\gamma$ secreted from MDDC. In this study, MDDC were exposed to poly I:C for 24 hours and a previous study of smoke and poly I:C exposure in human PBMC showed minimal $IFN\gamma$ following 24 hours and a significant

increase observed at 48 and 72 hours (Mian, Lauzon, Stampfli, Mossman, & Ashkar, 2008). Time course experiments need to be conducted.

Cigarette smoke consists of two components: vapour and particulate phases. CSC provides a model of the particulate phase of smoke. There is some controversy that CSC is an appropriate model for smoke exposure and the need for a particle only control in order to distinguish specific versus non-specific effects of particulates. Most of the chemicals and nicotine associated with smoke are contained in the particulate phase of smoke. It is this component of smoke primarily responsible for the immunosuppressive effects of smoke (Nouri-Shirazi & Guinet, 2006; Sopori, 2002) and is therefore an appropriate model for *in vitro* study. One cannot argue the value of controls but due to the thousands of chemicals, including tar and nicotine contained in CSC it can be argued that treatment effects observed are most likely due to chemicals rather than particulates.

Future Directions

Further investigation into the time course effects of cigarette smoke on DC will be important to understand acute versus late phase immune responses. Other surface markers such as CCR7, CD40/OX40L and cytokines such as TSLP/TGF β should also be explored to elucidate the effects of migration, TC stimulation and Th2/Treg induction, respectively. In addition, MLR experiments should be conducted with naïve TC at various responder ratios in order to determine true TC polarization. Findings from this study can inform clinical research. One example would be to isolate MDDC from smokers and non-smokers and those isolated cells exposed to cigarette smoke.

EC play a critical role in innate immunity by recognizing invading pathogens and secreting antimicrobial substances such as defensins and inflammatory/chemotactic

mediators such as cytokines, chemokines and leukotrienes (Bals & Hiemstra, 2004). Further study should focus on how smoke exposed EC not only regulate inflammation and the immune response but how they interact with DC through cell to cell contact and soluble mediators (Polito & Proud, 1998; Schleimer et al., 2007; Stick & Holt, 2003). This knowledge will be critical in understanding the pathophysiology of many lung diseases and would provide many potential therapeutic targets. Overall, this study provides a sound basis for future investigation into the complex effect of smoke on DC and lung disease.

Significance to Nursing

Although this study is based on basic science experiments, much of the knowledge obtained can be applied to nursing education, research and practice. Information obtained in nursing and medical research is shared. In nursing education, one of the foundational courses is pathophysiology which includes immunology concepts therefore information obtained in this study applies to the foundation of nursing education. With regards to nursing research, by nurses becoming familiar with immunology techniques they can incorporate these concepts in to clinical research and impact the progress of nursing science. Nursing practice is affected by research through the incorporation of research findings into evidence-based practice. The main goal of all research is to increase knowledge in order to improve our understanding of disease and positively affect patient care. By understanding more about the effects of cigarette smoke not only do we increase pathophysiological knowledge of diseases affected by smoking but we indirectly impact patient care through improved diagnostics, therapeutics, interventions and evaluation.

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Appendices

Appendix A: Definitions

Adaptive immunity: the ability of an antigen to induce antigen-specific responses in lymphocytes. Adaptive immunity is characterized by an increase in response with exposure, memory and clonal selection of lymphocytes.

Allergen: an antigen that has the ability to induce a hypersensitivity or allergic reaction.

Allogeneic: different individuals of the same species.

Antigen: any molecule that can bind to an antibody. Some have the ability to stimulate antibody production.

Chemokine: small proteins that induce the migration and/or activation of cells.

Cigarette smoke condensate (CSC): commercially available particulates obtained from burned cigarettes that are dissolved in dimethyl sulfoxide and contains many chemicals including nicotine and tar. In this study, CSC was used to mimic the particulate phase of smoke.

CD4: cluster of differentiation 4 is present on thymocytes, monocytes, macrophages and is a co-receptor of MHC Class II. In this study, T cells used in mixed lymphocyte reactions were CD4 positive.

CD11c: cluster of differentiation 11c is present on myeloid cells and binds fibrinogen. In this study, CD11c was used to identify dendritic cells in flow cytometry experiments.

CD80: cluster of differentiation 80 is present on dendritic cells and is a ligand for CD28 on T cells. It is important in the activation of naïve T cells through co-stimulation (signal 2).

CD83: cluster of differentiation 83 is present on dendritic, B and Langerhans' cells. It is a marker for activated dendritic cells and may assist in antigen presentation or cellular interactions that follow lymphocyte activation

CD86: cluster of differentiation 86 is present on dendritic, monocytes and activated B cells and it is a ligand for CD28 on T cells. It is important in the activation of naïve T through co-stimulation (signal 2).

CD54: cluster of differentiation 54 is present on hematopoietic and non-hematopoietic cells. Also known as intercellular adhesion molecule (ICAM-1) and is involved in adhesion of neutrophils and is a receptor for rhinovirus.

Cluster of differentiation (CD): a monoclonal antibody that identifies surface molecules, used to identify various immune cells.

Cytokine: proteins made by cells that affect the function of other cells or the cell itself.

Dendritic cell (DC): professional antigen presenting cell and the most potent activator of T and B cells. They are involved in linking the innate and adaptive immune systems and located in tissues exposed to the external environment such as the skin, lungs and intestines. In this study, monocyte-derived dendritic cells isolated from human peripheral blood were used in all experiments.

Dimethyl sulfoxide (DMSO): a chemical compound that is a polar aprotic solvent. It is an excellent solvent and cryoprotectant. In this study, CSC is dissolved in DMSO therefore DMSO is used as a vehicle control in all experiments.

Flow cytometry: a research method that uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells. It is a technique for counting and examining cells or particles based on size, granularity and fluorescence.

Homotypic cluster score: clustering of dendritic cells with each other that is an indicator of upregulation of adhesion molecules and chemokines/chemokine receptors. In this study, the scoring of homotypic clustering is a semi-quantitative score of 0-8.

Human leukocyte antigen DR (HLA-DR): human leukocyte antigen encoded major histocompatibility complex, MHC class II, cell surface receptor ligand for T cell receptor. It is present on antigen presenting cells with the primary function to present peptide antigens to the immune system for the purpose of eliciting an immune response.

Innate immunity: earliest host response to invading pathogens such as bacteria, virus and allergens that includes various recognition systems such as toll-like receptors. The innate immune response does not increase with repeated exposure.

Interferon gamma (IFN γ): cytokine that can induce cells to resist viral replication. It is produced by CD4+ Th1 cells and is a type II interferon and has antiviral, immunoregulatory, and anti-tumour properties.

Interferon-inducing protein 10 (IP10): chemokine that selectively attracts Th1 lymphocytes and monocytes, and inhibits cytokine-stimulated hematopoietic progenitor cell proliferation.

Interleukin 2 (IL2): a T cell derived cytokine that stimulates growth and differentiation of T cells, B cells, NK cells, monocytes, macrophages. A central cytokine in the development of an adaptive immune response.

Interleukin 4 (IL4): a cytokine that is secreted by Th2 cells. It enhances both secretion and cell surface expression of IgE and IgG1. An important cytokine in allergic disease and has overlapping functions with IL13.

Interleukin 5 (IL5): secreted by Th2 and mast cells that is a key mediator in eosinophil activation. An important cytokine in allergic disease.

Interleukin 6 (IL6): both a pro-inflammatory and anti-inflammatory cytokine important mediators of fever and of the acute phase response and secreted in response to

activation of the innate immune system. High IL6 It is associated with both Th1 and Th2 responses and low IL6 is associated with Treg responses.

Interleukin 8 (IL8): inflammatory chemokine produced by many cell types. Mainly functions as a neutrophil chemoattractant.

Interleukin 10 (IL10): secreted by Th0 cells blocks cytokine synthesis by Th1 cells

Interleukin 12p70 (IL12p70): Th1 polarizing type cytokine secreted mainly by dendritic cells. Induces IFN γ production and proliferation/differentiation of Th1 cells.

Interleukin 13 (IL13): secreted by Th2 cells, involved in the upregulation of IgE secretion by B cells. An important cytokine in allergic disease.

Ligand: general term for a molecule recognized by another structure such as a receptor.

MHC Class II: major histocompatibility complex class II present on antigen presenting cells with the primary function to present peptide antigens, both self and non-self, to lymphocytes for the purpose of eliciting an immune response. Specifically, MHC class II presents antigen fragments to T-helper cells by binding to the CD4 receptor on the T-helper cells.

Mixed lymphocyte reaction (MLR): When lymphocytes for two different individuals are cultured together. In this study, CSC/poly I:C exposed dendritic cells were co-cultured with allogeneic CD4⁺ T cells for 4-5 days.

Monocyte-derived dendritic cells (MDDC): dendritic cells obtained by culturing CD14⁺ monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL4). Used in this study as the dendritic cell model.

Normal human bronchial epithelial cells (NHBE): commercially available primary cell culture of bronchial epithelial cells from human donors. Used in this study as the bronchial epithelial cell model.

Phagocytosis: the engulfment of particles, bacteria, cell debris, etc. by cells. A characteristic function of cells such as macrophages and dendritic cells. In this study, phagocytosis is assessed by FITC-dextran uptake by dendritic cells and analyzed by flow cytometry. Data is presented as mean fluorescence intensity (MFI). A low MFI is associated with a mature dendritic cell and a high MFI is associated with an immature dendritic cell.

Polyinosinic-polycytidylic acid (Poly I:C): a commercially available synthetic double stranded RNA. A toll-like receptor 3 ligand that mimics viral infection and induces a Th1 response. In this study, poly I:C is used as a positive control for dendritic cell maturation

Polarizing cytokine: a cytokine that induces a specific phenotype in the target cell.

T cells (TC): thymic lymphocytes that developed in the thymus.

T cell proliferation: In this study, TC proliferation is assessed by CFSE staining and analyzed by flow cytometry. Data are presented as percent proliferated.

Naïve T helper lymphocyte (Th0): T lymphocytes that have never engaged a specific antigen. They are different from effector and memory T cells.

T helper type 1 lymphocyte (Th1): T lymphocyte characterized by the cytokines they produce (IFN γ). An increase in Th1 cytokines are associated with autoimmune diseases.

T helper type 2 lymphocyte (Th2): T lymphocyte characterized by the cytokines they produce (IL4, IL5, IL13). An increase in Th2 cytokines are associated with allergic diseases.

T helper type 17 lymphocyte (Th17): T lymphocyte characterized by the cytokines they produce (IL17).

Toll-like receptor 3 (TLR3): one of the innate immune systems recognitions systems. It is one of the toll-like receptors that recognizes double stranded RNA of viruses.

T regulatory lymphocyte (Treg): T lymphocyte characterized by the cytokines they produce (IL10, TGF β). They have the ability to inhibit T cell responses and induce tolerance.

Tumor necrosis factor alpha (TNF α): a pleiotropic inflammatory cytokine involved in apoptotic cell death/proliferation, differentiation, inflammation, tumor growth, and viral replication. It is important in both Th1 and Th2 polarization, acute phase reactions and fever.

Viability: to be alive. In this study, viability was assessed by 7-AAD staining and analyzed by flow cytometry. Data are presented at percent live.

About the Author

Alison Jones Montpetit was born and raised in Decatur, Alabama. She attended the University of North Carolina at Wilmington where she obtained a Bachelor's degree in nursing in 2001. She practiced nursing in the Medical Intensive Care Unit at New Hanover Regional Medical Center. In 2002, she moved to Tampa, Florida to pursue the BS-PhD program at the University of South Florida. During her tenure, she completed a Master's in nursing (2004) and attended the Summer Genetics Institute at the National Institute of Nursing Research (2004). She is married to Marty Louis Montpetit, PhD and expects her first child in August of 2008.