CIGARETTE SMOKE ALTERS INFLUENZA INDUCED IMMUNE RESPONSES OF
THE RESPIRATORY EPITHELIUM

Katherine Maurine Horvath

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Approved By:

Dr. David Diaz-Sanchez

Dr. Ilona Jaspers

Dr. Steven Kleegberger

Dr. Terry Noah

Dr. David Peden

ABSTRACT

KATHERINE MAURINE HORVATH: Cigarette Smoke Alters Influenza Induced Immune
Responses of the Respiratory Epithelium
(Under the direction of Ilona Jaspers)

Epidemiological evidence demonstrates that smokers are at increased risk for and
suffer greater morbidity and mortality from influenza infection but the mechanism
underlying this susceptibility is poorly understood. Previous work from our laboratory
confirmed that smokers have increased markers of influenza infection using both *in vivo* and
in vitro models of influenza infection. In this dissertation, the differential nasal immune
responses to influenza infection were explored in nonsmokers and smokers. In the *in vitro*
model of influenza infection, nasal epithelial cells (NEC) obtained from nonsmokers and
smokers were differentiated *ex vivo* and co-cultured with monocyte-derived dendritic cells
(mono-DCs) from nonsmokers to determine the effect of cigarette smoke (CS) exposure on
the ability of NEC to communicate with underlying DCs. These co-cultures were then
infected with influenza A virus. Both NEC from smokers and mono-DCs co-cultured with
smoker NEC had decreased expression of antiviral mediators interferon regulatory factor 7
(IRF7) and Th1 cell chemokine interferon gamma-induced protein 10 kDa (IP-10) with
increased expression of Th2 chemokine thymic stromal lymphopoeitin (TSLP). Thus, CS
exposure altered antiviral defense mechanisms in both NEC and mono-DCs and changed the
nature of communication between these two cell types. In the *in vivo* model of human

influenza infection, nonsmokers and smokers were administered live attenuated influenza virus (LAIV) and the resulting localized nasal immune responses were monitored using nasal lavages and nasal biopsies. Natural killer (NK) cell cytotoxic responses and chemokines important for NK cell activation were suppressed in smoker nasal lavages. This data was intriguing because 1) it was the first documentation of NK cells in the nasal lavage cell population and 2) decreased NK cell activity in smokers could contribute to delayed influenza virus clearance. These data were also the first to show that γδ T intraepithelial lymphocytes, a rare immune cell type, migrated to the nasal mucosa following LAIV inoculation in both nonsmokers and smokers. Together these data demonstrate that CS exposure suppresses NK, NEC, and DC specific immune responses of the respiratory mucosa and contribute to the mechanism of increased susceptibility to respiratory viruses observed in CS exposed populations.

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LIST OF ABBREVIATIONS AND SYMBOLS

ADAM – a disintegrin and metalloproteinase

ANOVA – analysis of variance

AP-1 – activator protein 1

APC – allophycocyanin

AUC – area under the curve

BAL – bronchoalveolar lavage

BMI – body mass index

BPDE – benzo[a]pyrene-7,8-diol-9,10-epoxide

CCR7 – c-c chemokine receptor 7

CD – cluster of differentiation

COPD – chronic obstructive pulmonary disease

COX-2 – cyclooxygenase-2

CS – cigarette smoke

CSE – cigarette smoke extract

CXCL – chemokine motif ligand

CXCR3 – chemokine receptor for IP-10

DAPK – death-associated protein kinase

DC – dendritic cell

DC-LAMP – DC-lysosome associated membrane protein

DE – diesel exhaust

DEP – diesel exhaust particles

DNA – deoxyribonucleic acid

DNMT1 – DNA (cytosine-5)-methyltransferase 1

DNMT3a – DNA methyltransferase 3a

EGCG – epigallocatechin gallate

EGFR – epidermal growth factor receptor

ELISA – enzyme-linked immunosorbent assay

ERK – extracellular signal-related kinases

FBS – fetal bovine serum

FEV1 – forced expiratory volume in 1 minute

FHIT – fragile histidine triad

FITC – fluorescein isothiocyanate

FSC – forward scatter

GM-CSF – granulocyte-macrophage colony stimulating factor

GSH – reduced glutathione

GSSG – oxidized glutathione

H&E – hematoxylin and eosin

H_2O_2 – hydrogen peroxide

HA – hemagglutinin

HAU – hemagglutinin units

HBSS – hank's buffered salt solution

HLA – human leukocyte antigen

HRP – horse radish peroxidase

HRV – human rhinovirus

HtrA3 – HtrA serine peptidase 3

IFN – interferons

IGF1 – insulin-like growth factor 1

IL – Interleukin

IP-10 – interferon gamma-induced protein 10 kDa

IRF – interferon regulatory factor

ISRE – interferon response element

Jak – janus kinase

LAIV – live attenuated influenza virus

LPS – lipopolysaccaride

LRI – lower respiratory tract infection

MCP-1 – monocyte chemotactic protein 1, CCL2

MDC – monocyte derived chemokine

MDCK – Madin-Darby canine kidney

MGMT – methylguanine-DNA methyltransferase

MHC – major histocompatibility complex

MIC – MHC class I polypeptide-related sequence

MIP – macrophage inflammatory protein

MOI – multiplicity of infection

Mono-DCs – monocyte-derived dendritic cells

NAC – n-acetylcysteine

NEC – nasal epithelial cells

NF-κB – nuclear factor κB

NHBEC – normal human bronchial epithelial cells

NK – natural killer

NKG2D – NK cell activating receptor

NKp44 – natural killer cell related protein 44

NKp46 – natural killer cell related protein 46

NLF – nasal lavage fluid

NRF2 – nuclear factor receptor 2

OVA – ovalbumin

PAHs – polyaromatic hydrocarbons

Pb – lead

PBMC – peripheral blood mononuclear cells

PCR – polymerase chain reaction

PE – phycoerythrin

PM – particulate matter

Po – polonium

poly I:C – polyinosinic:polycytidylic acid

PRR – pattern recognition receptors

q-RT-PCR – quantitative RealTime PCR

RAET1 – retinoic acid early transcripts-1

RANTES – regulated upon activation, normal T cell expressed and secreted, CCL5

RAR-β – retinoic acid receptor β

RIG-I – retinoic acid inducible gene-1

RNA – ribonucleic acid

ROS – reactive oxygen species

RSV – respiratory syncytial virus

RUNX3 – runt-related transcription factor 3

SEM – standard error of the mean

SHS – second hand smoke

SSC – side scatter

STAT – signal transducers and activators of transcription

TARC – thymus and activation-regulated chemokine, CCL17

TBS – tris buffered saline

TCR – T cell receptor

TGFβ – transforming growth factor β

Th1 – T helper cell 1

Th2 – T helper cell 2

TLR – Toll-like receptor

TNFα – tumor necrosis factor α

TSLP – thymic stromal lymphopoeitin

ULBP – UL16 binding protein

V – variable region of TCR chain

α – alpha

β – beta

γ – gamma

γδ – gamma delta

δ – delta

ΔΔ Ct – delta delta Ct

Ω – omega

CHAPTER 1

INTRODUCTION

Host Defense at the Respiratory Mucosa

The respiratory epithelium functions as a barrier against the outside world and is the first line of defense against airborne environmental stimuli, including pollutants, pathogens, and allergens. Respiratory epithelial cells form a ciliated, polarized, and pseudostratified columnar epithelium. The apical side of the cell is exposed to the outside environment whereas the basolateral side of the cell communicates with underlying lamina propria and, in the case of a pathogenic infection or pollutant exposure, can secrete mediators to attract immune cells to airways under assault (see Figure 1). Respiratory epithelial and immune cells participate in complex crosstalk to orchestrate innate and adaptive inflammatory responses in the respiratory epithelium. Innate host defense is characterized by non-specific immune cells including neutrophils, macrophages, and dendritic cells (DCs) that recognize general pathogens and environmental stimuli and direct the initial stages of immune responses. Innate immune responses transition into adaptive immune mechanisms that generate pathogen specific T and B lymphocytes to eliminate the invading pathogen at its source, destroy infected and/or damaged host tissue, and regulate both innate and adaptive immune responses to maintain homeostasis. Respiratory epithelial cells communicate with both innate and

adaptive arms of the immune system to both alert the immune system to environmental stimuli and repair host damage.

The respiratory mucosa contains resident immune cells that monitor the airways for foreign antigens. These immune cell types will be introduced briefly below, and their specific functions during influenza infection and pollutant exposures will be detailed later in this chapter. Respiratory DCs lie basolateral to respiratory epithelial cells in the lamina propria throughout the airways. DCs maintain constant contact with respiratory epithelial cells, and their "fingerlike" projections can penetrate between tight junction epithelial cells barriers to directly sample the airways for pathogenic antigens (1; 2). Intraepithelial lymphocytes, or γδ T cells, are present within the epithelium and can play both innate and adaptive roles in respiratory infections. Immune cell phenotypes of the airway lumen itself vary from the proximal to distal airways. Neutrophils (3), acute inflammatory cells that migrate to the site of infection to kill invading pathogens are the major cell types in the nasal lavage, or saline washes of the nasal cavity. Eosinophils, basophils, and mast cells have also been identified in the nasal lavages of allergic individuals (4). Within the bronchial airways, there may be a different range of leukocytes including monocytes/macrophages, neutrophils, natural killer (NK) cells, and DCs (5). In particular, alveolar macrophages are major cell types present at baseline within the lower airways that clear the alveloli through phagocytosis of foreign particles and antigens (6; 7). Thus, although respiratory DCs are located throughout the basolateral epithelium, the cell types within the airway lumen that patrol to react against foreign particles and pathogens may vary from the upper to lower airways.

Besides initiating direct responses to pathogens, the cells of the respiratory mucosa participate in a complex crosstalk. This interplay between the airway epithelium and resident immune cells plays a crucial role in modifying the epithelium's response to antigens and pollutants. As the main surface cell type within the respiratory mucosa, respiratory epithelial cells act as "switchboards" which through physical cell-cell interactions and the secretion of soluble immune mediators initiate and regulate immune defense throughout the airways. This communication is not one way, and regulatory cytokines secreted by activated immune cells act in turn upon epithelial cells and other immune cells to affect the balance between beneficial inflammatory and antiviral immune activation, and bystander damage to the airways.

Respiratory Mucosal Responses to Influenza Infection

Epithelial Cells Initiate and Regulate Influenza Immune Responses

Respiratory epithelial cells are the preferential host cell type targeted by influenza virus and activate signaling cascades that both transmit "danger" to nearby epithelial cells and secrete chemokines to attract immune cells to the site of infection. Influenza virus is a negative strand RNA virus from the orthomyoxviridae family. Hemagglutinin (HA), an influenza viral surface protein, is cleaved by either soluble or membrane bound respiratory serine proteases (8). Cleavage of HA allows: 1) its subunit HA1 to bind to α2-6 linked sialic acids on ciliated respiratory cells and 2) its subunit HA2 to fuse the viral envelope with the cellular membrane. Thus, the influenza virus enters the cell through endocytosis and proceeds through its life cycle (8). Influenza activates the innate immune signaling pathways of the respiratory epithelium and stimulates the generation of cytokines and chemokines (9). Pattern recognition receptors like toll-like receptor (TLR)

3 and retinoic acid inducible gene-1 protein (RIG-I) recognize viral RNA and activate nuclear transcription factors like nuclear factor κB (NF-κB), interferon regulatory factor (IRF) 3, and IRF7 in epithelial cells. Activation induces the production of anti-viral Type I interferons (IFNs) α/β that help prevent further viral infection in epithelial cells. The secretion of antiviral type I IFNs is a broad spectrum signal that spans several cell types and can alert neighboring epithelial cells, DCs, and T cells (10) alike to the presence of a viral infection. See Figure 2. Epithelial cells also secrete cytokines and chemokines such as regulated upon activation, normal T cell expressed and secreted (RANTES, CCL5), interferon gamma-induced protein 10 kDa (IP-10, CXCL10), interleukin (IL) 1β (IL-1β), interleukin 6 (IL-6), and IL-8 which recruit additional immune cells such as neutrophils, NK cells, and T cells that clear respiratory viruses. Mechanisms of eliminating viruses from the airways include neutralizing the pathogen directly through antibody-mediated opsonization and phagocytosis by neutrophils and macrophages. Pathogens are also neutralized indirectly by killing virus infected host cells through antibody-dependent cellular cytotoxicity (NK cells, cytotoxic T cells), lysis of cells through complement activation, and recognition of specific virus sequences on host cells and subsequent induction of apoptosis (cytotoxic T cells). Both innate and adaptive immune cells work together to eliminate viruses and repair respiratory epithelia.

DCs Act As Liasons Between Innate and Adaptive Immune Systems During Influenza Infection

DCs are professional antigen presenting cells that monitor host cells for display of foreign antigens from infected host cells. DCs phagocytose and process these antigens to fulfill the pivotal task of mobilizing both innate and adaptive immune cells by secreting

chemokines CXCL16, macrophage inflammatory protein 2α (MIP2α, CXCL2), CXCL1, macrophage inflammatory protein 2β (MIP2β, CXCL3) and IP-10 to attract neutrophils, cytotoxic T cells and NK cells, and by secreting IL-8, RANTES, and IP-10 to attract memory T cells (11). Activated DCs also upregulate expression of maturation receptors CD80, CD86, and HLA-DR to aid in antigen presentation and provide co-stimulatory signals to T cells during influenza infection (12). DCs migrate to peripheral lymph nodes to initiate an adaptive T cell response by activating influenza specific CD4+ and CD8+ T cell clones and supporting clonal expansion of naïve T cells via autocrine IL-2 production (13). DCs can also enhance T cell responses through production of IFN regulated chemokines such as CXCL9, IP-10, and CXCL11 to drive a Th1 CD4 T cell response and expand activated cytotoxic CD8 T cell pools (13). Along with non-professional antigen presenting cells, such as B lymphocytes, DCs activate memory T cell pools for specific antigens (14). However, these memory T cells require less co-stimulatory activation and generally proliferate faster than naïve T cells to attain their effector functions of cytotoxicity and cytokine secretion within 24 h (14). Along with the B cell antibody response, the T memory cell response to influenza infection constitutes the adaptive immune response to vaccination and protects against subsequent infections.

NK Cells Communicate with Other Respiratory Mucosal Cells During an Influenza Infection

NK cells perform essential functions such as killing virus infected epithelial cells and secreting cytokines to regulate innate and adaptive immune responses (15). NK cells have inhibitory activation receptors that recognize "normal" self antigen on host cells (15). Absence of these normal antigens on epithelial cells will reverse the receptors'

inhibitory effects and lead to NK cell activation (15). In this way, NK cells are on

constant patrol in the epithelium for virus-infected as well as transformed tumor cells.

NK cells play important roles in respiratory viral infections. Young mice depleted of NK

cells prior to influenza infection have increased weight loss and higher lung viral titers

(16). In addition, studies have shown that NK cell activating receptor Ncr1 on NK cells

protects against lethal influenza infections in mice (17). In addition, the loss of NK cell

function by genetic defects is associated with recurrent viral and bacterial respiratory

infections in humans (5). NK cells interact directly with epithelial cells, DCs, and

intraepithelial lymphocytes of the respiratory epithelium during both homeostatic

conditions as well as during an influenza infection. Cytotoxic NK cell activity is directly

modulated by both the influenza virus, which binds to natural killer cell related protein 44

and 46 (NKp44 and NKp46) receptors on NK cells (18), as well as by cell-cell

interactions and exogenous cytokines. In particular, NK cells and DCs engage in a

mutually activating crosstalk (19). Type I IFNs secreted by DCs activate NK cells, and

IFNy secreted by NK cells activates DCs. The receptors UL16 binding protein (ULBP) 1-

3 on DCs ligate and activate NK cell activating receptors (NKG2D), indicating that direct

cell-cell communication can enhance NK cell cytotoxic activity. In addition, ULBP1 and

ULBP2 on DCs can be upregulated by TLR3 stimulation (20), suggesting that DCs may

respond to influenza infections by increasing their ability to communicate with NK cells.

NK cell activation during influenza infection is also dependent upon "wireless"

communication, i.e. cytokine secretion from DCs and epithelial cells (19; 21). It has been

suggested that CD56^{dim} CD16(+) cytotoxic NK cells in the respiratory epithelium arise

from CD56^{bright} CD16(-) cytokine secreting NK cells that partially mature on exposure to

a pathogen through activation by DC-derived Type 1 IFNs, IL-12, and IL-15 (22). For example, DC-dependent production of IFNα and IL-12 increased NK cell cytolysis, upregulated CD69 expression, and increased IFNγ production in NK cells (23). Thus, NK cell cytotoxic and cytokine secreting activities may play important roles in respiratory infections. My own research has demonstrated that NK cells in the nasal cavity play important roles during responses to respiratory viral infections.

At baseline and during influenza infection, epithelial cells, NK cells, DCs, and intraepithelial lymphocytes can communicate through NKG2D signaling. See Figure 2. During immune responses, epithelial cells as well as DCs upregulate "stress" induced NKG2D ligands like MHC class I polypeptide-related sequence A (MICA) and B (MICB) as well as ULBP1-4 that signal to both NK cells and γδ T cells (24). Expression of MICB is also upregulated on influenza infected macrophages (25). This NKG2D mediated activation induces additional secretion of chemotactic or proinflammatory cytokines to induce NK cell targeted killing (24). Normal human bronchial epithelial cells (NHBEC) express little MICA/B or ULBPs1-4 on the extracellular surface until treatment with 0.3mM H_2O_2, an inducer of oxidative stress, possibly through the activation of extracellular signal-related kinases (ERK) signaling pathways (26). In contrast, other studies have shown that NKG2D ligand expression is constitutive on healthy cell types including T cells, monocytes, and DCs (27). In fact, others have shown that NHBEC at baseline have significant expression of MICA/B which mediates allogeneic cytolysis by CD8+ T cells (28). Tumor cells have developed mechanisms to avoid NK cell and cytotoxic lymphocyte immune-surveillance by cleavage of membrane bound MICA by a disintegrin and metalloproteinase (ADAM) proteases (29). In this way,

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soluble MICA has been associated with tumors. NKG2D signaling is a common activation mechanism that links epithelial cell, DC, NK cell, and T cell immune responses.

γδ T cells Play Important Roles in Initiating and Regulating Immune Responses During an Influenza Infection

γδ T cells, or intraepithelial lymphocytes, are at their highest percentages in epithelial tissues. Like DCs and NK cells in the respiratory epithelium, γδ T cells act as bridges between innate and adaptive immunity by regulating both arms of the immune system. A recent review by Bonneville et al summarizes current knowledge about γδ T cell locations, activation, and effector functions (30). Their presence in mucosal epithelia allow them to quickly respond to assaults and sense "danger" from nearby cells through recognition of stressed cell ligands and activation by proinflammatory cytokines in the epithelia microenvironment (30). γδ T cells recognize these signals through TLR, T cell receptor (TCR), or NK cell receptor (i.e. NKG2D) signaling. γδT cells express NKG2D, and therefore can be activated by NKG2D ligands like MICA/B and ULPBs (31) on either stressed epithelial cells or activated respiratory DCs. For example, ULBP4 binds to Vγ9/Vδ2 TCR and induces cytolytic activity (32). Some stress ligands recognized by the γδ TCR itself include CD1c on DCs or macrophages and viral glycoproteins (30). γδ T cells can be indirectly activated by TLR stimulation on DCs (in the case of influenza infection, TLR3 stimulation) through the DC mediated secretion of type I IFNs, TNF, and IL-12 (30). Functions of γδ T cells include immediate killing of virus infected or tumor transformed epithelial cells through either conventional T cell cytolysis (Fas signaling) or NK cell cytolysis (perforin/granzymes) (30). The ability of γδ T cells to kill in a T cell

mediated fashion without thymus selection for specific antigens emphasize that these transitional T cells defy traditional innate or adaptive labels (30). Like other innate immune cells, upon stimulation by epithelial cells or DCs, γδ T cells secrete cytokines and chemokines such as IL-17, IFNγ and monocyte chemotactic protein 1 (MCP-1, CCL2) to promote neutrophil, DC, and macrophage activation (30). γδ T cells can regulate immune responses in the respiratory epithelium to promote homeostasis. γδ T cells can either directly kill or, through secretion of IL-10 and transforming growth factor β (TGFβ), can suppress activated DCs, macrophages, neutrophils, as well as conventional T cells (30). γδ T cells also regulate epithelial cell repair. γδ T cells in the epithelium express epithelial cell tight junction proteins like E-cadherin and occludin that are upregulated by TCR engagement (30). During wound repair, intradermal γδ T cells secrete epithelial cell growth factors insulin-like growth factor 1 (IGF1). In this manner, γδ T cells can regulate and enhance barrier function in the epithelium (30). This function could be particularly important in oxidant air pollutant exposure which disrupts respiratory epithelial cell tight junctions (33-35).

γδ T cells are necessary for successful resolution of many respiratory infections. γδ T cell deficient mice infected with *Nocardia asteroides* have decreased survival by 14 days post infection (36) whereas in wildtype mice *Nocardia asteroides* infection induces γδ T cell infiltration into the lung within 5 days (37). Mice infected with influenza virus show infiltration of γδ T cells into the BAL by 10 days post infection (38; 39) and these T cells are positive for variable (V) γ chains 1,2, and 4 (40). Vγ9Vδ2 TCR cells isolated from the peripheral blood of humans are capable of killing influenza-infected macrophages (41). γδ T cell deficient mice depleted of CD4 and CD8 T cell populations

have decreased survival following H3N2 influenza immunization compared to γδ T cell deficient mice with no conventional T cell depletion, indicating that γδ T cells and conventional T cells may have functional redundancy (42). Therefore, γδ T cells play important roles as cytotoxic lymphocytes within the airways during respiratory infections.

Through cytokine secretion and receptor mediated interaction, the epithelial and immune cells of the respiratory mucosa communicate during influenza infections. These interactions are summarized below in Figure 3. The following will detail current knowledge on the effects of cigarette smoke (CS) exposure on respiratory immune cells and their responses to influenza infection.

Cigarette Smoking Increases Risk of Influenza Infection

Cigarette smoke exposure is associated with an increased risk of viral infections, including influenza (43)(44)(45). Following the 1968 influenza A Hong Kong epidemic, Finklea et al. reported that smokers who smoked more than 21 cigarettes per day were subject to a 21% increase in clinical influenza incidence and had increased illness severity compared to nonsmokers (46). Subsequent epidemiological studies have demonstrated that cigarette smoking leads to increased incidence and severity of influenza infection in multiple populations including female US military recruits (43), male Israeli soldiers (44) and senior citizens in assisted living communities (45; 47). A 2004 meta-analysis confirmed that along with influenza infections, smokers have an increased risk for invasive pneumococcal disease and tuberculosis (48). Combined with evidence that smokers have lower influenza vaccination rates than nonsmokers, the increased influenza infection risk becomes an even more significant public health issue

(49). Because CS exposure is associated with susceptibility to respiratory infections, smokers and nonsmokers exposed to secondhand smoke (SHS) represent populations vulnerable to formidable illnesses like pandemic influenza and constitute a significant public health burden. My dissertation research adds to the body of literature that demonstrates cigarette smoke affects influenza induced immune responses.

Although it is recognized that CS is a risk factor for influenza infection, the underlying mechanism is likely multifactorial and is not well understood. Because both respiratory viruses and pollutants like CS interact initially with mucosal immune cells in the airway epithelium, the following will focus on the effects of CS on mucosal immunity in the respiratory tract during an influenza infection.

Effects of Cigarette Smoke on Influenza Infection in the Respiratory Mucosa

Cigarette Smoke Exposure Modifies Overall Influenza Immune Response

In animal models, CS exposure alters influenza induced immune responses. BALB/c mice exposed to 9 cigarettes per day for 4 days followed by influenza infection have increased inflammatory responses, greater viral titers and worse lung pathology, but decreased IL-6, IL-1β, IP-10, granzyme B, and granzyme K immune responses (50). In a similar study, C57BL/6J mice exposed to 1 cigarette per day for 1 week and then exposed to 3 cigarettes per day for an additional week followed by stimulation with polyinosinic:polycytidylic acid (poly I:C), which mimics double stranded RNA, have enhanced airway cellular infiltration, IL-18, IFNγ, Type I IFNs and IL-12/23p40 (51). CS alone may have differential effects in the upper and lower airways. C57BL/6 mice exposed to subacute levels of CS have increased inflammatory responses and neutrophil

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influx in both the upper and lower airways (52). However, following chronic CS exposure, inflammatory responses decrease in the upper airways but remain elevated in the lower airways (52). Thus, the nature of CS induced effects in whole animal models may vary based on length of exposure, exposure regimen, as well as the airways sampled.

Cigarette Smoke Exposure Modifies Epithelial Cell Antiviral Defenses

CS exposure alters inflammatory responses in human airway epithelial cells. Our laboratory has previously shown that nasal epithelial cells from smokers infected with influenza both *in vivo* and *ex vivo* had suppressed influenza induced activation of IRF7 and subsequent IFN-stimulated responses (53). Other studies have demonstrated that CS exposure augments human epithelial cell responses to other common respiratory virus infections. NHBEC pretreated with CS extract (CSE) and infected with human rhinovirus (HRV) 16 have suppressed IP-10 (54; 55) and RANTES (55) production. Janus kinase/signal transducers and activators of transcription (Jak/STAT) activation could play a role in this mechanism because CSE inhibits STAT1 phosphorylation in response to poly IC (55) and IFNγ (56). In contrast, CSE enhances rhinovirus-induced secretion of IL-8 from airway epithelial cells (54; 57). CS decreases apoptosis and increases necrosis in airway epithelial cells in response to respiratory syncytial virus (RSV) infection (58) which could explain the enhancement of proinflammatory cytokine IL-8. Thus, CS exposure can suppress antiviral but increase proinflammatory responses to respiratory viruses in respiratory epithelial cells.

Cigarette Smoke Exposure Modifies DC and Downstream Adaptive T Cell Function

CS exposure alters respiratory DC function. BALF DCs from smokers have increased expression of CD80, CD86, and CD1a with a decreased expression of the

lymph node homing receptor c-c chemokine receptor 7 (CCR7) (59). In contrast, sputum DCs from smokers exhibit decreased maturation markers CD83 and DC-lysosome associated membrane protein (DC-LAMP) compared to both before smoking cessation programs as well as to never smokers (60; 61). Smokers with chronic obstructive pulmonary disease (COPD) also have decreased numbers of bronchial mucosal DCs compared to nonsmoking controls (62; 63). The effect of CS exposure on pulmonary DC numbers in mice is unclear. Mice chronically exposed to 5 cigarettes per day, 5 days per week for 24 weeks had increased DCs in the airways and lung parenchyma with increased expression of CD40 and CD86 that correlated with alveolar wall destruction (64). Conversely, mice exposed to 4 cigarettes per day, 5 days a week for 4 weeks had decreased numbers of DCs in the lung but similar antigen induced migration to the lung (65). Although whether or not absolute numbers of DCs in the lung are augmented by CS exposure is not understood, CS exposure may potentially alter DC co-stimulatory molecules.

CS exposure alters normal T cell function by decreasing pathological T helper cell 1 (Th1) responses and increasing T helper cell 2 (Th2) allergic responses. CS extract inhibited lipopolysaccaride (LPS) induced Th1 stimulation of DCs by decreasing IL-12p70 secretion (66) and LPS-induced expression of CD40, CD80, CD86, and CCR7 on DCs (67). Treatment with antioxidants n-acetylcysteine (NAC) and catalase reversed this IL-12p70 suppression (66). CS exposure in mice also suppressed ovalbumin induced CD80, CD86, and MHC Class II maturation of lung DCs as well as decreased the ability to these cells to stimulate IL-2 production and CD4 T cell proliferation *ex vivo* (65). CS exposure potentially alters T cell proliferation through DC modification.

CS exposure may enhance cytotoxic CD8 T cell function and NKG2D ligand expression. CSE exposure of mouse bone marrow DCs increased CD8 and decreased CD4 T cell proliferation in a mixed lymphocyte reaction (68). CS exposure of mice also may enhance cytotoxic lymphocyte activity by enhancing expression of NKG2D ligand retinoic acid early transcripts-1 (RAET1) (69). In humans, smoking and COPD enhances NKG2D ligand MICA in the bronchial epithelium (69). Consequently, conventional cytotoxic lymphocytes in the bronchial epithelium positive for CD3, CD8, and NKG2D were enhanced in smokers compared to nonsmokers (70). What this role of sustained NKG2D mediated CD8 cytotoxic lymphocyte activation plays in smokers during the course of a respiratory virus infection is unknown, especially because enhanced NKG2D ligands on the airway epithelium could activate NK cells as well as T cells. Thus, CS may alter the CD8 T cell activity either through DC or NKG2D ligand mediated changes.

The effects of CS exposure on adaptive memory response to influenza infection are unclear. The generation of appropriate memory T and B cell responses depend on DC mediated antigen presentation to naïve T cells. However, the effects of CS exposure on specific memory T cell responses are unknown. CS exposure may not alter the B cell memory response as the levels of circulating influenza specific antibodies in either humans (71-73) or animals (74) exposed to CS prior to influenza infection remain unchanged. Therefore, it is likely that CS-induced alterations in innate immune responses may play a greater role than adaptive memory responses in susceptibility to viral infections.

Cigarette Smoke Exposure Modifies NK Cell Function

CS exposure impacts NK cell immune activity. NK cell numbers and activity were decreased in cigarette smokers (75-79), and smokers who quit smoking for 31 days had elevations in cytotoxic NK cell levels (80). CS exposure suppressed NK cell activation and decreased cytolytic activity and CD69 expression in mice (81). In a melanoma tumor challenge model, CS exposure decreased NK cell activation and cytolytic activity, resulting in increased tumor incidence (81). CS-conditioned media decreased NK cell cytotoxicity and perforin expression in peripheral blood mononuclear cells (PBMC) (82). In addition, CS-conditioned media decreased poly I:C induced increases in NK cell cytotoxicity and CD69 expression in PBMC (83). This NK cell suppression was IL-15 dependent, indicating that IL-15 producing DCs or monocytes within the PBMC population could be responsible for this suppression, underscoring how communication between immune cells can affect antiviral responses (83). Decreased NK cell activity in humans is linked to recurrent pathogenic infections (5) and could be partially responsible for why individuals exposed to cigarette smoke have overall decreased immune responses to respiratory viral infections (48). However, in the context of COPD, which is associated with chronic cigarette smoking, the role of NK cells is unclear. Lung leukocytes from chronic CS exposed mice stimulated with poly I:C *ex vivo* had enhanced NK cell production of IFNγ compared to air controls (84). This could be due to CS-induced upregulation of NKG2D ligands on pulmonary mouse epithelium (69). Thus CS exposure may have opposing effects on cytolytic and NKG2D activation on NK cells. Whether and how NK cells are modulated in smokers in the context of viral infections, specifically influenza, is unknown.

Effects of Other Air Pollutants on Influenza Infection

Similarities Between Cigarette Smoke, Second Hand Smoke, Diesel Exhaust, and

Biomass

Besides cigarette smoke, there are many other air pollutants sharing similar chemical features that can affect respiratory health. CS, diesel exhaust (DE), and biomass fumes (dung, wood smoke) are all products of incomplete combustion of organic materials. Nonsmokers exposed to SHS and active smokers are exposed to analogous chemical pollutants, such as polyaromatic hydrocarbons (PAHs), although the doses and exact compositions can vary (85). Thus, many air pollutants share similar chemical compositions that include PAHs, benzene, and carbon monoxide (86-88). These pollutants also contribute to the heterogenous composition of particulate matter (PM), which contains metals (Cr, Co, Ni, Mn, Zn, V, Cu, and Fe) as well as PAHs like benzo[a]pyrene and naphthalene (89). These different air pollutants can adversely affect human health through induction of oxidative stress and by increasing inflammation (89).

Effects of Secondhand Smoke Exposure on Respiratory Viral infections

Studies examining the effects of SHS exposure on respiratory immune responses to influenza and other respiratory viruses are not as numerous as CS studies but in general suggest that exposure to SHS is deleterious to respiratory health (90). Our own data demonstrated that nonsmokers routinely exposed to SHS prior to inoculation with live attenuated influenza virus (LAIV) had suppressed IP-10 and IL-6 responses that were intermediate between nonsmokers and active smokers, indicating that CS exposure may have a "dose dependent" effect on immune suppression (3). SHS exposure either prenatally, during infancy, or during both time periods decreased *in vitro* secretion of the immunoregulatory cytokine IL-10 from peripheral blood monocyte derived DCs (91).

SHS exposure also increased RSV infection in children (92). A study examining the relationship between SHS exposure and acute lower respiratory tract infection (LRI) in hospitalized young children indicated that while SHS was not associated with increased risk of RSV-LRI, infants exposed to SHS with RSV-LRI were more likely to have desaturating oxygen levels (93). Another study showed that RSV hospitalized infants exposed to SHS postnatally also had decreased oxygen saturation, which could increase RSV morbidity (94). In addition, smoking in the household is considered a risk factor in RSV-related hospitalization (95). Thus, like CS, SHS exposure can impact immune responses to respiratory viral infections.

Effects of Diesel Exhaust and Particulate Matter on Influenza Infection

Diesel exhaust, a component of PM, also alters immune responses to respiratory viral infections. Epidemiological studies have also indicated that increases or fluctuations in ambient PM levels can increase risk of respiratory infections as well as mortality from influenza and pneumonia (96). Animal models examining the effects of PM on respiratory viruses, particularly RSV, demonstrate that PM does indeed increase virus mortality and alters immune responses possibly through decreasing antiviral mediators, increasing inflammation, and inhibiting macrophage phagocytosis (96). DE is similar in size to fine PM, can easily travel deep into the lungs, and is a significant contributor to overall PM levels (96). There is a wealth of animal studies confirming that DE enhances susceptibility to viral infections, including data from our own group (97; 98). Taken together, these data suggest that like CS exposure, other ambient air pollutants have negative effects on respiratory virus infections.

Effects of Particulate Matter on DC and Adaptive T Cell Function

Exposure to PM impacts DC and T cell respiratory responses. PM increased

expression of CD40, CD80, and CD86 (99) and decreased TLR2 and TLR4 expression

(100) on DCs. Stimulation of allogenic CD4+ T cells with PM treated DCs produced a

Th2-like response with a decreased IFNy:IL-13 ratio compared with LPS stimulated DCs

(99). In addition, PM exposed pulmonary DCs from mice stimulated naïve CD4 T cells in

a Th2 response (101). Ultrafine carbon black particles and ovalbumin (OVA) instillation

also enhanced DC numbers, DC co-stimulatory molecules, and T cell proliferation in

draining lymph nodes (102). Oxidative stress as well as nuclear factor receptor 2 (NRF2),

a master regulator of the antioxidant response, may play a role in these pollutant-induced

changes. DCs from (NRF2)-/- mice following PM exposure had increased H_2O_2

production and decreased responses of antioxidant genes (103). As a result, NRF2-/- DCs

had a decreased ability to phagocytose antigen with an increased release of

proinflammatory cytokines IL-18 and TNFα (103). PM exposure to NRF2-/- DCs

resulted in enhanced secretion of Th2 cytokines IL-5 and IL-13 compared to wildtype

mice (103). Thus PM can affect DC and downstream T cell responses in an oxidative

stress dependent manner.

Effects of Diesel Exhaust on DC and Adaptive T Cell Function

There are extensive data on the effects of DE particles (DEP) on DC function

using both *in vivo* and *in vitro* exposure models. DEP modify characteristic DC

responses. DEP alone increased expression of co-stimulatory molecules CD40, CD80,

CD83, and CD86 and increased phagocytic capability and expression of the endocytosis

receptor CD206 on DCs (104) although others have reported no changes in co-

stimulatory marker expression (105). DEP also modulate DC responses to LPS. DEP

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alone decreased LPS induced TLR2 and TLR4 expression on DCs (104). DEP suppressed

LPS induced expression of CD83 and MHC Class II on DCs and secretion of IL-12

through activation of the Nrf2 signaling pathway (106) although others have reported that

DEP enhanced LPS induced CD83 expression on DCs (105). Thus DEP exposure

modifies DC maturation markers and LPS induced DC activation.

DEP exposure modulates not only expression of DC maturation markers but also

DC activation and downstream T cell responses. DCs when pretreated with DEP and then

stimulated with CD40 ligand and IFNy secreted less IL-12 and TNFα in an

immunosuppressive effect (107). DEP also modified DC ability to orchestrate a T cell

response. In co-cultures with DEP treated DCs and allogenic CD4+ T cells, DEP

enhanced IL-13, IL-12, and IFNγ (104), and IL-10 (106) from stimulated T cells. DEP

exposure models increased Th2 cytokine responses. Exposure to both DEP and

ovalbumin in mice enhanced the ability of splenic mononuclear cells to generate IL-4, IL-

5, and IL-15 following *ex vivo* antigenic stimulation (108). The combination of repeated

exposures to both DEP and OVA increased MHC Class II markers and DC maturation

markers CD11c, CD80, and CD86 as well as B cell marker CD19 on whole lung cells

(108). Therefore, DEP can affect the ability of DCs to initiate a T cell response.

The mechanism underlying DEP enhancement of Th2 allergic responses has been

studied using co-culture systems of DCs and airway epithelial cells (2; 109; 110).

Cultured DCs are applied basolaterally to human airway epithelial cells grown on

membrane support and cab access deposited particles in the epithelium through inter-

epithelial projections (110). Fine particle deposition increases DC projections into the

lumen (2). Exposure of pollutants to the epithelium induced the release of factors

promoting DC development. Ambient PM enhanced release of MIP-3α, a chemokine that causes DC migration, from bronchial epithelial cells (111). DEP exposure to human bronchial epithelial cells increased DC CD83 expression and enhanced T cell proliferation in a granulocyte-macrophage colony stimulating factor (GM-CSF) dependent manner (112). Thymic stromal lymphopoeitin (TSLP) release from DEP treated human bronchial epithelial cells induced Th2 DC polarization (113). Therefore, DEP can induce the release of cytokines from airway epithelial cells that activate DCs in a Th2 dependent manner.

Effects of Biomass on Respiratory Infections

Besides SHS, other indoor pollutants can contribute to deleterious health effects. Solid fuels, such as biomass, which includes wood, dung, and crop remains, and coal are primarily used for both cooking and heating in up to 90% of households in rural areas of developing countries and are significant contributors to indoor air pollution (114). These indoor kitchens are poorly ventilated and typically expose women and young children to high levels of PM, carbon monoxide, nitrogen oxides, and PAHs for 3 to 7 hours daily for years, and the overall health consequences of these exposures are comparable to SHS (114; 115). A meta-analysis determined that although the link between solid fuel exposure and RSV is unclear, solid fuel exposure increases the risk of pneumonia in young children (116). Solid fuel exposure is also associated with diseases like COPD in women (117; 118), chronic bronchitis in women (118) and acute respiratory infections in children (117; 118). Consequently, reducing biomass exposures in these rural populations could benefit respiratory health.

Effects of Other Air Pollutants on NK and T Lymphocyte Function

Data on the effects of other oxidant airway pollutants such as DEP, PM, and biomass on NK and T cell function are limited. BALB/C mice injected with DEP followed with LPS treatment had decreased splenic NK and NKT cell mediated production of IFNγ (119). Daily wood smoke exposure for 6 months did not alter expression of CD3, CD4 or CD 8 on T cells, Mac-1 on macrophages, CD19 on B cells, or CD16 on NK cells (120). However, the effects of wood smoke on concanavalin A induced T cell proliferation were inconclusive as low levels of wood smoke enhanced proliferation (100 μg/m^3) whereas high levels of wood smoke (<300 μg/m^3) suppressed proliferation (120). The phenomenon that low doses of PAHs enhance immune responses whereas high doses of PAHs are immunosuppressive has been reported in mouse uterine NK cells from placental exposed PAHs (121). Thus, the effects of DEP and wood smoke on lymphocytes in the respiratory epithelium beg elucidation and perhaps share common mechanisms with CS induced immune suppression.

Summary

It is evident that ambient air pollutants, especially CS, increase susceptibility to respiratory viruses. This susceptibility most likely is due to CS mediated suppression of innate immune antiviral responses. Suppression may initiate in respiratory epithelial cells and be transferred to other respiratory immune cells like DCs, NK cells, and γδ T cells. In the following chapters, I will describe my research into how CS may impact respiratory mucosal responses to influenza infection in these specific immune cell types. These data underscore the need to limit CS exposure as well as devise therapeutics to prevent CS induced disease.

Apical
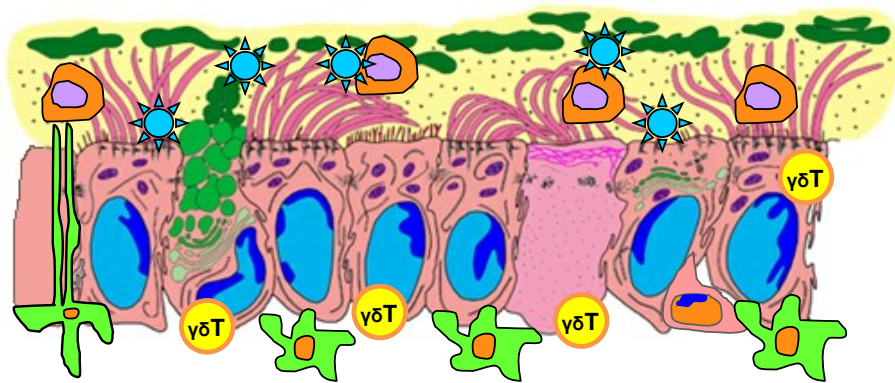
Basolateral

☀ **Respiratory Viruses**	🦠 **Dendritic Cell (DC)**
🔬 **Respiratory Epithelial Cell**	γδT **Intraepithelial γδ T cells**
🔶 **Airway Leukocytes**	

Figure 1. Immune Cells of the Respiratory Epithelium

In the apical airway lumen, leukocytes patrol the airways and interact with respiratory viruses. Underlying respiratory epithelial cells communicate directly with these viruses as well as with activated airway leukocytes. Basolateral to respiratory epithelial cells lie dendritic cells that patrol the epithelium for foreign antigens and can directly sample the airways with dendritic-like projections. Intra-epithelial γδ T lymphocytes are located throughout the respiratory mucosa and regulate immune responses.
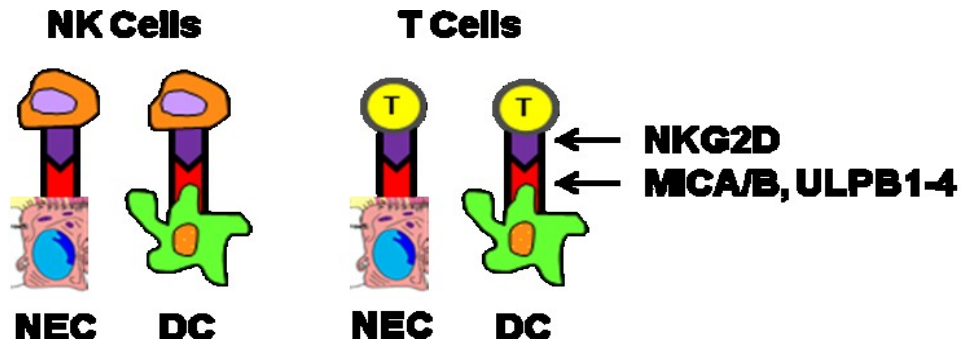
Figure 2. Respiratory Immune Cells Communicate Via NKG2D Signaling.

NEC and DCs express NKG2D ligands MICA, MICB, and ULPB1-4 at baseline and during cellular stress, including viral infections. NK cells and T cells recognize these ligands via NKG2D activating receptors to enhance cytotoxicity and cytokine secretion.
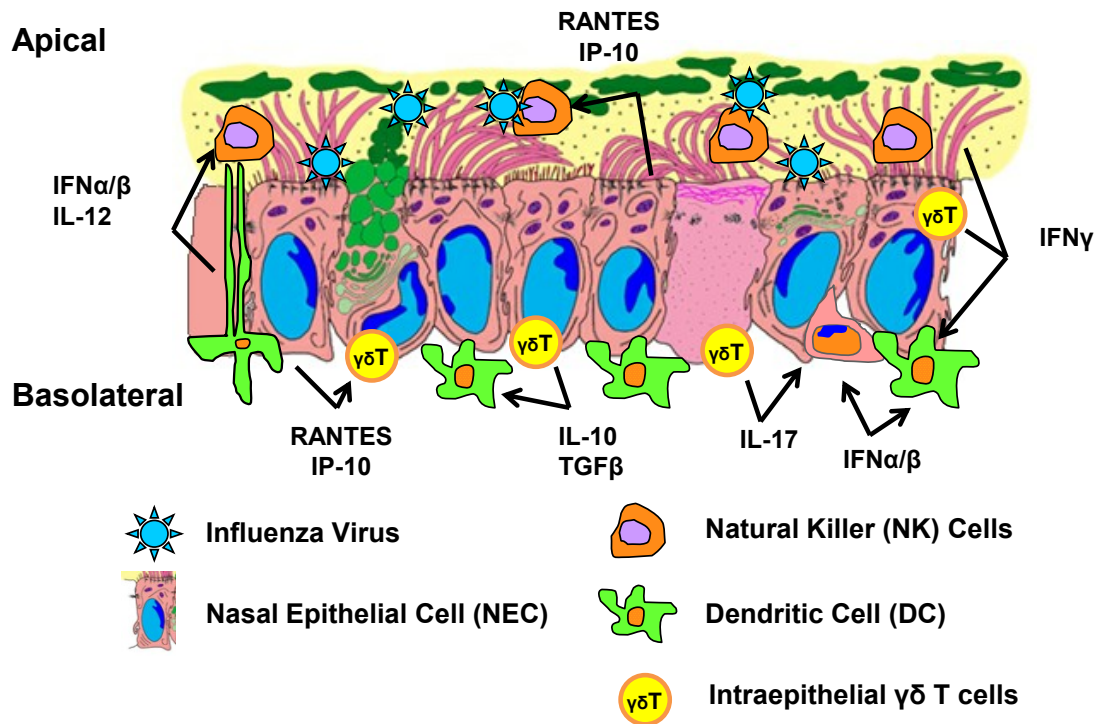
Figure 3. Nasal Mucosal Cells Communicate During Influenza Infections.

In the apical airway lumen, NK cells patrol the airways for pathogens such as the influenza virus and monitor nasal epithelial cells (NEC) for markers of infection. NEC secrete chemokines RANTES and IP-10 to attract and activate additional NK cells. Respiratory DCs lie basolateral to NEC and monitor NEC for display of viral antigens. Their "dendritic-like" projections can directly sample the airways. DCs secrete type I IFNs α/β to activate both NEC and NK cells. In turn, NEC-derived IFN α/β and NK cell-derived IFNγ activate DCs. NEC secrete T cell chemokines RANTES and IP-10 to attract intraepithelial $\gamma\delta$ T cells. During an immune response, $\gamma\delta$ T cells can secrete IL-10 and TGFβ to suppress DC immune responses or secrete IL-17 to enhance NEC inflammation.

CHAPTER 2

EPITHELIAL CELLS FROM SMOKERS MODIFY DENDRITIC CELL RESPONSES
TO INFLUENZA INFECTION

Abstract

Rationale: Epidemiological evidence suggests that cigarette smoking is a risk factor for
influenza infection, but the mechanisms underlying this susceptibility remain unknown.
To ascertain if airway epithelial cells from smokers have a decreased ability to
orchestrate an influenza-induced immune response, we established a model utilizing
differentiated NEC (nasal epithelial cells) from nonsmokers and smokers and then co-
cultured NEC with peripheral blood monocyte-derived dendritic cells (mono-DCs) from
nonsmokers.

Methods: NEC/mono-DC co-cultures were infected with influenza A virus and analyzed
for influenza-induced immune responses 24 h post infection.

Results: We demonstrated that NEC from smokers, as well as mono-DCs co-cultured
with NECs from smokers, have suppressed influenza-induced interferon related proteins
IRF7, TLR3, and RIG-I, likely due to suppressed IFNα production from smoker NECs.
Furthermore, NEC/mono-DC co-cultures using NEC from smokers have suppressed
levels of T cell/NK cell chemokine IP-10 following influenza infection, indicating that

NECs from smokers may skew early influenza-induced Th1 responses. In contrast, we demonstrated that NEC/mono-DC co-cultures using NEC from smokers had increased influenza-induced levels of the Th2 chemokine TSLP. In addition, NEC from smokers cultured alone had increased influenza-induced levels of the Th2 chemokine TARC.

Conclusions: Using this model, we have demonstrated that following influenza infection, NEC obtained from smokers create an overall cytokine microenvironment that suppresses the interferon-mediated Th1 response and enhances the TSLP-TARC mediated Th2 response to potentially modify DC responses. Smoking-induced alterations in the Th1/Th2 balance may play a role in developing underlying susceptibilities to respiratory viral infections as well as promote the likelihood of acquiring Th2 pro-allergic diseases.

Introduction

Epidemiological studies demonstrate that smokers are at increased risk for influenza infections (43-46). However, mechanisms mediating enhanced susceptibility to viral infections seen in smokers are not known. In human (71-73) and animal (74) models of influenza, adaptive humoral immunity, as measured by influenza specific antibody production is unaffected by CS exposure. Therefore, this chapter focuses on the effects of CS exposure on innate immune mechanisms during an influenza infection.

It has been shown that CS exposure suppresses innate immune responses of the respiratory epithelium (53). Influenza virus infects epithelial cells by binding via hemagglutinin and entering cells via sialic acid residues utilizing endocytosis. Soon after infection, influenza activates the innate immune system of the respiratory epithelium and stimulates the generation of cytokines and chemokines (9). Pattern recognition receptors (PRRs) such as TLR3 and RIG-I recognize viral RNA and activate nuclear transcription factors like NF-κB, IRF3, and IRF7 in epithelial cells. Synthesis and binding of IRFs to interferon response element (ISRE) promoter regions induces the production of antiviral type I IFNs, for example IFNα/β, that help prevent further viral infection. We have previously shown that IRF7 expression is decreased in nasal epithelial cells (NEC) from smokers following influenza infection, yielding suppressed type I IFN production in these cells (53). Similarly, CS conditioned media has been shown to inhibit IRF3, IRF7, and NF-kB responses to poly I:C in lung epithelial cells and fibroblasts (122).

The interplay between the airway epithelium and resident immune cells such as DCs is crucial in mobilizing respiratory immune responses. Activated DCs fulfill the pivotal task of mobilizing both innate and adaptive immune cells by secreting

chemokines to attract neutrophils, cytotoxic and memory T cells, and NK cells (11).

Specifically, DCs produce chemokines such as RANTES, CXCL9, IP-10, and CXCL11

and drive a Th1 CD4+ T cell response to expand activated cytotoxic T cell pools (13).

Direct infection of DCs with influenza virus has been shown to activate DC antiviral

defenses including RIG-I, TLR3, and IRF7 (123), but whether and how signals derived

from the microenvironment, such as surrounding epithelial cells, modifies these effects is

not known.

Respiratory DCs are capable of responding to not only Th1 cytokines produced

during a viral infection, but also to Th2 chemokines produced by a dysregulated

respiratory epithelium. TSLP is an epithelial cell-derived Th2 chemokine that

communicates with and induces Th2 responses in DCs. TSLP acts directly on DCs to

increase production of TARC (124), which in turn coaxes naïve T cells to secrete pro-

allergic cytokines such as IL-5, IL-4, IL-13, and TNFα which have the potential to foster

the development of an allergic, Th2 CD4 T cell phenotype (125). Although it has been

demonstrated that normal human bronchial epithelial cells up regulate TSLP in response

to stimulation with the viral dsRNA mimetic poly I:C (126), it is not known how virus-

induced TSLP or TARC expression may be altered during an influenza infection in

smokers.

In vitro studies that treat DCs directly with CS may not provide the most realistic

model system because signals derived from the respiratory epithelium are important in

generating the proper microenvironment for DC maturation in vivo. To address the

limitations of current human in vitro airway epithelia models, co-culture systems of

airway epithelial cells and DCs have been developed to study the effects of particles on

the airway epithelium (1; 2; 127). These models have the potential to explore the

mechanisms of airway epithelial and immune cell communication during immunological

responses, including respiratory viral infections. We expanded upon these existing

models to develop a co-culture system of human NEC obtained from nonsmokers and

smokers and monocyte-derived DCs (mono-DCs) obtained from healthy nonsmokers to

determine how smoking-induced changes at the level of the epithelium affects

communication with resident immune cells.

Methods

Culture of NEC

NECs were obtained from smoking and nonsmoking healthy human volunteers and differentiated *in vitro* on 0.4 μM pore size membrane support as described previously (53). The selection criteria for subject recruitment were similar to those described previously (53; 128). Smoking status was assessed via questionnaire and confirmed through urine cotinine measurements (128). All of the smokers recruited for the study were current smokers.

Culture of Mono-DCs, NEC-Mono-DC Co-Culture System and Influenza Infection

Peripheral blood monocytes were isolated from healthy nonsmoking volunteers. Monocyte-derived DCs (mono-DCs) were generated by culturing peripheral blood monocytes with 30 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ) and 30 ng/ml IL-4 (Peprotech) for 5-7 days. Differentiation of mono-DCs was confirmed using flow cytometry to identify expression of DC markers CD86, CD40, CD209, HLA-DR, and CD11c as described in supplemental data (BD Biosciences, San Jose, CA). Our NEC-mono-DC co-culture system is based upon a three dimensional cell culture model described previously (1). 1.5×10^5 mono-DCs were applied to the basolateral side of inverted differentiated NEC grown on membrane support of approximately 1.13 cm^2 (see Figure 4). Mono-DCs were adhered for two hours after which NECs were infected with influenza A/Bangkok/1/79 (H3N2 serotype) as described before (129). All samples were collected 24 hrs post infection unless otherwise indicated.

Supernatant Cytokine Levels

The apical surfaces of the co-culture NECs were washed with HBSS. Apical washes and basolateral supernatants were collected and analyzed for cytokine secretion of IP-10 (BD Biosciences), RANTES, TARC, and TSLP (all R&D Systems) using commercially available ELISA kits.

Analysis of mRNA From NEC and Mono-DCs

NEC and mono-DCs were removed from the membrane and added to Trizol® (Invitrogen, Carlsbad, CA) for isolation of total RNA. Real-time qRT-PCR was performed as described previously (129), using commercially available primers and probes for TLR3, RIG-I, IRF7, IP-10, and RANTES (Applied Biosystems, Foster City, CA)

Western Blotting

Whole cell lysates were prepared and analyzed as described by us before (53), using specific antibodies to IRF7 (Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (1:2000, USBiological, Swampscott, MA).

Visualization of Co-Culture System

Co-cultures were fixed with ice cold methanol for 20 minutes. Antibodies to CD11c (ebioscience, San Diego, CA) and acetylated α-tubulin (Abcam, Cambridge, MA) were used to identify mono-DCs and cilia of NEC, respectively, followed by incubation with Alexa-488-conjugated and Alex-596-conjugated secondary antibodies (Invitrogen). Samples were visualized using a Nikon C1Si laser scanning confocal microscope and

images were processed using the EZ-C1 FreeViewer software (Nikon Instruments, Melville, NY).

Flow Cytometery of Mono-DCs

Mono-DCs were removed from the membrane using a cell scraper into RPMI media supplemented with 10% FBS. Mono-DCs were resuspended in flow staining buffer (DPBS supplemented with 1% FBS and 0.09% sodium azide) and stained at a density of 1.5×10^5 cells/100 ul total volume. Mono-DCs were stained according to manufacturer's instructions (BDBiosciences) with CD45 (Leukocyte marker), CD86, CD209, CD11c, CD11b and HLA-DR (DC markers), washed with flow staining buffer, fixed with 0.5% paraformaldehyde, and analyzed by flow cytometry within 24 h.

Statistical Analysis

mRNA and mono-DC maturation data from co-culture experiments are expressed as fold induction over non-infected control to determine influenza-induced responses and analyzed using a Wilcoxon signed-rank test. Differences in influenza-induced and baseline responses between nonsmoker and smoker NECs were analyzed using a nonparametric Mann Whitney U test. Protein supernatant data were analyzed using a 2-way ANOVA followed by a Bonferroni's post-hoc analysis. All data were expressed as mean $\pm$ S.E.M with $p<0.05$ considered to be significant.

Results

Development and Visualization of Co-Culture Model

To develop mono-DCs, peripheral blood monocytes were incubated with IL-4 and

GM-CSF and analyzed for positive expression of characteristic DC surface markers. Prior

to co-culture with NECs, mono-DCs were analyzed by flow cytometry and shown to have

positive expression of DC maturation markers CD11b, CD11c, CD86, CD209, and HLA-

DR (data not shown).

To determine whether mono-DCs cells form networks on the basolateral side of

NECs in our co-culture model, we visualized the different cell types using confocal

microscopy. In Figure 5, the top panels show *en face* visualization of the apical border of

the epithelium (left) and the mono-DCs on the basolateral side. To stain the cilia of NECs

we used mouse anti-acetylated alpha tubulin followed by an alexa-596-conjugated

secondary antibody (red), as demonstrated previously (129; 130). DCs were identified

using mouse anti-CD11c followed by an alexa-488-conjugated secondary antibody

(green). The bottom panel shows a *x-y-z* optical cross section of the co-culture model

with the mono-DCs lying basolaterally to the nasal NECs, a polarization that resembles

their *in vivo* orientations.

Mono-DC Maturation in Co-Culture Model Following Influenza Infection

To determine whether surface markers indicating DC maturation are changed in

mono-DCs cultured with NEC from smokers and non-smokers, we analyzed surface

marker expression of mono-DCs by flow cytometry following influenza infection. Cells

were gated (P1) for positive expression of leukocyte marker CD45 (Fig. 6A). Mono-DCs

co-cultured with NECs had positive baseline expression of CD11b, CD40, CD209, CD11c, and CD86 (representative histograms in Figure 6B-F). Figures 6G-I show that influenza infection did not change expression of CD11b, CD40, and CD209 in mono-DCs cultured with NECs from either smokers or nonsmokers. In contrast, expression of CD11c was only upregulated after influenza infection in mono-DCs derived from co-cultures using NEC from non-smokers (Fig. 6J). CD86 was enhanced after influenza infection in mono-DCs derived from both nonsmoker and smoker co-cultures (Fig. 6K). However, changes in CD11c and CD86 did not appear to be robust.

Smoker NECs Have Suppressed Anti-Viral Responses

To determine how antiviral defense responses are modified, NEC/mono-DC co-cultures using NECs from nonsmokers and smokers were infected from the epithelial side with 1 MOI influenza A virus. Total RNA was collected separately from both cell types 24 hrs after infection and analyzed for antiviral responses. To determine whether our previous observation of suppressed influenza-induced IRF7 expression in NEC from smokers (53) could also be observed in this co-culture model, we analyzed IRF7 mRNA (Fig. 7A) and protein (Fig. 7B) expression in NEC from co-cultures. Similar to our previous study (53), influenza-induced IRF7 expression was suppressed in NEC from smokers. In addition to IRF7, influenza-induced expression of pattern recognition receptors (PRRs) is important for antiviral defense responses. Influenza infection did not induce TLR3 mRNA expression in NEC from smokers (Fig. 7C) and that influenza-induced RIG-I mRNA expression was suppressed in NEC from smokers (Fig. 7D). Baseline expression of RIG-I, TLR3, and IRF7 was similar in nonsmoker and smoker NECs as shown in Table 1.

Smoker NECs Suppress Anti-Viral Responses In Mono-DCs

To determine whether the changes in antiviral defense responses seen in NEC from smokers also lead to suppressed responses in the mono-DCs, we analyzed the same antiviral defense markers in the mono-DCs of the co-cultures. Influenza infection increased IRF7 (Fig. 8A) and RIG-I (Fig. 8B) mRNA levels in mono-DCs co-cultured with either NEC from nonsmokers or smokers, but this induction was significantly greater in mono-DCs cultured with NEC from non-smokers. Influenza infection tended to increase TLR3 mRNA levels of mono-DCs co-cultured with NEC from nonsmokers, albeit it did not reach statistical significance ($p=0.06$) (Fig. 8C).

Activation of TLR3 and RIG-I prompts the production of inflammatory and immune mediators, including chemokines such as IP-10 and RANTES (131; 132), which are important for immune cell migration. Therefore, we examined the expression of these two chemokines in the co-cultures. Baseline expression of RANTES and IP-10 mRNA was similar in nonsmoker and smoker NECs from co-cultures (See Table 1). Influenza induced IP-10 mRNA expression was suppressed in NECs (Fig. 9A) and mono-DCs (Fig. 9B) from co-cultures using NEC from smokers. The use of transwell inserts allows for separate analysis of protein secretion from the apical (upper) and basolateral (lower) compartments of the mono-DC/NEC co-culture system (See Figure 4), as reported previously (130; 133). In apical washes (Fig. 10A) and basolateral supernatants (Fig. 10B) IP-10 secretion in influenza infected co-cultures using smoker NECs was suppressed compared to nonsmoker controls. Influenza infection induced RANTES expression in NEC from both nonsmokers and smokers (Fig. 9C), but not mono-DCs derived from the co-cultures (Fig. 9D). Interestingly, RANTES protein secretion was

increased in apical washes and basolateral supernatants from co-cultures using influenza infected NECs from nonsmokers but not from smokers. Overall, these data indicate that influenza-induced IP-10 was suppressed in both NEC and mono-DCs co-cultures using NEC from smokers.

Influenza Infection Increases Th2 Chemokines In Smoker NECs

It has been shown that CS exposure can lead to a Th2 phenotypic immune response (134). Therefore, after demonstrating that co-cultures using NEC from smokers show suppressed expression of chemokines associated with Th1 responses (i.e. IP-10), we determined whether chemokines associated with Th2 phenotypes were altered. We first analyzed the expression of TSLP, a Th2 chemokine that is secreted by NECs and is upregulated in mouse lungs after CS extract exposure (135). Influenza infection increased TSLP secretion into the basolateral supernatant in co-cultures using NEC from smokers compared to nonsmokers (Fig. 11A). TSLP is not detectable in the apical compartment (data not shown). TSLP acts upon DCs to stimulate TARC production (136). Therefore, we determined whether the changes in TSLP expression were reflected in increased TARC expression in basolateral supernatants from co-cultures using NEC from smokers. Overall, influenza infection did not increase TARC levels in the basolateral supernatants from NEC/mono-DCs co-cultures using NEC from nonsmokers or smokers (Fig. 11B), and TARC levels were below detection in the apical compartment (data not shown). Considering that mono-DCs from healthy individuals have high constitutive levels of TARC expression (137), it is likely that mono-DCs are heavily contributing to the overall high TARC levels in the basolateral supernatants. Previous studies have shown that in addition to DCs, TARC can be expressed in other respiratory cell types, including

36

epithelial cells (138). To further determine if influenza-induced TARC expression is altered in NECs from smokers alone, we designed experiments in which NECs in the absence of mono-DCs were analyzed. Figure 11 shows that influenza infection significantly increased TARC production in NECs from smokers, but not in NECs from nonsmokers.

Discussion

Communication between DCs and other cell types during an immune response is crucial for DC activation, and single cell culture models using DCs cannot study this interaction. Our two cell co-culture model of NECs and DCs allows us to determine the interplay between the virally infected epithelium and DCs and also how smoking may interrupt this communication. Using this model, we have demonstrated that during an influenza infection, NEC obtained from smokers have altered communication with underlying mono-DCs, creating an overall cytokine microenvironment that suppresses the interferon mediated Th1 response and enhances the TSLP-TARC mediated Th2 response.

We have shown here that co-culturing mono-DCs with NEC from smokers alters the mono-DC response to influenza infection. The nature of this communication is likely through "soluble mediators or cytokine secretion, although other possibilities exist. Activated DCs have been shown to release exovesicles, small membrane bound vesicles that can contain cytokine and immune receptors (139). These exovesicles are capable of activating TNFα pathways in airway epithelial cells in a co-culture model (140). Thus, it is possible in our model that NEC and mono-DCs are communicating through exovesicle mediated pathways. In fact, RNA-containing exovesicles have been detected in human NLF (141). It has also been suggested in a co-culture model of lung epithelial A549 cells and mono-DCs that DCs are capable of forming intraepithelial cell projections through the pores of the cell culture membrane to engage in direct epithelial cell-DC contact (2). Although we cannot rule such interactions out, due to the smaller pore size we must use for efficient culture of differentiated NEC (0.4 μM vs. 3.0 μM size used by Blank et al (2)), we doubt mono-DC projections are playing a large role in their activation and did

not find projections in our immunohistochemical models. Also, mono-DCs used in our studies do not show any markers of direct influenza infection (no detectable influenza HA RNA; data not shown), suggesting that they may not have access to the apical surface where infection is occurring. Therefore, we believe it is possible that in our model antiviral mediator and cytokine expression are initiated by epithelial derived cytokines and chemokines acting on mono-DCs. For example, NEC derived IFNs are secreted by infected epithelial cells (53) and can activate nearby NEC in an autocrine fashion to induce the expression of interferon related gene products, including TLR3 (130). Similarly, influenza infected NEC could also activate nearby DCs in a paracrine fashion via IFNα secretion to act upon mono-DC IFNα receptors, culminating in the transcription of IRF7, TLR3, RIG-I, and IP-10. Thus, it is possible that the reduced IFNα expression seen in NEC from smokers, as shown previously (53), leads to suppressed IFN-induced antiviral defense responses in underlying immune cells, such as DCs. Overall, these data demonstrate that in addition to type I IFN release and IRF7 expression (13), the expression of PRRs, such as RIG-I and TLR3 are suppressed in smoker NECs, potentially resulting in suppressed activation of resident immune cells that communicate with the respiratory epithelium during an influenza infection.

Our data demonstrate that influenza-induced IP-10 expression is reduced in both NECs from smokers as well as mono-DCs co-cultured with NECs from smokers, thereby leading to overall reductions in IP-10 protein secretion in both the apical and basolateral compartment of the co-culture systems. During an influenza infection, IP-10 is released from NECs to attract lymphocytes and T cells to the site of infection (142). Smoke exposed mice infected with influenza have suppressed levels of whole lung IP-10 mRNA

with modified profiles of CD4/CD8 T cells in the BAL and draining lymph nodes (50). hawse have shown that smokers inoculated with LAIV have reduced IP-10 levels in nasal lavage fluid compared to non-smoker controls (3). Besides T cells, suppressed IP-10 production by the respiratory epithelium could lead to suppressed recruitment of other immune cells like NK cells. NK cells express the IP-10 receptor CXCR3 and migrate to the respiratory epithelium during an influenza infection (143). Decreased IP-10 responses could be responsible for decreased cytotoxic NK cell numbers and could explain why levels of granzymes B and K, important mediators of cytotoxic NK cells, are reduced following influenza infection of mice exposed to CS (50).See chapter 3. Thus, reduced IP-10 production by either NEC or DCs after viral infections could have a significant impact on the ability to fight and clear the infection. Influenza infection increases RANTES secretion into the apical and basolateral compartments in nonsmokers not in smokers, although there was no overall difference in RANTES secretion between groups. Like IP-10, RANTES is an important chemokine that is released during influenza infection (142). A lack of IP-10 response in smokers may indicate that smokers NECs have decreased communication with immune cells and highlights the importance of non-immune cells in orchestrating both innate and adaptive immune responses to viral infections.

Cigarette smoke exposure has been shown to shift immune responses from a Th1 to a Th2 phenotype (134). TSLP, a Th2 chemokine, is secreted by epithelial cells and triggers TARC expression in nearby DCs (124). TSLP in epithelial cells as well as TARC in DCs is upregulated with exposure to airborne pollutants including diesel exhaust (113) and CS extract (135). CS exposure has also been shown to upregulate TARC mRNA

expression in whole lungs in mice (144). We have shown here that influenza infection upregulates TSLP secretion in co-cultures using NECs from smokers, but not nonsmokers, suggesting that in smokers the activation of the TSLP pathway may be occurring in concert with suppression of Th1 chemokines, such as IP-10. Smoker NECs themselves also have elevated TARC levels, which have also been shown in the broncheoalveolar lavage of current and ex-smokers (145). TSLP and TARC were undetectable in the apical washes (data not shown), which also suggests that differentiated NECs may have polarized protein secretion patterns. These data are the first examples of TSLP and TARC upregulation in smoker epithelial cells during an influenza infection and suggest that in smokers Th2 chemokines that alter the viral immune response from a predominant Th1 phenotype to a Th2 phenotype may originate from both airway epithelial cells as well as myeloid cells, like DCs.

In our model, infected NECs can communicate with underlying immature mono-DCs through cytokine secretion. Immature DCs develop from monocyte precursors after treatment with GM-CSF and IL-4 and only progress to maturation through two steps: 1) exposure to a combination of inflammatory cytokines from the respiratory epithelium and 2) after the acquisition of antigen with associated increases in co-stimulatory molecules that engage naïve T cells (146). Our model of infected NEC does produce inflammatory and immune activating cytokines like type I IFNs and IL-6 (53) but may not provide other signals like TNFα that are necessary to induce IL-12 production from mature DCs (147). In the case of an influenza infection, DCs are thought to process antigen through the phagocytosis of apoptotic cells (146). It is possible that in our model the cell culture membrane that separates NECs from mono-DCs impairs antigen capture by the DCs.

This could provide a possible explanation for the limited influenza-induced changes in maturation markers CD11c and CD86 and the lack of influenza-induced changes in CD40, CD209, and CD11b that we observed during influenza infection in both nonsmoker and smoker co-cultures despite drastic differences in antiviral responses. In addition, our model compared the ability of NECs from smokers vs. non-smokers to communicate with mono-DCs . It has been shown that peripheral blood mononuclear cells (PBMC) from smokers have an overall suppressed type I IFN response to poly I:C, a mimetic of double stranded viral RNA, (148). Thus, generating mono-DCs using monocytes from smokers in this co-culture model could provide information on how signals emanating from influenza-infected NEC result in different activation and maturation of mono-DCs obtained from smokers.

Nasal epithelial cells are a useful model for studying innate immune responses of the airways. During a respiratory viral infection, secretion of antiviral and immune activating mediators from the respiratory epithelium engages accessory cells (like NK cells, DCs, monocytes, etc) to induce an innate immune response (149). Mucosal epithelial cells in the nasopharynx act as a first line of defense where they must differentiate between harmless and disease causing pathogens such as influenza and will therefore set the stage for a respiratory immune response (149). Compared to bronchial cells of the lower airways, NEC have similar profiles of baseline as well as cytokine-stimulated inflammatory mediators (150). Recent genome-wide expression analyses have shown that gene expression patterns of epithelial cells from the nose and bronchial region overlap significantly at baseline and that similar smoking –induced changes are reflected in both cell types (151). In our own publications, we have shown that nasal and bronchial

epithelial cells have comparable influenza-induced IFNα, IFNβ, and IFNΩ expression albeit with differing magnitudes of response (130), further supporting the hypothesis that the smoking-induced changes we observed in NEC are likely to be present in the lower airways as well, at least qualitatively

As we discussed previously (53), NEC from smokers and non-smokers when differentiated *in vitro* appear to resemble their *in vivo* counterparts, including the persistence of high MUC5B expression overtime (53), and smoking-induced changes in phenotypes of NEC were associated with epigenetic changes in these cells. Specifically, we have previously shown that suppression of influenza-induced IRF7 expression in NEC from smokers was associated with enhanced DNA methylation of the *IRF7* gene in NEC (53) which also correlates with our findings here. In addition to *IRF7*, DNA methylation patterns of other genes are altered in airway epithelial cells from smokers, and will be further discussed in Chapter 5. Determining the effects of these epigenetic changes may elucidate mechanisms of immune suppression in smokers.

Table 1. Baseline Antiviral and Chemokine mRNA Expression in Co-Culture NECs is Similar Between Nonsmokers and Smokers.

NECs from nonsmoker and smoker NEC/mono-DC co-cultures were harvested 24 h post influenza infection. Baseline NEC mRNA expression levels for antiviral mediators and chemokines are depicted. Data are mean ± SEM and analyzed using a nonparametric Mann-Whitney U Test.

	Nonsmoker n =6	Smoker n=6	p
IRF7	19.0 ± 6.64	21.2 ± 8.79	0.937
TLR3	0.640 ± 0.293	0.138 ± 0.009	0.093
RIG-I	0.845 ± 0.251	1.39 ± 0.439	0.240

	Nonsmoker n=5	Smoker n=5	p
IP-10	1.40 ± 0.669	1.70 ± 0.761	0.841
RANTES	0.932 ± 0.393	0.576 ± 0.236	0.691
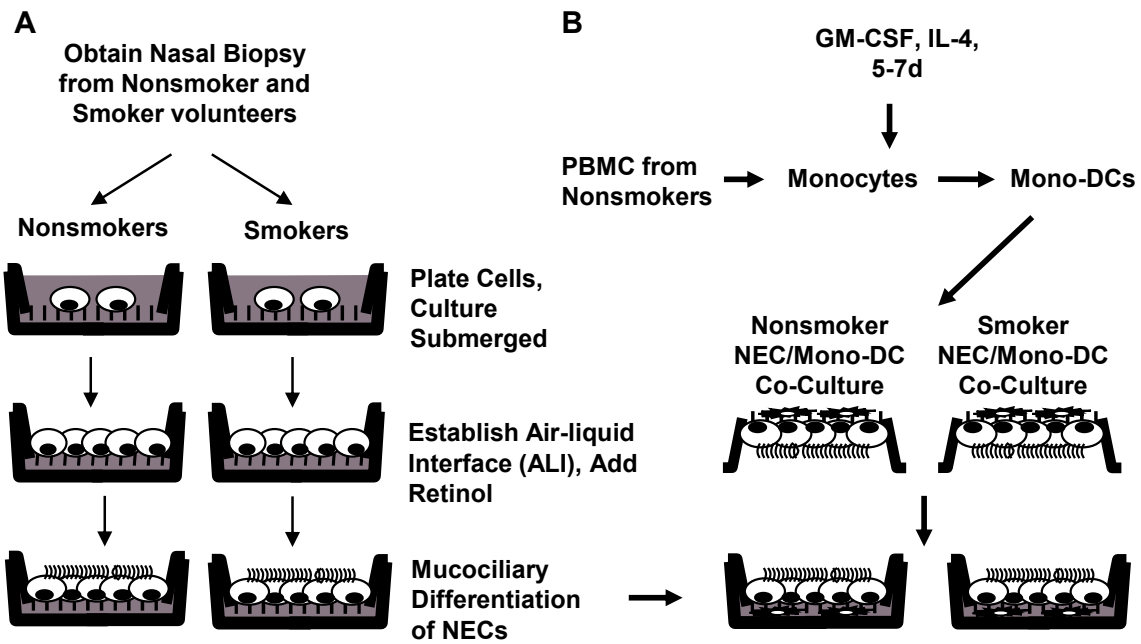
Figure 4. Development of NEC/Mono-DC Co-Culture Model.

A) NEC were obtained from healthy nonsmoker and smoker human volunteers and plated on cell culture inserts. After cells become confluent, an air liquid interface was established and retinol was added to promote mucociliary differentiation. NEC were fully differentiated after 3-4 weeks. B) Monocytes were harvested from peripheral blood mononuclear cells (PBMC) from normal, non-asthmatic, nonsmokers. Monocytes were treated with 30 ng/ml GM-CSF and 30 ng/ml IL-4 for 5-7 days. Mono-DCs were harvested and applied to the basolateral side of differentiated NEC grown on membrane support for 2 hours. Non-adherent mono-DCs were removed, and the NEC/mono-DC co-cultures were placed in basolateral media.
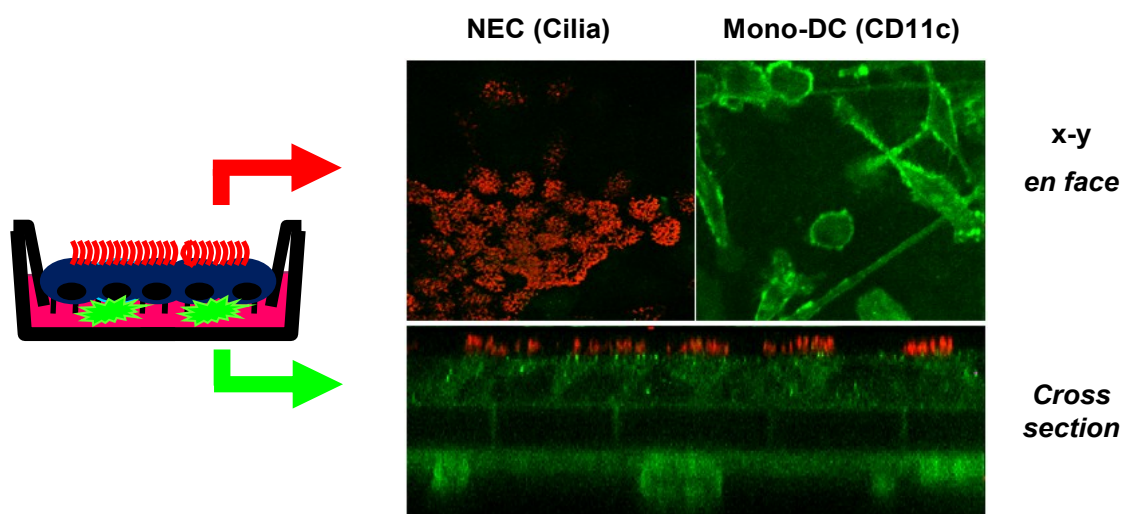
Figure 5. Visualization of the NEC/Mono-DC Co-Culture Model.

Co-culture membranes were fixed in ice cold methanol for 20 minutes and stored in 70% ethanol until analysis. Antibodies to CD11c and acetylated α-tubulin were used to identify mono-DCs and cilia of NEC, respectively. Following incubation with secondary Alexa 596 (NEC, red) and Alexa 488 (mono-DCs, green) conjugated antibodies samples were visualized using confocal microscopy. Z-stack analyses were used to visualize cross-sections of the co-culture system.
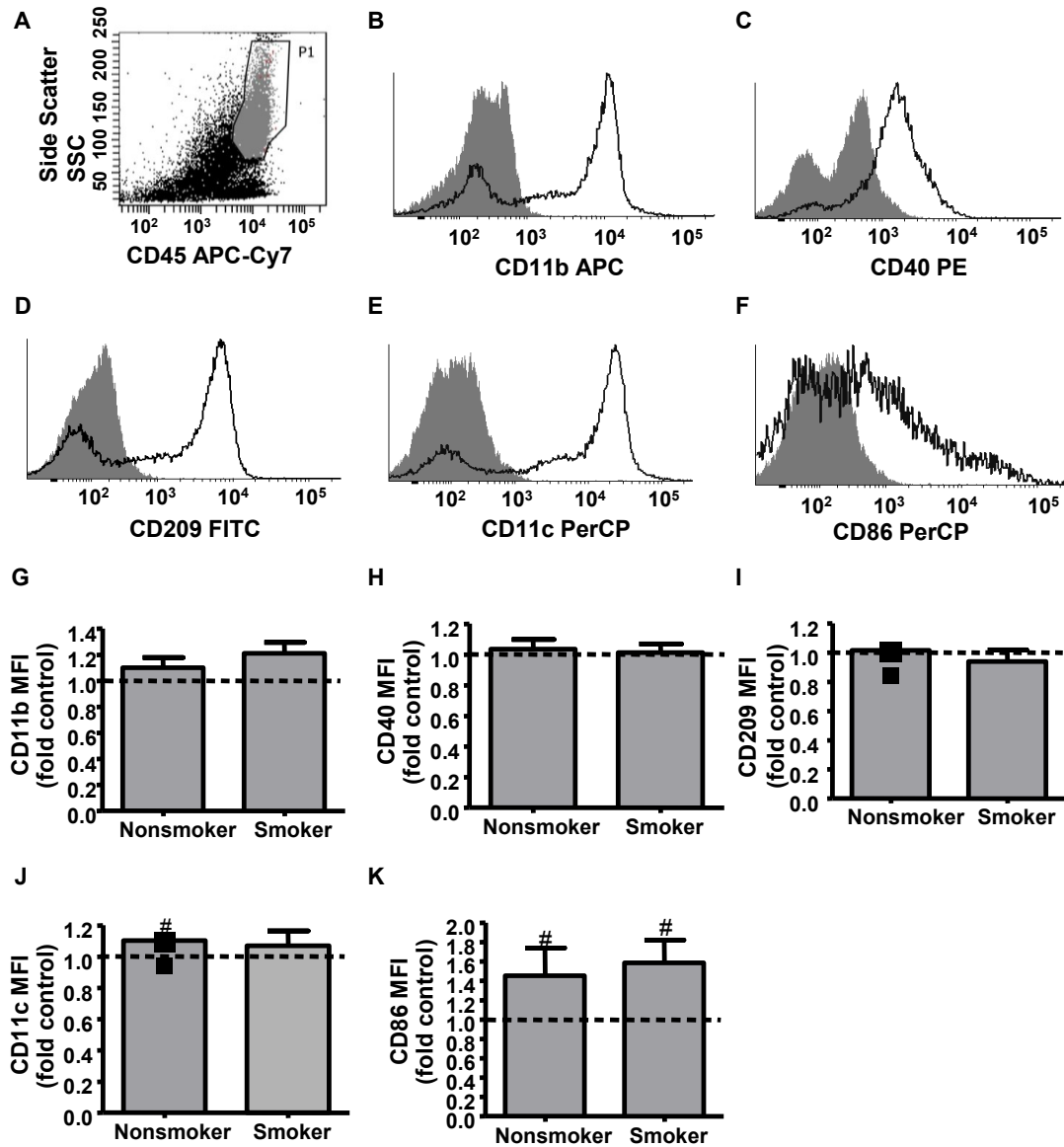
Figure 6. Mono-DCs Co-Cultured With Either Nonsmoker or Smoker NEC Have Similar Influenza-Induced Changes in DC Maturation Markers.

Mono-DCs were isolated from peripheral blood monocytes and applied to the basolateral side of differentiated NEC from either nonsmoker or smoker for 2 h followed by infection with influenza from the apical compartment. 24 h post infection, mono-DCs were harvested from co-cultures by mechanical disruption and stained for DC markers using flow cytometry. A) CD45+ leukocytes are identified in the P1 gate. Representative histogram plots showing expression of DC markers B) CD11b C) CD40, D) CD209, E) CD11c, and F) CD86 and DC marker response to influenza (fold induction) G) CD11b, H) CD40, I) CD209, J) CD11c, K) CD86 are shown. CD11b, CD40, CD209, CD11c nonsmoker n=6, smoker CD86 nonsmoker n=7, smoker n=6. Data are expressed as fold

induction over non-infected control and as mean ± SEM. # p<0.05 vs non-infected control.
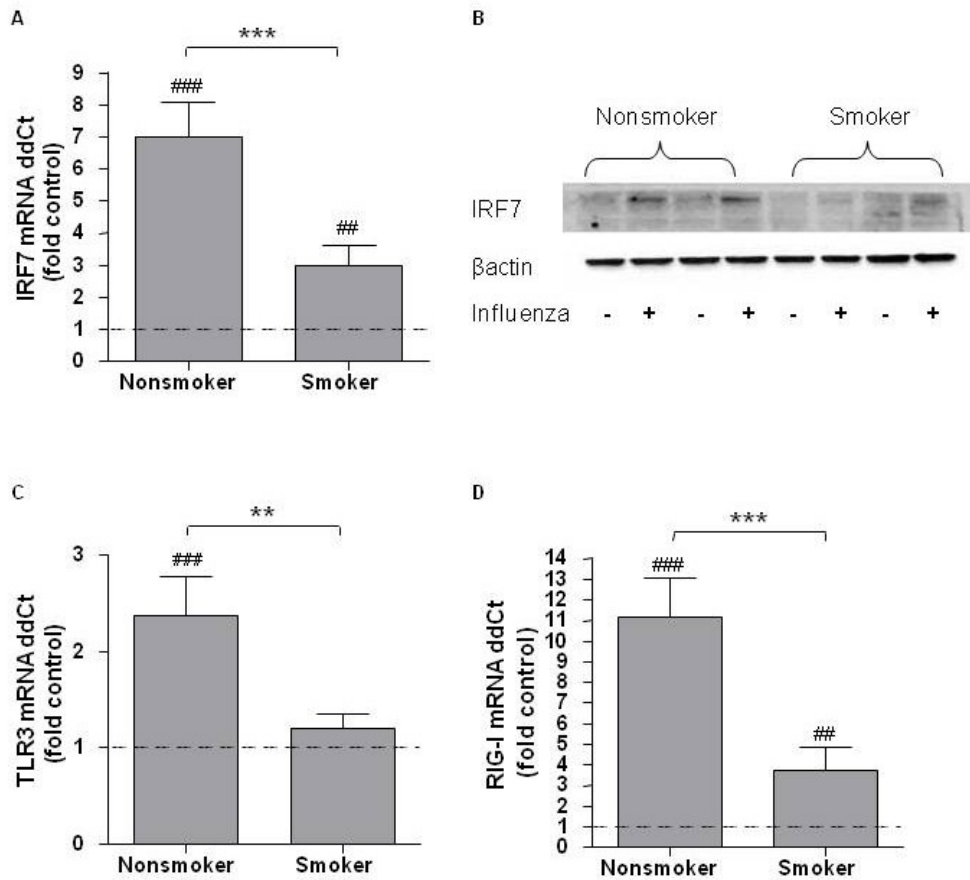
Figure 7. NEC From Smokers Have Suppressed Influenza-Induced Antiviral Responses.

NEC total RNA and whole cell lysates from nonsmoker and smoker NEC/mono-DC co-cultures were collected 24 h post influenza infection. A) Real time qRT-PCR was performed for IRF7. B) NEC whole cell lysates were analyzed by western blotting for IRF7 and then stripped and probed for βactin. Real time qRT-PCR was performed for C) TLR3 and D) RIG-I. mRNA expression of targets was normalized to β-actin, quantified using the ΔΔ Ct method, and expressed as fold induction over non-infected control. mRNA data are expressed as mean ± SEM: nonsmoker n=6, smoker n=6. For western blots, representative immunoblots are shown: nonsmoker n=2, smoker n=2. ##p<0.01, ###p< 0.001 vs non-infected control, **p<0.01, ***p<0.001 nonsmoker vs smoker.
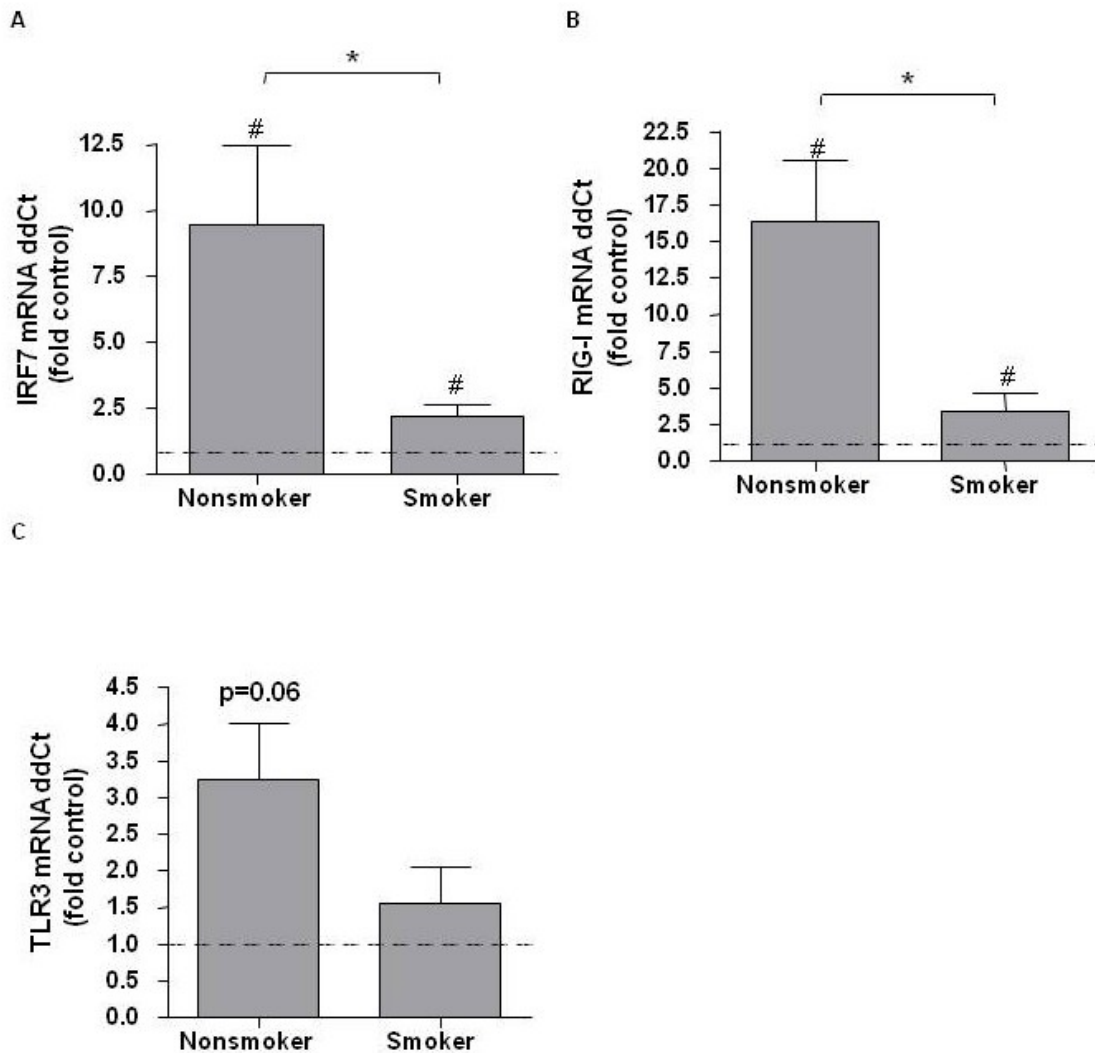
Figure 8. Mono-DCs Co-Cultured With Smoker NEC Have Suppressed Influenza-Induced Antiviral Responses.

RNA isolated from co-culture mono-DCs was collected 24 h post influenza infection. Real time qRT-PCR was performed for A) IRF7, B) RIG-I, and C) TLR3. mRNA expression of targets was normalized to β-actin, quantified using the ΔΔ Ct method, and expressed as fold induction over non-infected control. Data are expressed as mean ± SEM. Nonsmoker n=5, smoker n=5. #p<0.05 vs non-infected control, *p<0.05 nonsmoker vs smoker.
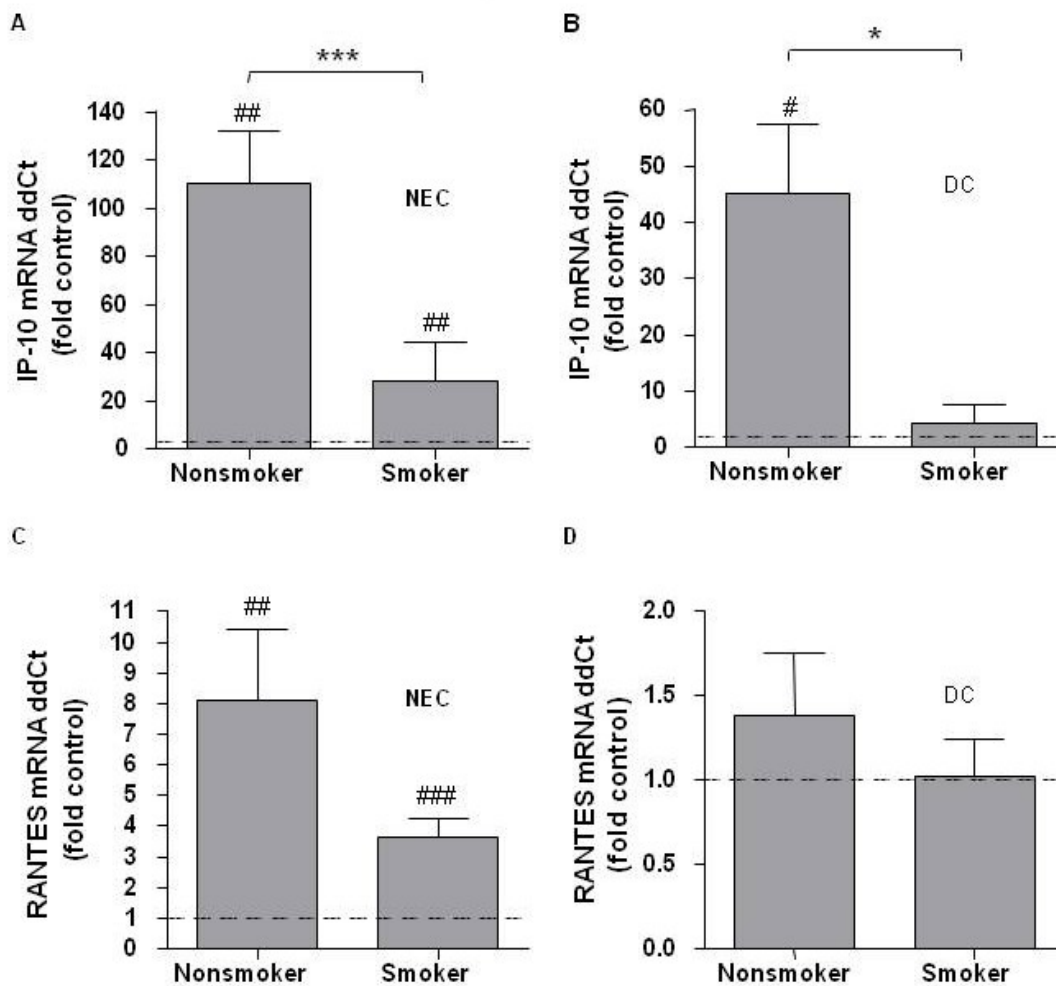
Figure 9. NEC From Smokers As Well As Mono-DCs Derived From Co-Cultures Using Smoker NEC Have Suppressed Influenza-Induced Th1 Responses.

NEC and mono-DC total RNA from nonsmoker and smoker NEC/mono-DC co-cultures was collected 24 h post influenza infection. Real time qRT-PCR was performed for IP-10 in A) NEC and B) mono-DCs. Real time qRT-PCR was also performed for RANTES in C) NEC and D) mono-DCs. mRNA expression of targets was normalized to β-actin, quantified using the ΔΔCt method, and expressed as fold induction over non-infected control. Data are expressed as mean ± SEM. Nonsmoker n=5, smoker n=5. #$p<0.05$, ##$p<0.01$, ###$p<0.001$ vs non-infected control, *$p<0.05$, ***$p<0.001$ nonsmoker vs smoker.
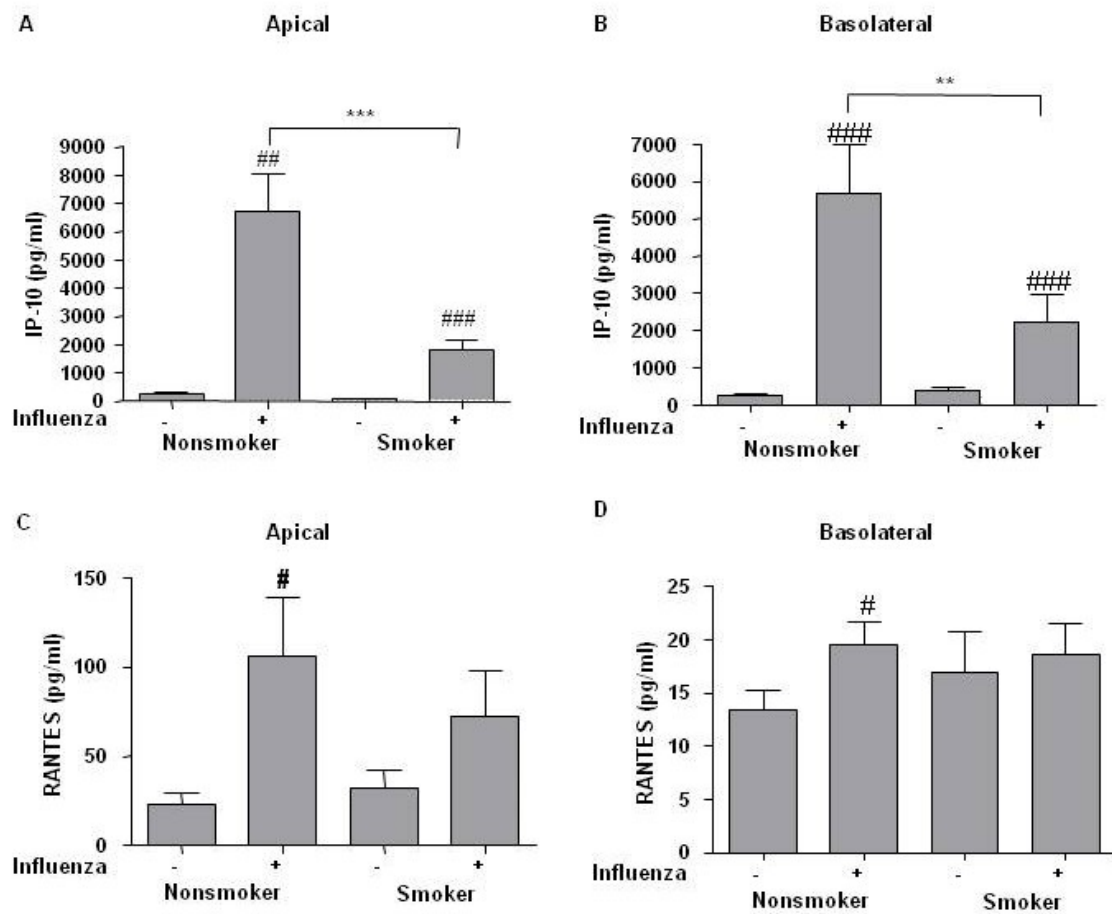
Figure 10. NEC/Mono-DC Co-Culture Apical Washes and Basolateral Supernatants Using NEC From Smokers Have Suppressed Influenza-Induced Th1 Chemokines.

NEC/mono-DC co-cultures using NECs from nonsmokers and smokers were harvested 24 h post influenza infection. IP-10 protein levels in A) apical washes (nonsmoker n=8, smoker n=7) and B) basolateral supernatants (nonsmoker n=5, smoker =7) and RANTES protein levels in C) apical washes (nonsmoker n=8, smoker n=7) and D) basolateral supernatants (nonsmoker n=9, smoker n=7) were measured by ELISA. #p<0.05, ##p<0.01, ###p<0.001 vs non-infected control, *p<0.05, ***p<0.001 nonsmoker vs smoker.
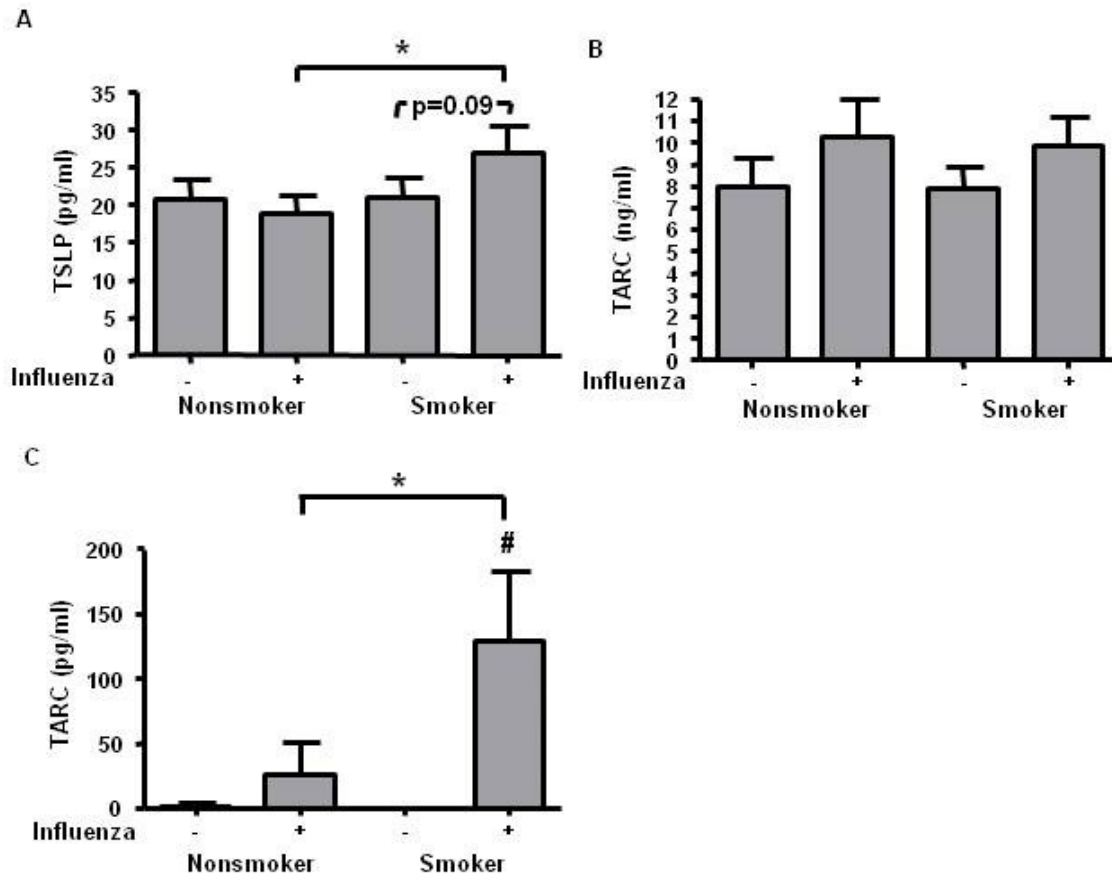
Figure 11. NEC/Mono-DC Co-Culture Supernatants Using NEC from Smokers Have Increased Influenza Induced Levels of the Th2 Chemokine TSLP.

Basolateral supernatants from NEC/mono-DC co-cultures were collected 24 h post influenza infection and were analyzed via ELISA for A) TSLP nonsmoker n=9, smoker n=8, and B) TARC nonsmoker n=10, smoker n=8. Data are expressed as fold induction over non-infected control and as mean ± SEM. #p<0.05 vs non-infected control, *p<0.05 nonsmoker vs smoker. C) Basolateral supernatants from NEC cultured alone were collected 24 h post influenza infection and were analyzed for TARC levels via ELISA. Data are expressed as mean ± SEM. Nonsmoker n=7, Smoker n=5. #p<0.05 vs non-infected control.

CHAPTER 3

NASAL NATURAL KILLER CELL FUNCTION IS SUPPRESSED IN SMOKERS

AFTER LIVE ATTENUATED INFLUENZA VIRUS

Abstract

Background: Modified function of immune cells in nasal secretions may play a role in the

enhanced susceptibility to respiratory viruses that is seen in smokers. Innate immune

cells in nasal secretions have largely been characterized by cellular differentials using

morphologic criteria alone, which have successfully indentified neutrophils as a

significant cell population within nasal lavage fluid (NLF) cells. However, flow

cytometry may be a superior method to fully characterize NLF immune cells. We

therefore characterized immune cells in NLF by flow cytometry, determined the effects

of live attenuated influenza virus (LAIV) on immune NLF cell function, and compared

responses in samples obtained from smokers and nonsmokers.

Methods: In a prospective observational study, we characterized immune cells in NLF of

nonsmokers at baseline using flow cytometry and immunohistochemistry. Nonsmokers

and smokers were inoculated with LAIV on day 0 and serial nasal lavages were collected

on days 1-4 and day 9 post-LAIV. LAIV induced changes of NLF cells were

characterized using flow cytometry. Cell-free NLF was analyzed for immune mediators

by ELISA and bioassay.

Results: CD45(+)CD56(-)CD16(+) neutrophils and CD45(+)CD56(+) natural killer (NK) cells comprised median 4.62% (range 0.33-14.52) and 23.27% (18.29-33.97), respectively, of non-squamous NLF cells in nonsmokers at baseline. LAIV did not induce changes in total NK cell or neutrophil percentages in either nonsmokers or smokers. However, following LAIV inoculation, cytotoxic CD16(+) NK cell percentages and granzyme B levels increased in nonsmokers, and these effects were suppressed in smokers. Smokers also showed decreased levels of NK cell chemokine thymus and activation-regulated chemokine (TARC) in NLF. LAIV induced expression of activating receptor NKG2D and chemokine receptor CXCR3 in peripheral blood NK cells from both nonsmoker and smoker *in vitro* but did not induce changes in cytotoxic CD16(+) NK cells or granzyme B activity in either group.

Conclusions: These data are the first to identify NK cells as a major immune cell type in the NLF cell population and demonstrate that mucosal NK cell cytotoxic function is suppressed in smokers following LAIV. Altered NK cell function in smokers suggests a potential mechanism that may mediate a heightened susceptibility to respiratory viruses.

Introduction

The nasal mucosa is the first site within the respiratory system to be exposed to pollutants and inhaled viral pathogens, including influenza. Therefore, nasal immune cells are likely to play important roles in early innate immune responses to these environmental stimuli. While macrophages and DCs have been identified in the nasal submucosa (152), and neutrophils have been identified in the nasal cavity (3), the overall immune cell populations within the nasal cavity have not been fully characterized. To phenotype nasal lavage fluid (NLF) cells, many researchers use cell differential analysis of cytocentrifuge slides stained with hematoxylin and eosin (H&E). Granulocytes are the easiest leukocytes to identify with H&E staining due to their polymorphic nuclei and are distinguished based on cytoplasmic staining: neutrophils have pale cytoplasm, eosinophils have a red granular cytoplasm, and basophils have a purple granular cytoplasm (153). T or B lymphocytes are smaller cells with dark, dense nuclei and little cytoplasm (153). NK cells are larger lymphocytes with a pale cytoplasm and are difficult to distinguish due to a lack of specific cellular morphology. In fact, NK cells appear similar to macrophages or monocytes after H&E staining (153). As a result, neutrophils, basophils, and eosinophils, but not NK cells, have been identified in NLF using cell differentials with H&E staining (154-156).

As an alternative to H&E staining, flow cytometry can be used to positively identify leukocytes in NLF. Flow cytometry has previously identified neutrophils in the NLF using CD16 expression (4; 157) but expression of CD56, the classical NK cell marker, has not been used to positively identify NK cells in NLF. However, flow cytometric analysis has positively identified CD56(+) NK cells as well as CD3(+) T

lymphocytes and HLA-DR(+) alveolar macrophages in the bronchoalveolar lavage of lung transplant recipients (158). Thus, NK cells have been identified in the airways of humans, (5) but whether NK cells are present in the nasal cavity and how they could function as a guard against inhaled pollutants or pathogens is not known.

Influenza infection induces the recruitment of immune cells into the lung, including NK cells (5). NK cells perform essential functions such as killing virus infected epithelial cells and secreting cytokines to regulate innate and adaptive immune responses (159). CD16, an FC receptor that induces antibody-dependent cell mediated cytotoxicity (160), is classic marker identifying neutrophils (161) but is also expressed on cytotoxic NK cells (159). CD16(+) cytotoxic NK cells also have dim CD56 expression and release cytotoxic granules containing perforin and granzymes to induce apoptosis in influenza infected cells (162). In contrast, CD16(-) NK cells have bright CD56 expression and are chiefly considered "cytokine secreting" NK cells as they secrete IFNγ that matures dendritic cells (DCs) (162).

NK cell activation during influenza infection is regulated by the microenvironment. In particular, the respiratory mucosa secretes RANTES and IP-10(19; 21) which bind to chemokine receptors CCR5 and CXCR3, respectively, on NK cells. In addition to IP-10 and RANTES, activated NK cells can secrete and respond to chemokines such as TARC and monocyte derived chemokine (MDC) (21). While multiple cell types produce these mediators, through secretion of these cytokines, NK cells engage in a positive feedback loop that enhances the NK cell pool (163). NK cells can also be activated by receptor mediated interactions. NK cells express many activating receptors, including NKG2D, which recognizes ligands induced during cellular stress. As

such, binding of ligands to the NKG2D receptor on NK cells enhances NK cell cytokine production as well as cytotoxic activity (19). In this manner, influenza infection in the respiratory tract may activate NK cells either through modification of activating surface and chemokine receptors or enrichment of the NK cell cytokine microenvironment. However, the role of NK cells in antiviral responses to influenza infection within the nasal passages has yet to be determined.

Airborne pollutants, such as CS, have been shown to increase susceptibility to respiratory viral infections, including influenza [18-20]. We have recently demonstrated that smokers' nasal epithelial cells have modified responses to influenza infections both *in vivo* (3) and *ex vivo* (53) resulting in increased markers of influenza infection. Because NK cells can control and regulate viral infections via killing of infected respiratory epithelial cells, altered NK cell functions in smokers could contribute to enhanced influenza infections. Although smoking has been shown to suppress peripheral NK cell activity *ex vivo* (75-77; 79; 164), the effects of smoking on respiratory NK cell functions are unknown. [24]

Our goals were to 1) phenotype immune cells in the nasal passages using flow cytometry, 2) determine the presence and function of these cells in the context of a viral infection, and 3) assess the effects of cigarette smoke exposure on nasal immune cell function. Nonsmokers and smokers were inoculated with the live attenuated influenza virus (LAIV) vaccine, similar to our previous study (3). Serial nasal lavages were used to compare immune cell, and specifically NK cell, function in the nasal cavity of smokers and nonsmokers. Our results show that NK cells are present in the NLF, NK cells in the NLF change after inoculation with LAIV, and NK cell responses are modified in smokers

following LAIV.

Methods

Effect of LAIV on Nasal Immune Responses: Study Design

This was a prospective longitudinal study comparing responses to LAIV between

cohorts of healthy young adult smokers and nonsmokers. The study design was as

described before (3). Baseline measurements were done at a screening visit and Day 0.

On Day 0 subjects received a standard nasal inoculum of the 2008-2009 formulation of

LAIV (FluMist®, MedImmune, Gaithersburg, MD; administered by study nurse

according to manufacturer's instructions) in both nostrils, then returned on Days 1, 2, 3, 4,

and 9 post-LAIV for serial nasal lavages. Subject exposure history questionnaires and

urine cotinine levels were used to estimate cigarette smoke exposure.

Study Subjects

Subjects were identified as described before (3) and included healthy young

adults between 18-35 years old in two groups: Group 1 = nonsmokers not regularly

exposed to secondhand smoke and Group 2 = self-described active cigarette smokers.

Informed consent was obtained from all subjects and the protocol was approved by the

UNC Biomedical Institutional Review Board. Exclusion criteria were as described before

(3). Table 2 details demographic and smoke exposure characteristics of the subjects

completing the study. Nonsmokers and smokers did not differ significantly for age, BMI,

or gender. One of 14 enrolled nonsmoker subjects and 5 of 20 enrolled smoker subjects

dropped out before completion of the study. Two smoker nasal lavage fluid sample sets

were compromised by freezer malfunction and therefore were not included in the

analysis. Self described smokers had significantly higher secondhand smoke exposure

and urine cotinine values compared to nonsmokers. No serious adverse events occurred among subjects completing the protocol. Informed consent was obtained from all subjects and the protocol was approved by the UNC Biomedical Institutional Review Board.

Nasal Lavage

Nasal lavage was performed using a method we have previously described (3; 165). In brief, 4 ml of saline was sprayed into each nostril in 100 ul repetitive sprays followed by periodic forceful expulsion of fluid into a collection cup. Fluid from both nostrils was pooled. The NLF was filtered using 40 μm cell strainer (BDBiosciences, San Jose, CA), and the NLF filtrate was pelleted by centrifugation. Cell-free NLF was stored in aliquots at -80°C until used in mediator assays. Contents of the cell strainer were treated with 5% dithiothreitol (DTT) solution (Sputolysin®, EMD Chemicals, Gibbstown, NJ). Filtered cells were combined with DTT treated cells to comprise the total NLF cell pellet. Cytocentrifuge slides were stained for differential cell counts and immunohistochemistry as described below The NLF cell pellet was processed for flow cytometry as described below to identify and quantify immune cells.

NLF Cell Differentials and Immunohistochemistry

Cytocentrifuge slides were prepared, fixed, and stained using a modified Wright stain for differential cell counts. For immunohistochemistry, cytocentrifuge slides were fixed with ice-cold methanol, washed with TBS and blocked with Powerblock (Biogenex, San Ramon, CA) for 1 hr at room temperature. Cells were then incubated with the following primary antibodies: mouse anti-human CD56 antibody (MAB24081 RnD, Minneapolis, MN) or mouse anti-human perforin (BD Biosciences) overnight at 4°C. The

slides were washed with TBS. Following incubation with an HRP (horse radish peroxidase)-conjugated secondary antibody, samples were washed with TBS and evaluated under light microscopy. Nonspecific staining was assessed by omitting the target-specific primary antibody.

Ex vivo NLF Cell Flow cytometry

The NLF cell pellet was suspended in flow cytometry buffer (PBS, 0.09% sodium azide, 1% heat inactivated FBS) and stained with antibodies to CD16 FITC (Beckman Coulter, Brea, CA), CD14 Pacific Blue (Biolegend, San Diego, CA) CD56 PE, CD4 Pe-Cy5, CD3 PerCP, CD45 APC-Cy7 (BD Biosciences) for 20 minutes at room temperature in the dark. Cells were washed with flow cytometry buffer, resuspended in 0.5% paraformaldehyde, and stored at 4°C in the dark. Samples were acquired within 24 hrs on a BDLSRII flow cytometer (BD Biosciences). Isotype-matched single color controls were used to control for nonspecific staining and to set analysis gates.

NLF Mediator and Urine Cotinine Assays.

NLF granzyme B activity was measured using a SensiZyme Granzyme B Activity kit (Sigma, St. Louis, MO). RANTES was quantified using commercially available ELISA (R&D, Minneapolis, MN) according to manufacturer's instructions. MDC, MCP-1, and TARC were quantified using a 9 assay multiplex ELISA platform (Meso Scale Discovery, Gaithersburg, MD). Urine cotinine was measured by ELISA (Bio-Quant, Inc., San Diego, CA) and expressed as a ratio to creatinine, measured by a colorimetric assay (Oxford Biomedical Research, Rochester Hills, MI).

In vitro NK Cell Stimulation Assays

Peripheral blood NK cells were isolated from PBMC from nonsmokers and smokers at baseline and stimulated *in vitro* with the 2008-2009 strain of LAIV. PBMC were isolated from nonsmokers and smokers using Lymphoprep™ (Axis-Shield, Oslo, Norway). NK cells were isolated from PBMC using negative selection for CD56 (Dynabeads® Untouched™ Human NK cells isolation kit, Invitrogen, Carlsbad, CA) according to manufacturer's instructions. $1x10^5$ NK cells were stimulated with 0.1 ul (2.56 HAU) LAIV (see below) in 100 ul of RPMI media containing 10% FBS, l-glutamine (Invitrogen), and penicillin:streptomycin (Invitrogen) for 24 h at 32°C in 5% CO_2. NK cells were centrifuged at 500 g for 5 min and processed for flow cytometry as described below. Cell free supernatant was aliquoted and stored at -80°C for assessment of granzyme B activity as described below.

LAIV Propagation in MDCK Cells in vitro

The 2008-2009 LAIV strain was propagated in Madin-Darby Canine Kidney (MDCK) epithelial cells *in vitro*. 0.05 MOI LAIV stimulated 90% confluent MDCK cells in serum free DMEM media supplemented with pen-strep, l-glutamine and 0.2% trypsin without EDTA (all Invitrogen) and incubated for 48 h at 32°C in 5% CO_2. The cell supernatant was gently aspirated and combined with 10% fetal bovine serum (Invitrogen) to inactivate trypsin. Cells and debris were pelleted by centrifugation at 500 g for 10 minutes. Cell free supernatant was concentrated by centrifugation with an Amicon Ultra-15 Centrifugal Unit (Millipore, Billerica, MA) using a 100,000 molecular weight cutoff. Smaller proteins (cytokines) fall through the filter whereas larger viruses (LAIV) are collected. To generate a vehicle control, MDCK cells were mock-infected with media and the supernatant was processed in the same fashion. Concentrated LAIV was aliquoted

and stored at -80°C until use. LAIV was titered using a hemagglutination assay as described before (166). The titer for the propagated virus was 25.6 HAU (hemagglutinin units)/ul, which was 8x higher than the original 2008-2009 LAIV strain (data not shown). MDCK-propagated LAIV was used in all *in vitro* assays.

Peripheral NK Cell Flow Cytometry

NK cells were washed and resuspended in flow cytometry buffer and stained with antibodies to CD16 FITC (Beckman Coulter), CD56 PE, CD3 APC-Cy7 (BD Biosciences), CXCR3 and NKG2D (Biolegend) for 20 minutes at room temperature. Cells were washed with flow cytometry buffer, resuspended in 0.5% paraformaldehyde, and stored at 4 C in the dark. Samples were acquired within 24 hrs on a BDLSRII flow cytometer (BD Biosciences).

Peripheral NK Cell Granzyme B Activity

Granzyme B activity from the *in vitro* LAIV NK cell stimulation was quantified as described before (167). Briefly, NK cell supernatants were combined 1:1 with 50 mM HEPES pH 7.5, 0.1% CHAPS, 10% sucrose, (all Sigma) and 400 uM colorimetric granzyme B substrate I (EMD4Biosciences, Merck, Darmstadt, Germany). Supernatants were incubated at 37°C in 5% CO_2 for 24 h. Absorbance was read using a plate reader at 405 nm wavelength. A standard curve of granzyme B (Sigma) with 1:1 serial dilutions was used to calculate specific activity.

Statistical Analysis

Baseline differences between NLF cell populations were determined using a Mann-Whitney U test. The effects of smoking status on NLF responses to LAIV were analyzed using a Kruskal-Wallis One-way ANOVA followed by Bonferroni's posttest to determine differences on individual days. An area under the curve (AUC) analysis followed by a Kruskal-Wallis One-way ANOVA was used as previously described (3) to determine the effects of smoking status on total immune NLF responses to LAIV. Within nonsmoker and smoker groups, Wilcoxon matched pairs tests were used to determine the effects of LAIV on peripheral NK cells *in vitro*. Data were shown as mean ± SEM or median (interquartile range).

Results

Characterizing Immune Cells in NLF In Nonsmokers At Baseline

A representative cellular differential of NLF cells was pictured in Figure 12. Squamous cells (black arrows) in the nasal lavage comprised 52.0% (37.3-62.5) of total NLF cells in nonsmokers at baseline. Neutrophils (dashed arrows) could also be identified by morphology in the nasal lavage and comprise 7.7% (2.1-16.5) of non-squamous NLF cells. We used flow cytometry to positively identify other non-squamous NLF cells. As shown in Figure 13, forward scatter (FSC) and side scatter (SSC) settings eliminated squamous epithelial cells from the view. Negative expression of CD45, a marker that stains only leukocytes, was used to discriminate between NLF non-squamous epithelial cells and NLF leukocytes of similar size. Figure 13 showed further analysis of CD45(+) NLF cells stained for surface marker expression of CD3 (T lymphocytes), CD4 (T helper lymphocyte) CD14 (monocytes), CD16 (neutrophils), and CD56 (NK cells). These data showed that of the markers tested, only subpopulations positive for CD16 and CD56 were identified in CD45$^+$ NLF cells.

To further characterize the immune phenotype of NLF cells, we focused our analysis on CD16 and CD56. In Figure 14A, a representative flow cytometric dot plot showed CD16 and CD56 co-expression on CD45(+) cells. Distinct CD56(+) NK cell (upper left quadrant) and CD56(-)CD16(+) neutrophil (lower right quadrant) populations were depicted. Also, CD16(-) NK cells appeared to have "brighter" CD56 expression compared to their CD56(+)CD16(+) NK cell counterparts. Figure 14B indicated that NK cells comprised a greater percentage [23.3% (18.3-34.0)] compared to neutrophils [4.6% (0.3-14.5)] of non-squamous NLF cells (p<0.01). Percentages of cytokine-secreting

CD16(-) NK cells [13.0% (10.4-20.3)] were greater than percentages of cytotoxic CD16(+) NK cells [8.8% (3.6-12.7)] in nonsmokers at baseline as shown in Figure 14C (p<0.05). None of the CD45(+) leukocytes in the nasal lavage were CD3(+), indicating that the CD56(+) cells were not natural killer T (NKT) cells.

We used immunohistochemistry to confirm the presence of NK cells in the NLF. Positive immunohistochemical staining for CD56 identified NK cells in Figure 15A. Cytotoxic NK cells were identified by immunohistochemical staining for perforin, a cytotoxic granule expressed in cytotoxic T lymphocytes and NK cells, as shown in Figure 15B.

Comparison of Nonsmokers vs. Smokers After LAIV

Smoker and nonsmoker groups did not differ by age, BMI, or gender (Table 2). As expected, smokers had significantly greater average urine cotinine levels than nonsmokers, indicating that the smokers continued to actively smoke during the study period.

Using flow cytometry, percentages of NK cells and neutrophils were quantified in the non- squamous NLF cell population of nonsmokers and smokers before (Day 0) and after inoculation with LAIV (Days 1-4, 9). See schematic in Figure 16. There were no statistically significant differences in neutrophil percentages associated with either LAIV or smoking status (see Figure 17A), which confirms our previous observations (3). Total NK cell percentages were also similar between groups and unchanged after LAIV (Figure 17B). To further characterize NK phenotypes after LAIV, proportions of cytotoxic NK cells within the total NK cell population were determined by assessing CD16(+) expression on NK cells using flow cytometry and cytotoxic NK cell activity was

determined by measuring granzyme B bioactivity in the NLF. CD16(+) NK cells

increased by day 2 after LAIV in nonsmokers, but this increase was significantly blunted

in smokers (Fig. 18A). Similarly, the rise in granzyme B seen in nonsmokers by day 3

was significantly suppressed in smokers (Figure 18B); as well as an overall suppression

of granzyme B response post LAIV in smokers, albeit not statistically significant

(p=0.09, Table 3).

Chemokines known to enhance NK cell function were quantified in the NLF.

RANTES and MCP-1 levels were similar in both nonsmokers and smokers following

LAIV inoculation (Figures 19A and 19B). MDC (Figure 19C) appeared to be suppressed

in smoker NLF at baseline, although this effect was not statistically significant. TARC

levels were suppressed in smoker NLF following LAIV both at Day 2 (Figures 19D) and

overall following LAIV (Table 3, AUC analysis). Overall AUC analysis determined

RANTES, MCP-1, and MDC levels were not affected by smoking status following LAIV

(data not shown).

To determine whether the effects of cigarette smoke exposure on NK cell function

are evident systemically, peripheral blood NK cells were isolated from nonsmokers and

smokers and stimulated *ex vivo* with LAIV. Percentages of peripheral cytotoxic CD16(+)

NK cells were not altered by *ex vivo* stimulation with LAIV in either nonsmokers or

smokers (Figure 20A). Interestingly, peripheral blood cytotoxic CD16(+) NK cells

composed a larger proportion of total NK cells [84.2% (79.3-90.4)] (Figure 20A) versus

mucosal NK cell populations [28.3% (22.2-46.2)] in nonsmokers at baseline (Figure 18A)

(p<0.0001). LAIV did not induce granzyme B secretion in peripheral NK cells from

smokers or nonsmokers (Figure 20B). To determine if LAIV stimulation *ex vivo* can alter

expression of peripheral NK cell activating and chemokine receptors (NKG2D and CXCR3), we assessed receptor expression by flow cytometry. LAIV increased NKG2D (Figure 20C) and CXCR3 (Figure 20D) expression on peripheral NK cells from both nonsmokers and smokers. However, there were no differences in peripheral NK cell receptor expression between smokers and nonsmokers.

Discussion

Characterizing innate immune cells within the nasal passages is an important step in understanding how pre-existing conditions, such as smoking, affect anti-influenza responses in the respiratory epithelium. Using a model of *in vivo* human influenza infection (3) and *ex vivo* flow cytometric methodology, we demonstrated that 1) NK cells are present in nasal secretions and constitute a significant portion of NLF immune cells, 2) the "normal" nasal NK cell response to LAIV involves an increase in activated cytotoxic NK cells, and 3) these LAIV-induced cytotoxic NK cell responses are suppressed in smokers.

The identification of NK cells as a prominent immune cell type in NLF is, to our knowledge, a novel finding and suggests that the study of innate immune responses in the upper airways should take NK cells into account. The use of cell differentials alone to phenotype NLF cells has likely overlooked NK cells (154-156). As shown in Figure 4, NK cells have non-descript morphologies and could be mistaken for NLF monocytes, macrophages or even basal epithelial cells. Using flow cytometry, other researchers have identified CD16(+) NLF cells. However, these CD16(+) cells were either classified as neutrophils (157) or the analysis gate was based on the relative size of a lymphocyte population (4), thus likely excluding NK cells. In our study, activated NK cells in NLF appeared to be of similar size and granularity as neutrophils as evidenced by the flow cytometric CD56 staining and SSC properties (see Figure 13). This is not surprising as both NK cells and neutrophils contain cytotoxic granules that should influence their SSC fluorescence. Figure 4 shows that NK cells positively identified in the NLF using immunohistochemistry are relatively large cells compared to T lymphocytes (153) and

have significantly greater cytoplasm/nucleus ratio. In addition, CD56 is essential to positively identify NK cells. A previous report did not observe any CD56(+) NK cells in NLF (168), but these studies used a significantly different study population (allergic rhinitics) and were focused on IL-4 producing lymphocytes, not NK cells, within the NLF (168). In addition, they, as well as others (4; 155-157), perform nasal lavages by administering a single bolus dose of saline which is held in the nasal cavity and then passively expelled (168). This is in contrast to our method, which uses repetitive spraying of smaller volumes of saline followed by forceful expulsion and collection. We speculate that compared to the bolus method of NLF collection, our method may be more mechanically disruptive to the nasal mucosa and thus produces higher numbers of NLF immune cells. However, in the NLF analyzed here, neutrophils, characterized both by cellular differential and flow cytometry analysis, were at levels similar to what has been described in bolus nasal lavages of normal human subjects at baseline by cell differential analysis (169).

Our data also show that NLF NK cells are activated during an influenza infection in healthy nonsmokers. Multiple signals contribute to NK cell activation including direct engagement of NK cell activating receptors by influenza virus (170), autocrine stimulation by activated NK cell derived chemokines (21; 171), and paracrine stimulation by other immune cells like DCs (172; 173). Therefore, NK cell activation is in part dependent on communication with other cell types. We have shown here that LAIV increases cytotoxic CD16(+) NK cell percentages and granzyme B activity in the NLF of nonsmokers, but did not observe similar increases in peripheral NK cells alone. This suggests that in the setting of infection, chemokines and mediators released by other cells

within the nasal mucosa assist in activating and maturing NK cells. We have previously shown that influenza-induced IP-10 levels are reduced in the nasal epithelium of smokers both *in vivo* (3) and *ex vivo* (174). IP-10 secreted from the nasal epithelium can induce chemotaxis and enhance cytotoxic activity in resting NK cells (21), suggesting that a decrease in IP-10 levels in the context of a viral infection may lead to the suppressed NK cell cytotoxic responses demonstrated here. Tobacco smoke-induced decreases in activating cytokines such as IP-10, MDC, and TARC could create a microenvironment unfavorable for NK cell maturation resulting in suppressed CD16(+) NK cell percentages, granzyme B activity, and suppression of IFNγ in the NLF, which we have shown previously (3). Interestingly, our *ex vivo* co-culture model of influenza infection introduced in the previous chapter demonstrated that smoker NEC had enhanced secretion of TARC, as opposed to suppressed secretion, following influenza, which is in contrast to the findings discussed in this chapter. Taken together these data suggest 1) not all conclusions drawn from different model systems are analogous and 2) TARC may have differential roles depending on the target cells involved (DCs vs. NK cells) during an influenza infection.

Our data suggest that peripheral NK cell responses are not affected by smoking status. CD56^{bright} CD16(-) cytokine-secreting NK cells predominate in lymph nodes and mucosal tissues, whereas CD56^{dim} CD16(+) cytotoxic NK cells are found in higher percentages in the peripheral blood (5). Because total NK cell percentages in NLF did not change following LAIV in either nonsmokers or smokers in our study, it is possible that the cytokine milieu within the nasal mucosa induced NK cell switching from a cytokine secreting to a cytotoxic phenotype (175). We did not observe this class switching in

peripheral NK cells inoculated with LAIV, which may be due to either differences in NK cell phenotypes or the lack of maturation cytokines from exogenous immune and respiratory cells present in the nasal mucosa. In addition, while stimulation with LAIV *ex vivo* did enhance activation markers NKG2D and CXCR3 on peripheral blood NK cells in both nonsmokers and smokers, LAIV did not induce granzyme B bioactivity in peripheral NK cells *ex vivo* in either group. This suggests that peripheral and mucosal NK cell phenotypes and responses are distinct and that NK cells require a combination of signals derived from direct infection and exposure to a maturation cytokine mixture to become fully activated and secrete cytotoxic granzymes.

Smokers are prone to respiratory microbial and viral infections, including pneumococcal pneumonia, legionellosis, meningococcal disease, rhinovirus, and influenza virus (48). We have shown here that smokers have decreased NK cell activity in the nasal passage, and this lack of functional NK cells patrolling the upper airways may contribute to increased respiratory infections. Interestingly, decreased NK cell cytotoxicity may also play a role in tumorigenesis in the respiratory system (15). NK cells from smokers have decreased anti-tumor action *ex vivo* (176), and decreased peripheral lymphocyte cytotoxicity *ex vivo* is associated with increased cancer risk (177). Thus, enhancement of NK cell function against infected cells or tumors could be an important therapeutic strategy for both smokers and cancer patients. For cancer patients, several NK cell therapies are already in clinical trials (178). Adiponectin treatment of NK cells exposed to cigarette smoke *ex vivo* partially restores NK cell cytotoxicity, suggesting that adiponectin may be an intriguing candidate for NK cell enhancement (179). Thus, suppressed NK cell activity in the nasal secretions of smokers may

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contribute to the suppressed anti-viral and anti-tumor function seen in the respiratory tracts of smokers.

NK cells in the nasal secretions may play important roles in nasal immunity through control of respiratory viral infections both in normal individuals and those with underlying respiratory conditions. Viral infections and inflammation within the nasal passages could affect immune responses in the lower airways, especially in individuals with underlying lower airway disease such as chronic obstructive pulmonary disease (COPD) or asthma. COPD is also associated with nasal inflammation and blockage of the upper airways (180). In asthmatic individuals, treatment of rhinitis and sinusitis improves asthma disease symptoms (181), indicating that nasal inflammation can affect asthma symptoms. Infections with viruses such as human rhinovirus are a major cause of both COPD and asthma exacerbations and the majority of these infections originate in the nose (182). Thus, innate immune cells, particularly NK cells, could play important roles in controlling viral infections and inflammation within the nose and prevent worsening of preexisting respiratory conditions. In summary, we have demonstrated that NK cells are present in nasal secretions, and that NK cells could play an important role in nasal innate immunity to viruses, as well as in the suppressed immune responses to respiratory infection seen in smokers. Further study of this unique mucosal immune cell population will be beneficial in assessing the effects of both pollutants and pathogens on upper respiratory immune responses in individuals with potentially enhanced susceptibility due to pre-existing diseases.

Table 2. Subject Characteristics and Tobacco Smoke Exposure

	Nonsmoker (N = 13)	Smoker (N = 13)
Age (yr)	25.3 ± 1.0	23.5 ± 1.2
Gender	5M/8F	7M/6F
BMI	25.1 ± 1.3	28.2 ± 2.3
Daily exposure		
Cigarettes smoked[1]	NA	8.5 ± 1.6
Urine cotinine[2]	0.0 ± 0.0	19.5 ± 7.1***

[1] Data shown as mean ± SEM for cigarettes smoked. Data were averaged from each subject's self reported estimates for study days 0 through 9.

[2] Data shown as mean ± SEM for mg cotinine (x 100) / mg creatinine. Data were obtained using screen urine values.

*** $P < .0001$ vs. nonsmoker

Table 3. Comparison of Total NLF Granzyme B and TARC Responses Post LAIV.

	Control	Smokers	P (Kruskal-Wallis)
	(N =13)	(N = 13)	
Granzyme B	225.0	111.5	0.09
	(68.7-519.3)	(14.9-310.0)	
TARC	76.4	45.7*	0.03
	(38.9-119.4)	(28.6-95.5)	

Data are shown as median (interquartile range). Area under NLF mediator quantity, Day 1-9 after LAIV inoculation. Mediators with p >0.1 are shown. * P < 0.05 vs. Nonsmoker
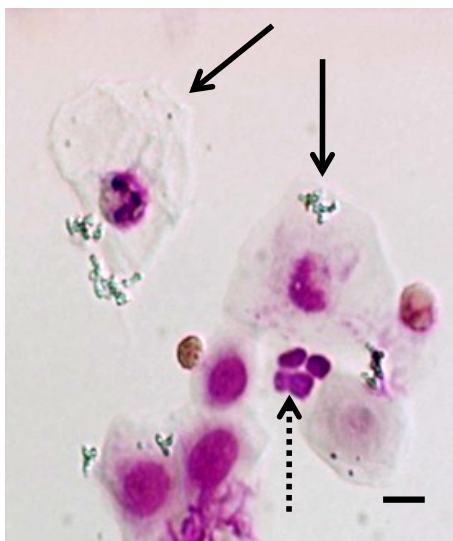
Figure 12. Nasal Lavage Cells

NLF cells from nonsmokers at baseline were stained with hematoxylin and eosin. Squamous epithelial cells (black arrows) and neutrophils (dashed arrows) can be positively identified. n=12. A representative image is shown. Bar = 10 μm.
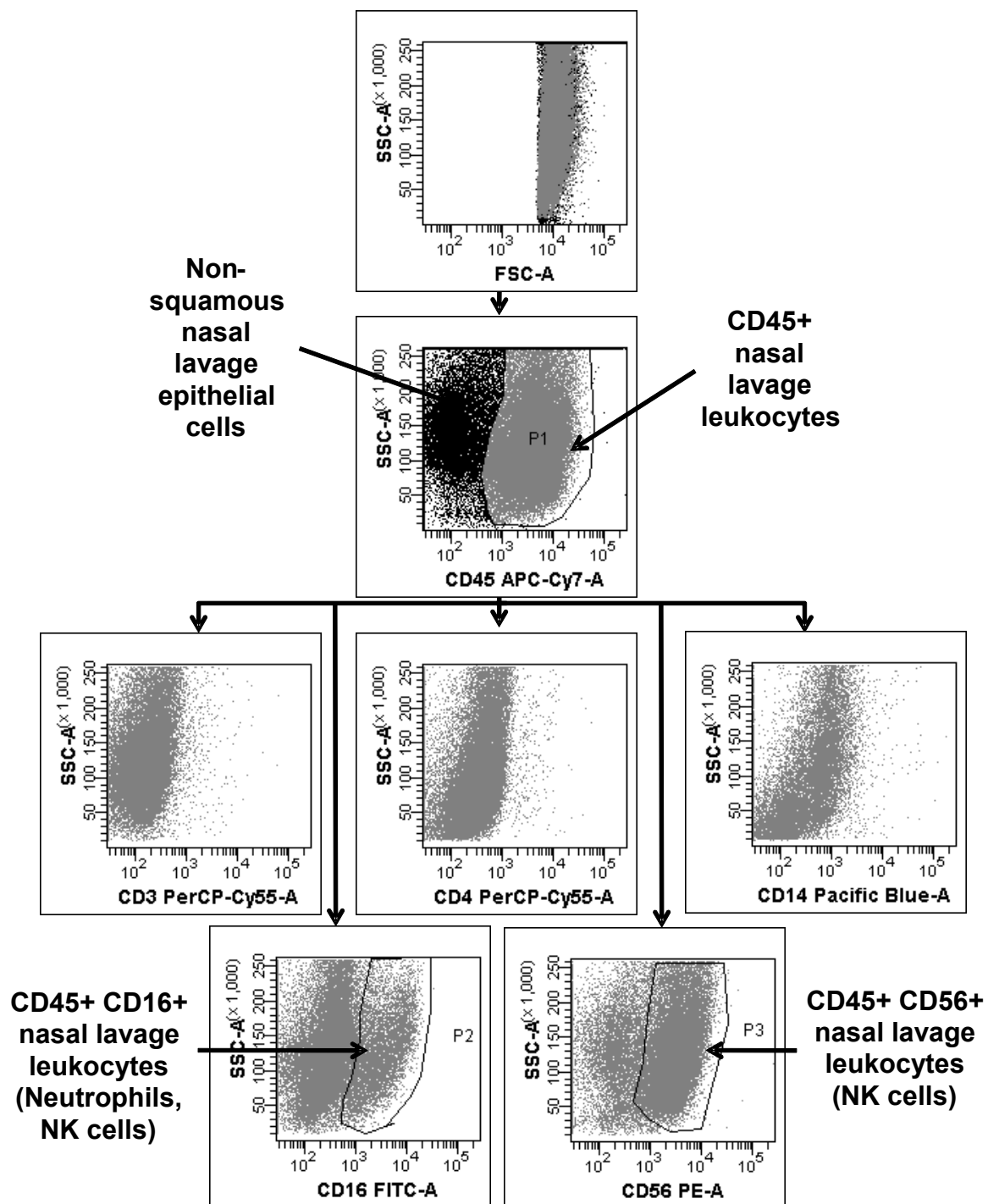
Figure 13. Nasal Lavage Immune Cell Flow Cytometry

NLF cells were collected from nonsmokers at baseline and analyzed by flow cytometry for leukocyte markers. The majority of NLF squamous epithelial cells are gated out by FSC and SSC settings. CD45+ NLF leukocytes are identified in NLF cells. CD45+ NLF cells are negative for surface markers CD3 (T lymphocytes) CD4 (Helper T lymphocytes), and CD14 (monocytes). CD45+ NLF cells contained populations positive for CD16 (neutrophils, NK cells) and CD56 (NK cells). A representative image at baseline is shown.
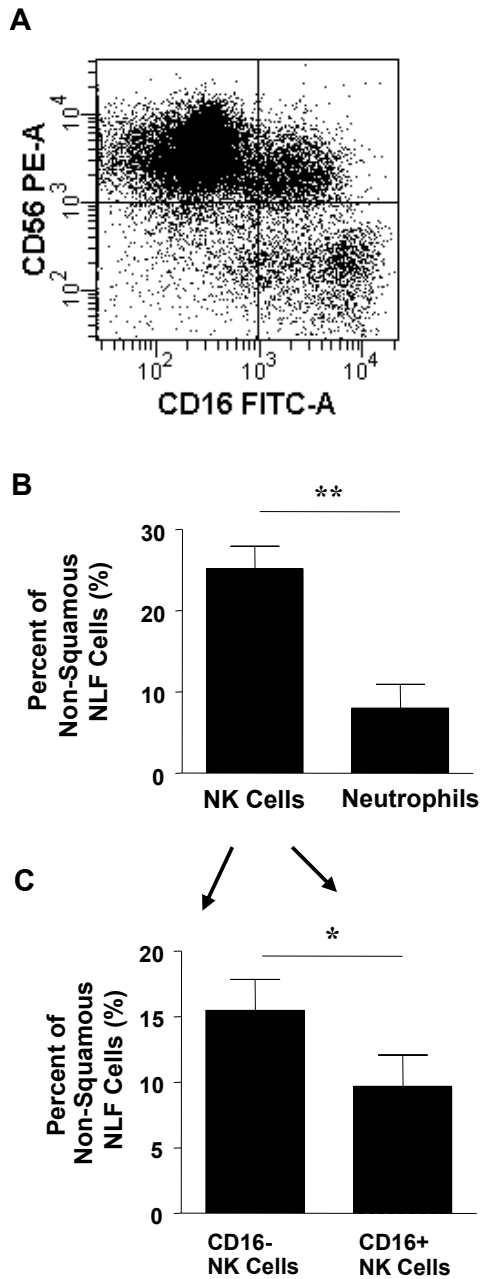
Figure 14. Characterization of NK Cells in the Nasal Lavage by Flow Cytometry.

NK cells were identified in CD45+ NLF cell populations of nonsmokers at baseline. A) Representative flow cytometric plot depicting NK cells (CD56+) and neutrophils (CD56-CD16+). B) Percentages of total NK cells are greater than neutrophils in non-squamous NLF cell populations. C) Percentages of CD16- NK cells are greater than percentages of CD16+ NK cells in non-squamous NLF cell populations. **p<0.01, * p<0.05. Nonsmoker n=11.
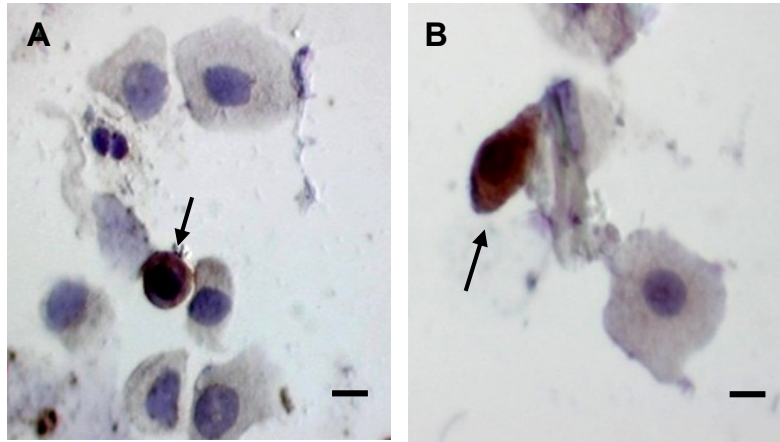
Figure 15. Characterization of NK cells in the Nasal Lavage by Immunohistochemistry.

NLF cells were characterized using immunohistochemistry. A) NLF cells are stained with anti-CD56-HRP to identify NK cells and B) NLF cells are stained with anti-perforin-HRP to identify cytotoxic NK cells. Bar = 10μm.
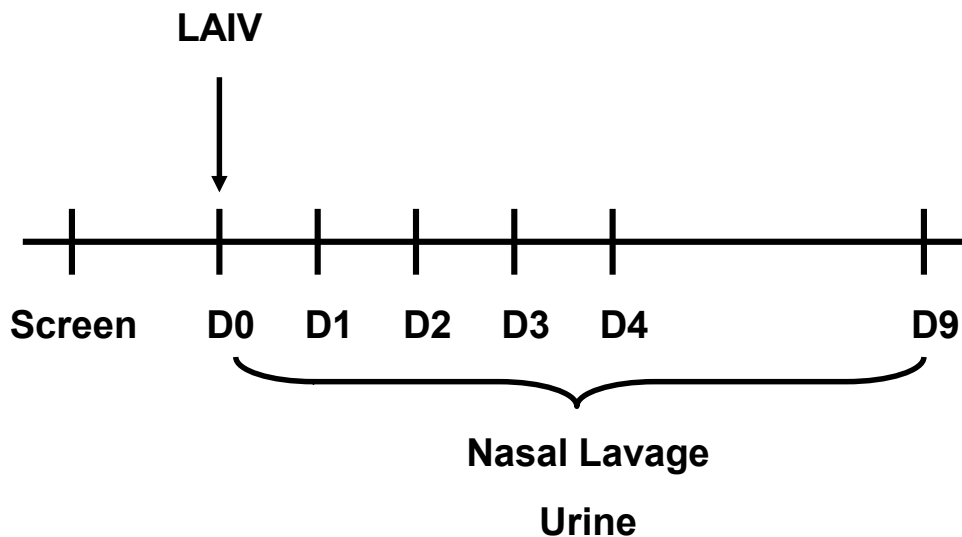
Figure 16. Schematic of LAIV Study Design.

Nonsmokers and smokers were given a baseline nasal lavage followed by inoculation with LAIV on Day 0. Serial nasal lavages were obtained on Days 1-4 and again on Day 9. Urine was collected throughout to study to measure cotinine, a metabolite of nicotine, as a marker of cigarette smoke exposure.

A
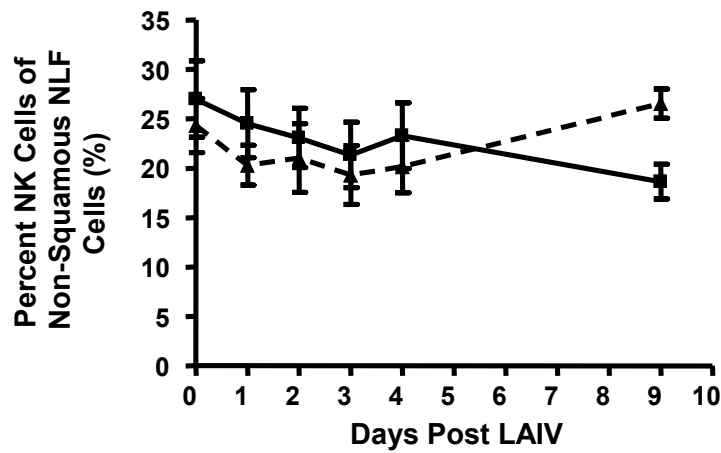
B
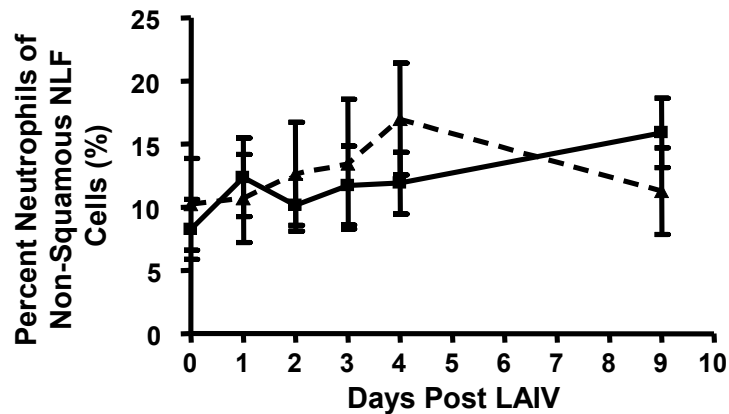
Figure 17. LAIV Does Not Significantly Affect Total NK Cell or Neutrophil Percentages in Nonsmokers or Smokers.

Using flow cytometry we identified NK cells and neutrophils in nonsmoker and smoker non-squamous NLF cells after LAIV inoculation. Neither A) NK cell nor B) neutrophil percentages in total NLF cells change following LAIV in either group. Nonsmokers n=12 (■, solid line), smokers n=9 (▲, dashed line).

A

B
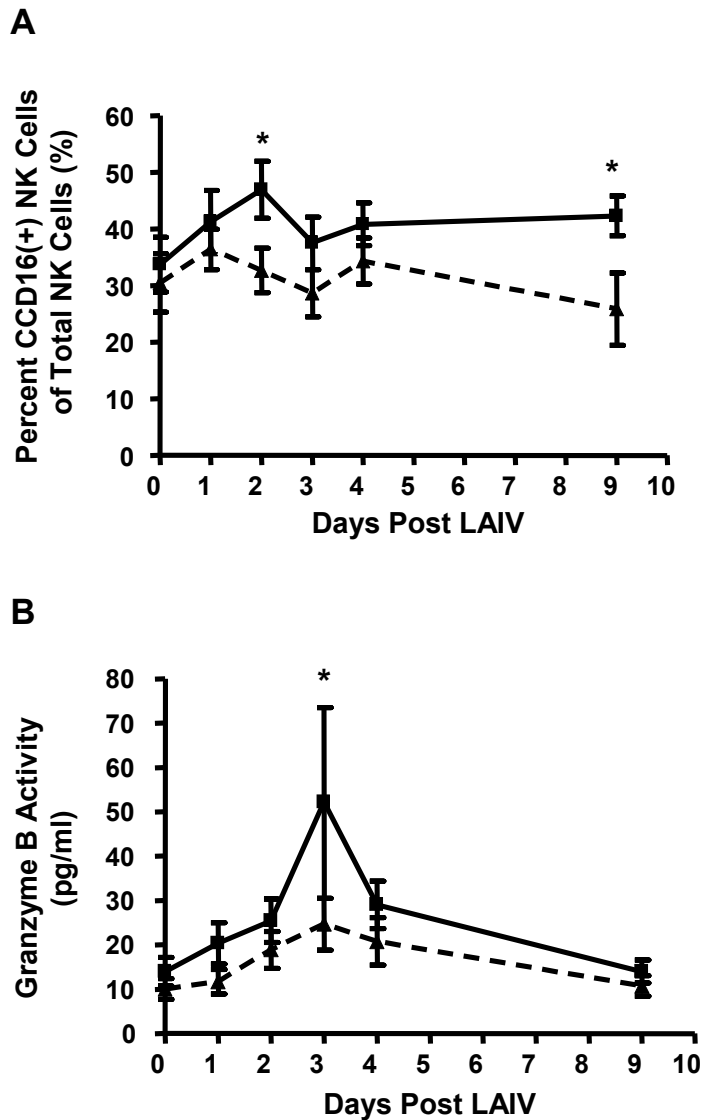
Figure 18. Cytotoxic NK Cell Activity is Suppressed in Smokers Following LAIV.

Cytotoxic NK cell percentages and activity were analyzed in NLF of nonsmokers and smokers after LAIV inoculation. A) CD16+ cytotoxic NK cell percentages of total NK cells were decreased in the NLF of smokers following LAIV. Kruskal-Wallis p=0.09, *p<0.05 nonsmoker vs smoker posttest. Nonsmokers n=12 (■, solid line), smokers n=9 (▲, dashed line). B) Granzyme B activity was decreased in NLF of smokers following LAIV inoculation. Kruskal-Wallis p<0.01, *p<0.05 nonsmoker vs smoker posttest. Nonsmokers n=13 (■, solid line), smokers n=13 (▲, dashed line).
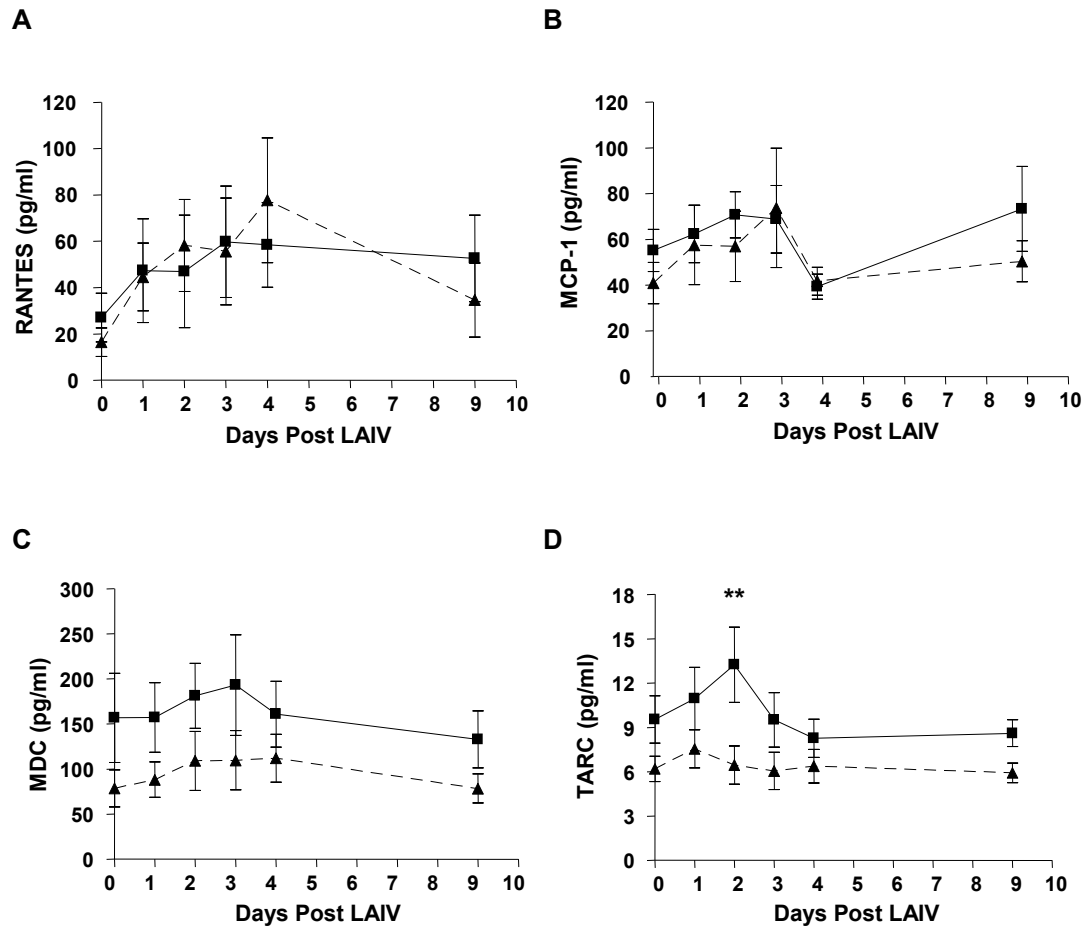
Figure 19. NK Cell Chemokine TARC is Decreased in Smoker NLF Following LAIV.

Chemokines known to enhance NK cell function were quantified in the NLF of nonsmokers and smokers after LAIV inoculation. A) RANTES, B) MCP-1, and C) MDC were similar in nonsmokers and smoker NLF whereas D) TARC is suppressed in smoker NLF. Kruskal-Wallis $p<0.05$, ** $p<0.01$ nonsmoker vs smoker posttest. Nonsmokers n=13 (■, solid line), smokers n=13 (▲, dashed line).
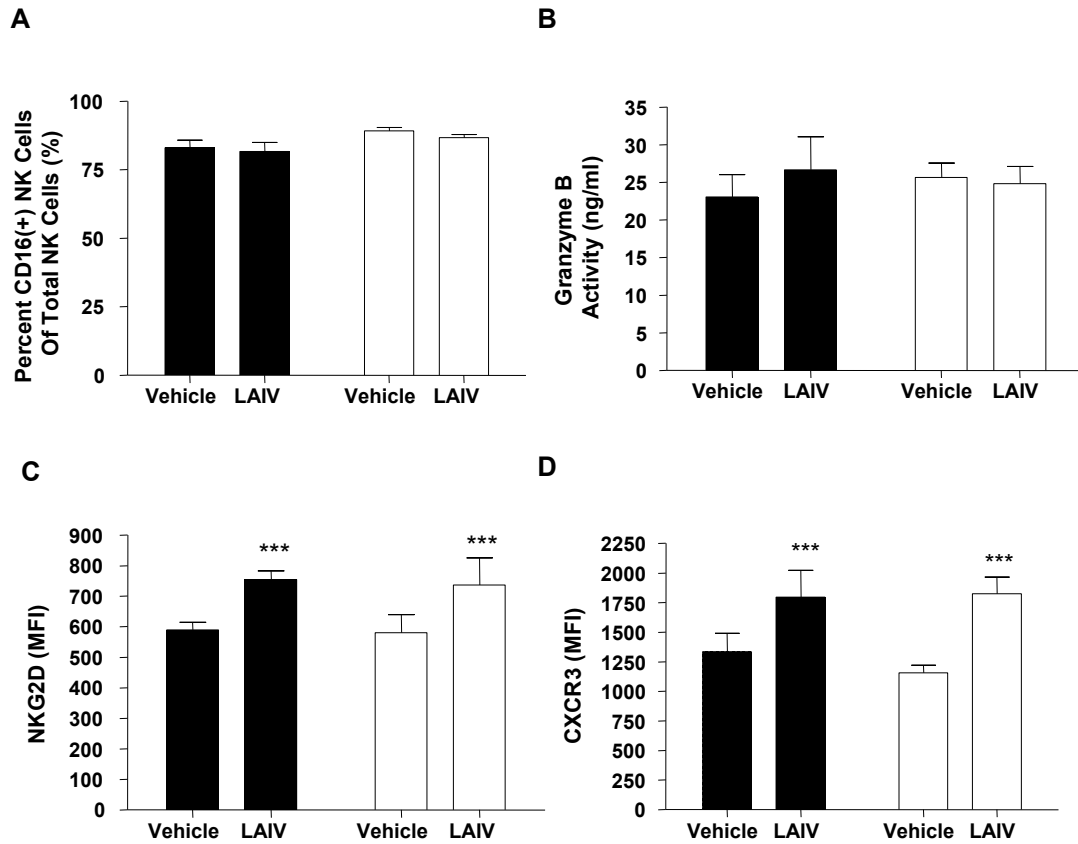
Figure 20. Effect of LAIV On Peripheral NK Cell Activity

Peripheral blood NK cells were isolated from nonsmokers and smokers and stimulated *in vitro* with LAIV. LAIV did not increase A) percent CD16+ cytotoxic NK cells or B) granzyme b activity in either nonsmokers or smokers. LAIV increased expression of C) NK cell activation receptor NKG2D and D) chemokine receptor CXCR3 in both nonsmokers and smokers. Nonsmokers n=6 (black bars), smokers n=6 (white bars). *** p<0.001 vs vehicle control.

CHAPTER 4

LIVE ATTEUNATED INFLUENZA VIRUS INDUCES MUCOSAL T CELL
RESPONSES IN NONSMOKERS AND SMOKERS

Abstract

Rationale: Epidemiological evidence demonstrates smokers are more susceptible to respiratory infections, including influenza. Research suggests smoking suppresses innate immune responses to influenza infection, but the effect of smoking on mucosal T cell function remains poorly understood. To describe how CS affects influenza-induced T cell responses in the nasal mucosa, we inoculated nonsmokers and smokers with LAIV and assessed nasal mucosal T cell populations. Analyses were conducted using two separate study years.

Methods: In the Year 2 study, mucosal T cell populations were harvested through nasal biopsy of the superficial inferior turbinate pre LAIV and on days 1 and 4 post LAIV from both nonsmoker and smoker subjects. Biopsies were digested with pronase and dispase to achieve a single cell suspension and analyzed using flow cytometry for CD45 leukocytes and were screened for cytotoxic lymphocytes (CD56), neutrophils and NK cells (CD16), macrophages and dendritic cells (CD1a and HLA-DR) CD16 (neutrophils, NK cells), CD1a and HLA-DR (macrophages, dendritic cells) and T cells (CD3,, CD4, CD8, and γδ TCR.) Results for specific γδ TCR chains were confirmed using stored nasal biopsy samples from Year 1 harvested on screen day and day 4 post LAIV.

Immunohistochemistry of nasal biopsy sections as well as qRT-PCR of nasal biopsies

was used to determine the presence of γδ TCR chains.

Results: CD45+ nasal biopsy cells were negative for CD16, CD56, CD1a, and HLA-DR

but were positive for CD3. LAIV induced changes in percentages of CD3+ T cells and γδ

T cells (CD3+CD4-CD8-γδTCR+) in the Year 2 study with no differences attributable to

cigarette smoking. T helper cell (CD3+CD4+) and cytotoxic T cell (CD3+CD8+)

populations were minimal in both nonsmokers and smokers by day 4 post LAIV. In the

confirmatory analysis from Year 1, γδTCR+ cells were identified in nasal biopsies using

immunohistochemistry on day 4 post LAIV. qRT-PCR analysis revealed that nasal

biopsies, but not cultured NEC, were positive for Vδ1 and Vγ3 chains although LAIV did

not upregulate TCR chain transcription.

Conclusions: CS exposure did not have an obvious effect on mucosal T cell phenotypes.

However, the infiltration of γδ T cells into the nasal mucosa post LAIV is a novel

finding, and γδ T cells may play important roles in early adaptive immune responses to

viruses in the respiratory mucosa.

Introduction

Smokers have increased risk of respiratory infections such as influenza but the mechanisms mediating this susceptibility are unknown. During influenza infection, the activation of the adaptive immune system, including T and B lymphocytes, is necessary to both 1) clear the initial infection and 2) generate a memory response. Conventional T lymphocytes expressing the αβ TCR are divided into either CD4+ helper T cells that in the context of a viral infection generally enhance immune cell activity or CD8+ cytotoxic T cells that kill virally infected cells (30). γδ T lymphocytes or intraepithelial lymphocytes are at their highest concentration in epithelial tissues where they act as "transitional" T lymphocytes that behave similar to cytotoxic CD8 T cells but acquire their effector mechanisms quicker (30). (183). γδ T cells migrate to the airways during respiratory infections (37) including influenza (38; 39) and play an important role in innate and adaptive immune responses. Most of the knowledge of γδ T cell function originates from mouse models, and the exact TCR subsets important in respiratory responses to influenza viruses in humans are unknown (30). Classifying the subsets of γδ T cells in human airways is important as they can have differential cytolytic or cytokine secreting activity (30). In the human nasal epithelium γδ T cells positive for the Vδ1Vγ1 chains have been identified in allergic rhinitics (184), and the Vδ1-3 and Vγ1-3 chains have been identified in normal individuals (185; 186). γδ T cells positive for the Vδ1 and Vγ1,2 chains (187) but not the Vδ2 chain have been also been identified in the bronchial epithelium of both smokers and nonsmokers. Therefore, γδ T cells are found in the respiratory epithelium of both nonsmokers and smokers. However, the role of γδ T cells

in the nasal epithelium during LAIV as well as the effects of CS exposure on γδ T cell activity remains unexplored.

While there do not appear to be differences in influenza-specific antibody production in smokers (71-73), the effect of smoking status on the ability of T cells to mount proper mucosal immune responses is unknown. Because, both conventional αβ and nonconventional γδ T cell activity is influenced by DC activation, we hypothesize that smokers have decreased T cell activation following LAIV inoculation compared to nonsmokers. To determine if smoking affects mucosal T cell activation, we administered nonsmokers and smokers LAIV as described previously (3). Naïve T cell activation post LAIV in the nasal mucosa was quantified in nonsmoker and smoker subjects using flow cytometry and PCR.

Methods

Study Design

This was a prospective longitudinal study comparing responses to LAIV between cohorts of healthy young adult smokers and nonsmokers. This study compiles data from two separate study years in order to obtain enough material for analysis. In Year 1, subjects were inoculated with the 2008-2009 LAIV strain, and subject characteristics are described in Chapter 3 Table 2. These data were used for nasal biopsy mRNA and immunohistochemical analyses. In Year 2, subjects were inoculated with the 2009-2010 LAIV strain and demographic and smoke exposure characteristics of the subjects completing the study are detailed in Table 4 of this chapter. These data were used for nasal biopsy flow cytometry analyses. The study design was as described before (3). Baseline measurements were done at a screening visit and Day 0. On Day 0 subjects received a standard nasal inoculum of LAIV (FluMist®, MedImmune, Gaithersburg, MD; administered by study nurse according to manufacturer's instructions) in both nostrils, then returned on day 4 post-LAIV for nasal biopsies in Year 1 and on days 1 and 4 post-LAIV for nasal biopsies in Year 2.

Study Subjects and Sample Estimate.

Subjects were identified as described before (3) and included healthy young adults between 18-35 years old in two groups: Group 1 = nonsmokers not regularly exposed to secondhand smoke and Group 2 = self-described active cigarette smokers. Exclusion criteria were as described before (3). Subject exposure history questionnaires were used to estimate CS exposure. In both Year 1 and Year 2, nonsmokers and smokers did not differ significantly in age, BMI, or gender. In both Year 1 and Year 2, self

described smokers had significantly higher secondhand smoke exposure compared to nonsmokers. No serious adverse events occurred among subjects completing the protocol. Informed consent was obtained from all subjects and the protocol was approved by the UNC Biomedical Institutional Review Board.

Nasal Biopsy

Nasal biopsies were harvested from the inferior turbinates of both nostrils using a RhinoProbe cuvette and were placed in RPMI media. Biopsies were pelleted and treated with 15μg/ml dispase (Roche, Indianapolis, IN) and 5μg/ml pronase (Sigma, St. Louis, MO) for 30 minutes. FBS (Invitrogen) at a final concentration of 10% was added to the cell pellet and filtered with a 40 uM cell strainer (BD Falcon, San Jose, CA) to remove larger epithelial cells. The cell pellet was resuspended in 100 ul of flow cytometry buffer (PBS plus 1% heat inactivated FBS plus 0.09% sodium azide) and stained with surface markers CD45 APC-cy7, CD3 PerCP, CD4 APC, CD8 PE, CD56 PE, HLA-DR alexa 700 (BDBiosciences), CD1a pacific blue (Biolegend, San Diego, CA), pan γδ TCR FITC ,and CD16 FITC (Beckman Coulter, Brea, CA) for 20 minutes at room temperature. Cells were washed with flow buffer, fixed with 0.5% paraformaldehyde, and analyzed on a BDLSRII flow cytometer within 24 hours. T cells were identified by gating using forward scatter (FSC) and side scatter (SSC) settings and gating on cells staining positive for CD45 and CD3.

Nasal Biopsy Immunohistochemistry

Nasal biopsies were obtained on screen day and day 4 post LAIV. Biopsy samples were fixed in 4% paraformaldehyde and embedded in paraffin. Five μm thick sections were placed on Superfrost/plus slides (Fisher Scientific, Pittsburgh, PA). Slides were

washed with TBS and blocked with Powerblock (Biogenex, San Ramon, CA) for 1 hr at room temperature. Following this, cells were incubated with mouse anti-human pan γδ TCR (ThermoScientific) overnight at 4°C. After incubation with primary antibody, cells were washed with TBS followed by incubation with HRP-conjugated secondary antibodies (Vector Labs, Burlingame, CA) for 1 hour at room temperature. Cells were washed with TBS and evaluated under light microscopy.

Nasal Biopsy PCR

Nasal biopsies were obtained on screen day and day 4 post LAIV. RNA from nasal biopsies was isolated as described before. Total RNA was extracted using TRizol (Invitrogen) as per the supplier's instruction. First-strand cDNA synthesis and real-time RT-PCR was performed as described previously (15, 16). The mRNA analyses for CD3γ, Vγ3 TCR, and Vδ1 TCR were performed using commercially available primer and probe sets (inventoried Taqman Gene Expression Assays) purchased from Applied Biosystems (Foster City, CA). NEC obtained from nasal biopsies and differentiated *ex vivo* as described before (188) were used as negative controls for T cell mRNA. PBMC were used as a positive control for T cell mRNA.

Statistical Analysis

The effects of smoking status on nasal biopsy responses to LAIV were analyzed using a Kruskal-Wallis one-way ANOVA followed by a Dunn's post hoc test. Data were shown as mean ± SEM or median (interquartile range).

Results

T cells Are Present In Nasal Biopsies At Baseline

Nasal biopsies were harvested at screen day and processed for flow cytometry. The majority of nasal biopsy cells were nasal epithelial cells, characterized by their larger size, high side scatter fluorescence, and non-specific (negative) staining for all immune markers. A distinct population of immune cells was identified based on CD45 staining and side scatter fluorescence as shown in Figure 21A. Using a panel of surface marker antibodies, these cells were negative for CD16 (neutrophils), CD56 (NK cells), CD1a (DCs), and HLA-DR (DCs, macrophages) (Figures 21B-E). These leukocytes were positive for T cell marker CD3 (Figure 21F). A representative image is shown.

LAIV Inoculation Modifies T Cell Populations in Nasal Biopsies

Nasal biopsies were harvested at screen day, day 1, and day 4 post LAIV and processed for flow cytometry. CD3+CD45+ T cells were quantified as percentages of total nasal epithelial cells in Figure 22. By day 4 post LAIV percentages of T cells increased from 0.1%(0.1-1.5) to 1.1%(0.9-4.7) in nonsmokers and 0.4%(0.2-0.9) to 1.2%(0.6-2.0) in smokers although only in nonsmokers was this increase statistically significant compared to baseline. Of the T cells that increase by day 4 post LAIV in Figure 23A, there were no CD4 T cells in nonsmoker [0.0%(0.0-0.1) or smoker [0.0%(0.0-0.0)] nasal biopsies and there were no statistical changes in CD4 T cell populations throughout the study. In addition, nominal percentages of CD8 T cells were identified in either nonsmoker or smoker groups in Figure 23B.

Overall, LAIV inoculation altered the percentages of CD4-CD8-γδ TCR (+) T cells in nasal biopsies (Figure 24), although these increases post LAIV compared to screen day did not quite reach statistical significance. We determined that these T cells are not conventional αβ T cells but were instead γδ T cells that were positive for the γδ TCR (Figure 24B). We also identified γδ TCR positive cells in nasal biopsies following LAIV using immunohistochemistry (Figure 25).

CD3 and γδ TCR Chains Are Identified In Nasal Biopsies By qRT-PCR

To identify the presence of γδ T cells we quantified mRNA expression within nasal biopsies before and after LAIV in both nonsmokers and smokers. Through determining positive expression of CD3γ we identified T cells (Figure 26) and γδ T cells by positive expression of the TCR variable γ3 (Vγ3) chain (Figure 27A) and the TCR variable δ1 (Vδ1) chain (Figure 27B). LAIV did not induce transcriptional upregulation of TCR chain mRNA in either nonsmokers or smokers.

Discussion

The modulation of γδ T cell populations in the nasal mucosa due to LAIV is a novel finding and is the first evidence that γδ T cells participate in LAIV-induced responses in humans. Like DCs and NK cells in the respiratory epithelium, γδ T cells can act as bridges between innate and adaptive immunity by regulating both arms of the immune system. Their location in nasal mucosal epithelia allow them to quickly respond to virus infections, and γδ T cells sense "danger" from nearby epithelial cells through recognition of stressed cell ligands that are upregulated by viral infections (31) and from activation by virus-induced proinflammatory cytokines in the epithelia microenvironment (30). γδ T cells migrate to the lung during influenza infections (38; 39) which correlates with the similar response we see in nasal mucosa. γδ T cells respond to viral infections both by killing virus infected epithelial cells similar to cytotoxic T and NK cells (30) and also by producing cytokines such as IFNγ, IL-17, and MCP-1 that induce immune cell chemotaxis (30). Therefore, γδ T cells and NK cells have some functional redundancy, and both cell types could contribute to LAIV induced levels of granzyme B (Chapter 3) as well as IFNγ (3) in the NLF, although it is likely that NK cells contribute more than γδ T cells to these NLF responses based on their location. We did not observe significant migration of CD4 or CD8 T cells to the nasal mucosa by day 4 post LAIV. This is likely because influenza specific lymphocytes like CD8 T cells do not migrate to the airways until day 7 post infection (189). Modifying our LAIV protocol to include later time points could capture influx of influenza specific CD4 or CD8 T cells. Therefore, γδ T cells play a significant role in nasal mucosal T cell activity during the early adaptive immune responses to LAIV.

γδ T cell migration to the nasal mucosa after LAIV is similar between nonsmokers and smokers. Because we did not observe a noticeable smoking effect, we speculate that intraepithelial lymphocyte activation in the nasal mucosa does not contribute to increased risk of influenza infections documented in smokers. In fact, differences in innate immune responses that we (3; 53; 174) and others have documented in the case of cigarette smokers may contribute to the underlying mechanisms of virus susceptibility rather than adaptive immune responses. In our studies we did not observe differences in T cell specific immunity to LAIV between nonsmokers and smokers. This is similar to other data that demonstrate humoral influenza vaccine memory is the same in nonsmokers and smokers (71-73). Thus, while innate immune defense responses are impaired in smokers, nasal mucosal adaptive immune responsesmay not be affected by smoking status.

Although we found detectable T cell specific receptor chain mRNA (CD3ε, Vγ3, Vδ1) in nasal biopsies, we did not observe LAIV-induced changes in receptor chain mRNA in either group despite finding increased percentages of T cells by day 4 post LAIV by flow cytometry. However, T cell activation may not always correspond with upregulation of T cell receptor chain transcription. In fact, activation of the TCR/CD3 complex by phorbol myristate acetate (PMA) and anti-CD3 in T cells leads to increased cytokine production and increased T cell proliferation, but actually inhibits the transcription of CD4, CD8, and TCR α,β, and γ mRNA as well as increases mRNA degradation (190; 191).While mRNA expression of T cell receptor chains may be used to classify γδ T cell subtypes (185; 186), mRNA expression may not necessarily correlate with T cell activation states. In addition, although both Vγ3 and Vδ1 TCR chains have

been identified in the nasal mucosa (185; 186), these chains may not be predominant in this particular γδ T cell population. A thorough analysis of variable TCR chains in LAIV infected nasal γδ T cells is necessary to characterize this population.

The ability to identify and characterize a relatively rare cell population (less than 5% of total nasal mucosa cells are γδ T cells) from human biopsy samples by flow cytometry could be a useful technique to examine immunotoxicological responses of other rare mucosal cell types. For example, γδ T cells are located at high concentrations in other mucosal tissues including the gut (192). These *in vivo* T cell assays could provide an approach to determine the effect of environmental factors on immune responses in mucosal tissues throughout the body and could prove useful to toxicologists and vaccine developers.

Table 4. Subject Characteristics and Tobacco Smoke Exposure: Year 2

	Nonsmoker (N = 13)	Smoker (N = 12)
Age (yr)	21.5 ± 0.7	25.3 ± 1.3
Gender	8M/5F	10M/2F
BMI	25.0 ± 1.6	24.8 ± 1.7
Daily exposure		
Smoked[1]	NA	11.2 ± 1.6

[1] Data shown as mean ± SEM for cigarettes smoked. Data were averaged from each subject's self reported estimates for study days 0 through 9.
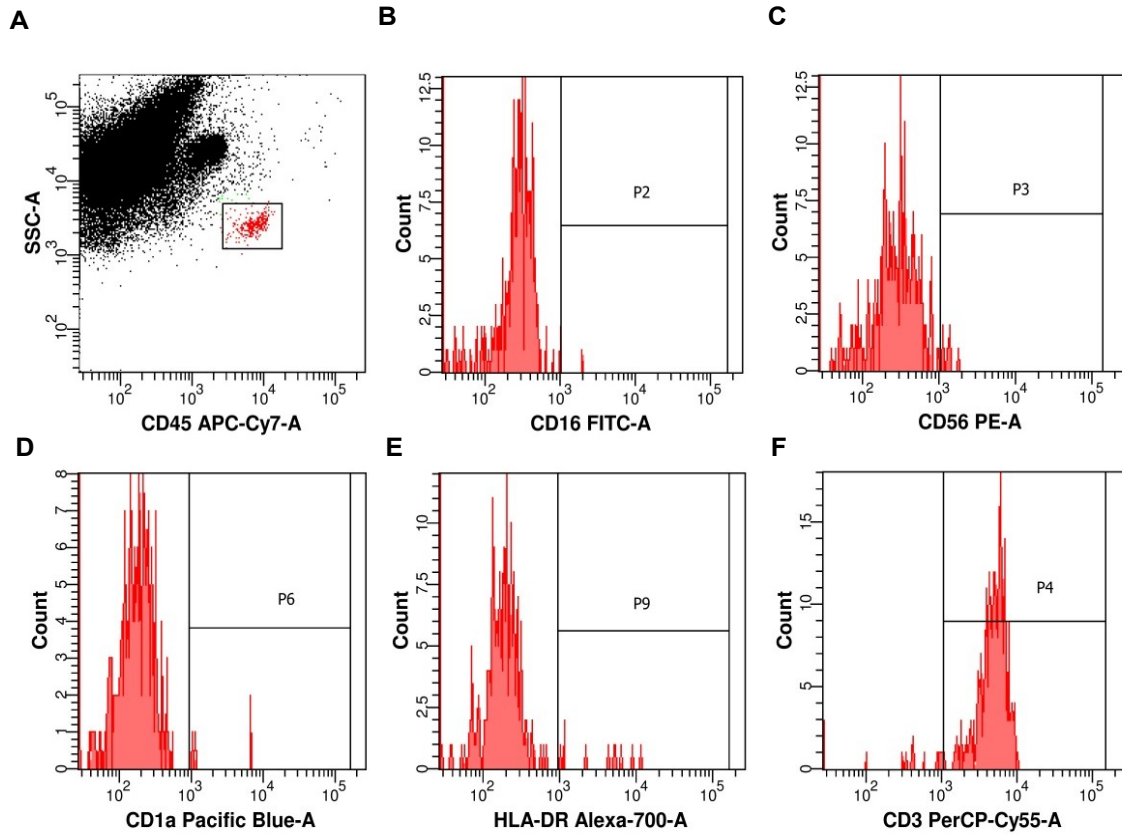
Figure 21. T Cells Are Present in Nasal Biopsies

Nasal biopsies obtained on screen day were processed and stained with immune markers for flow cytometric analysis. A) Nasal leukocytes were identified based on SSC voltages and positive staining for CD45. Expression of immune markers for B) neutrophils, CD16, C) NK cells, CD56, D) DCs, CD1a, E) DCs and macrophages, HLA-DR, and F) T cells, CD3. A representative image is shown.
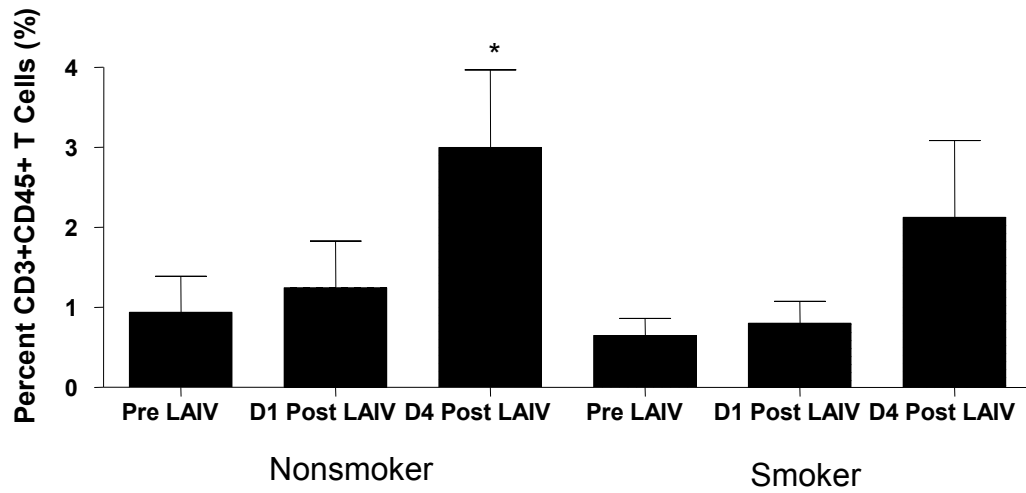
Figure 22. T Cells Increase in Nasal Biopsies Following LAIV.

Nasal biopsies obtained pre LAIV and day 1 and day 4 post LAIV were processed and stained with immune markers for flow cytometric analysis. CD45+CD3+ T cells were quantified in the nasal biopsies as percentages of total cells. Kruskal-Wallis $p<0.05$, * $p<0.05$ vs pre LAIV. Nonsmoker n=13, smoker n=12.
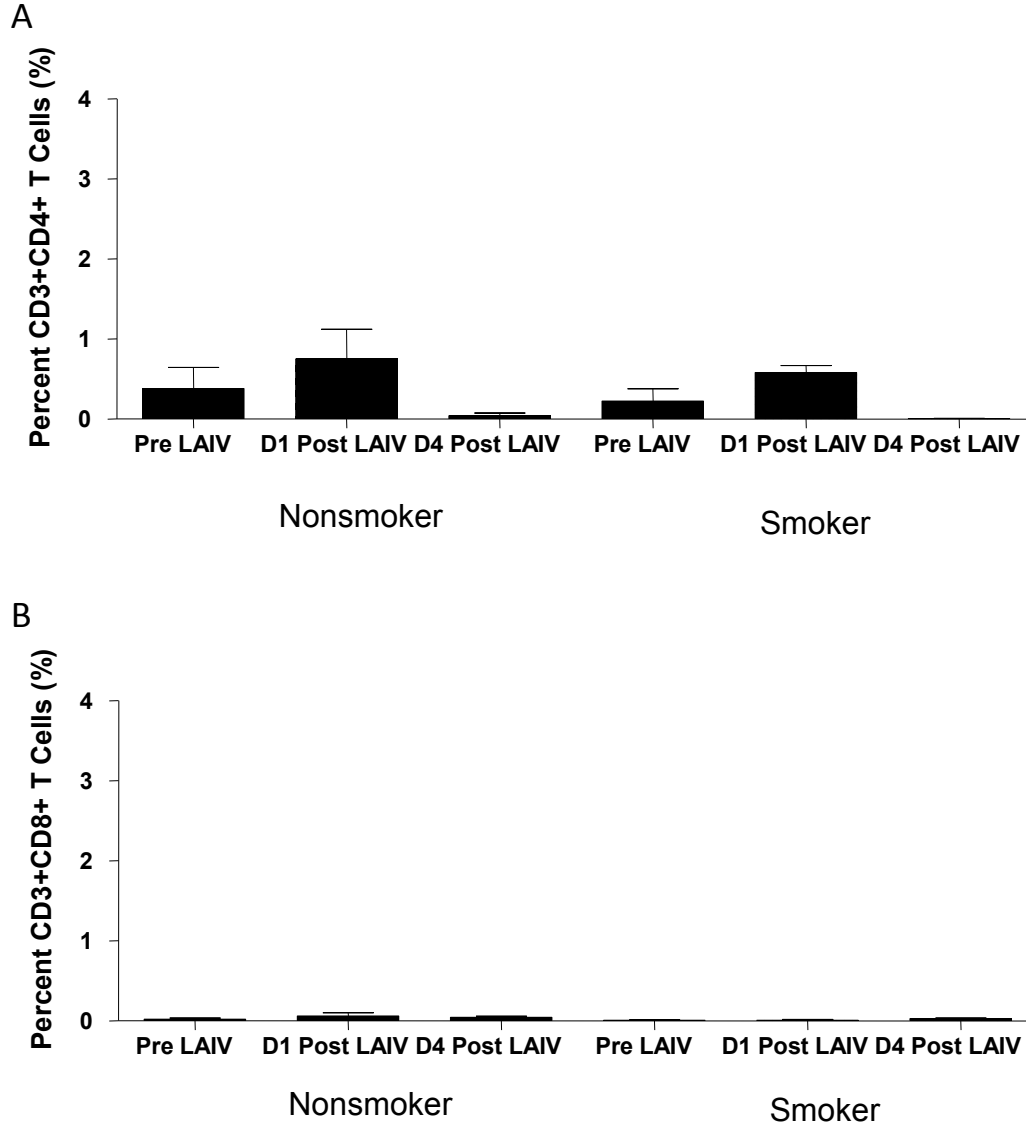
Figure 23. CD4 and CD8 T Cell Percentages Are Minimal Following LAIV.

Nasal biopsies obtained pre LAIV and day 1 and day 4 post LAIV were processed and stained with immune markers for flow cytometric analysis. A) CD45+CD3+CD4+ (Kruskal-Wallis p=0.0625) and B) CD45+CD3+CD8+ T cells were quantified in the nasal biopsies as percentages of total cells. Nonsmoker n=6, smoker n=5.
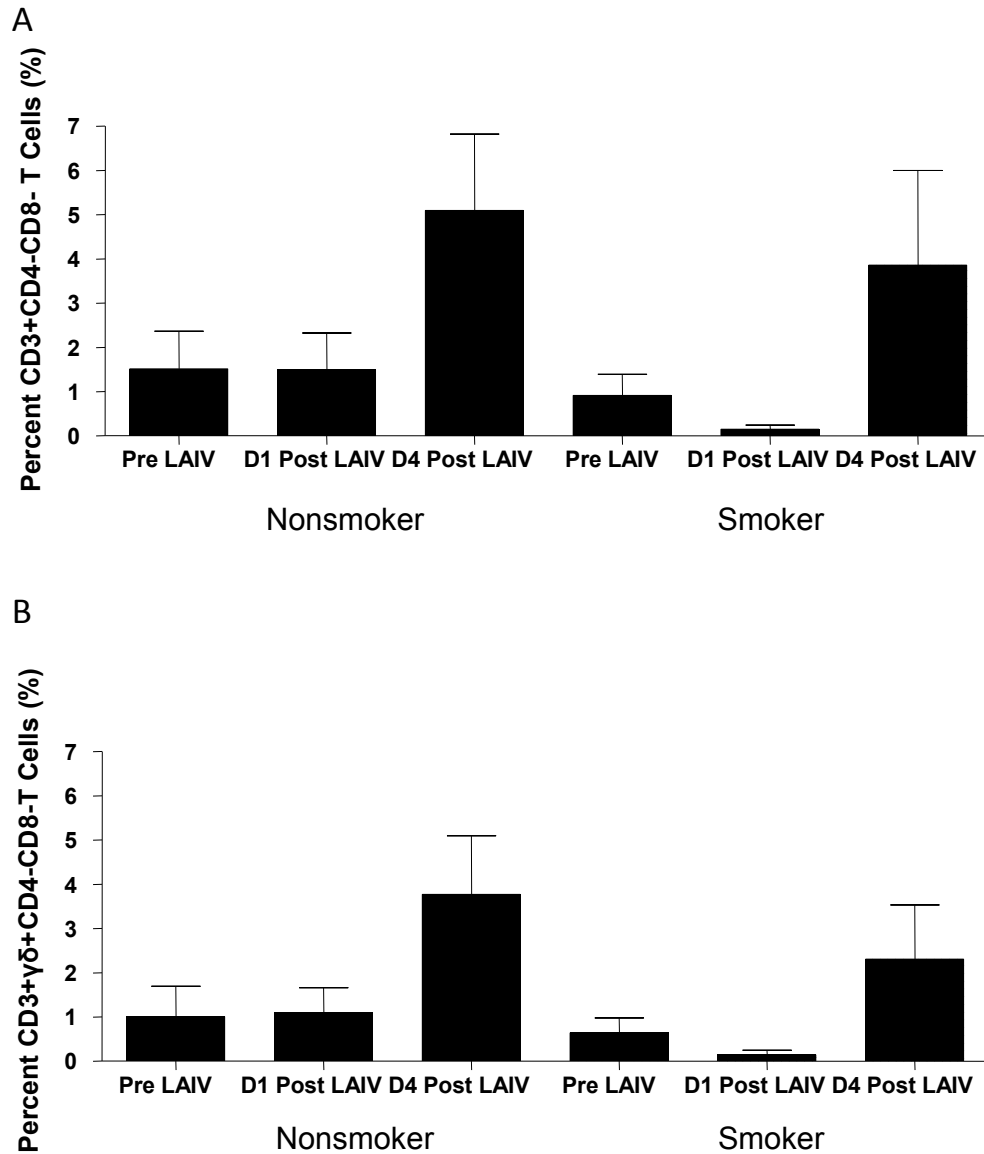
Figure 24. LAIV Modulates γδ T Cell Percentages in Nasal Biopsies in Nonsmokers and Smokers.

Nasal biopsies obtained pre LAIV and day 1 and day 4 post LAIV were processed and stained with immune markers for flow cytometric analysis. A) CD45+CD3+CD4-CD8- and B) CD45+CD3+CD4-CD8-γδ T cells were quantified in the nasal biopsies as percentages of total cells. Nonsmoker n=6, smoker n=5. Kruskal-Wallis p<0.05
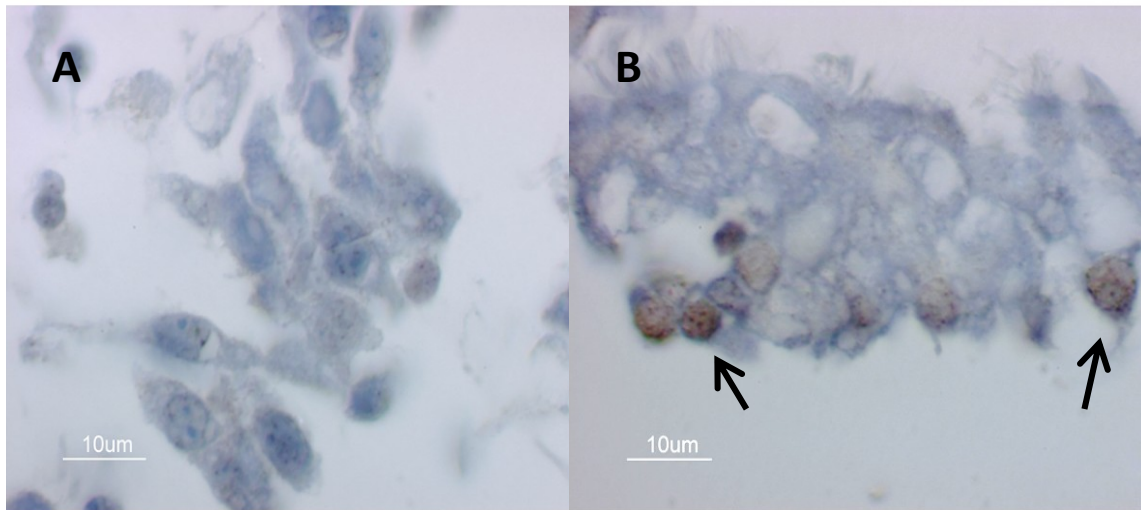
Figure 25. γδ T Cells Are Present In Nasal Biopsies: Immunohistochemistry.

Nasal biopsies were harvested before (A) and after (B) LAIV inoculation. Biopsies were fixed and embedded in paraffin. Biopsies were stained for immunohistochemistry with anti-γδ-TCR-HRP (black arrows) to identify γδ T cells. A representative image is shown.
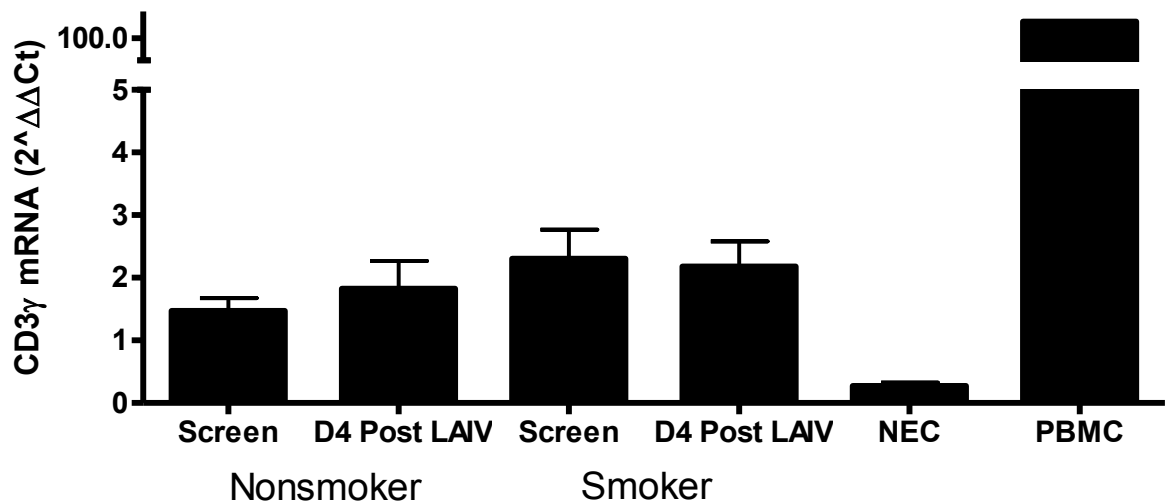
Figure 26. CD3γ mRNA is Present in Nasal Biopsies.

Nasal biopsies obtained pre LAIV and day 4 post LAIV were processed for mRNA analysis. Expression of CD3γ mRNA was quantified in nasal biopsies and did not change with LAIV or smoking status. Differentiated NEC and PBMC were used as negative and positive controls for T cell mRNA, respectively. Data are normalized to βactin mRNA levels. Nonsmoker n=10, smoker n=12.
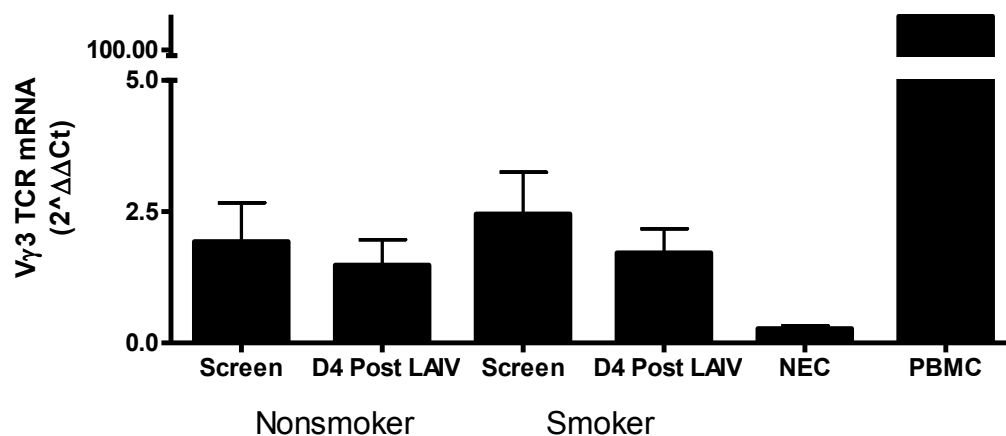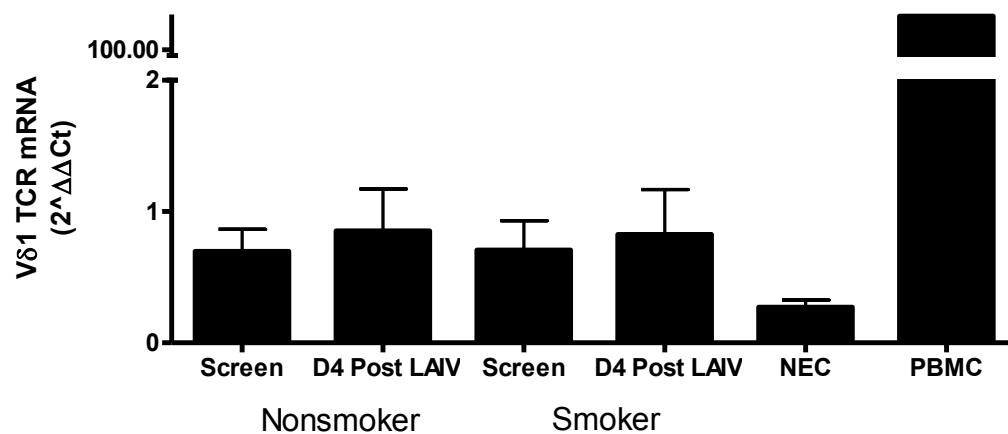
Figure 27. γδ TCR mRNA is Present in Nasal Biopsies.

Nasal biopsies obtained pre LAIV and day 4 post LAIV were processed for mRNA analysis. Expression of A) Vγ3 and B) Vδ1 mRNA was quantified in nasal biopsies and did not change with LAIV or smoking status. Differentiated NEC and PBMC were used as negative and positive controls for T cell mRNA, respectively. Data are normalized to βactin mRNA levels. Nonsmoker n=10, smoker n=10.

CHAPTER 5

GENERAL DISCUSSION

"Not smoking is the single most important thing you can do to prolong life."

(193) This quote is from the book *Know Your Chances: Understanding Health Statistics*,

which portrays the risk of dying from various causes such as vascular disease, cancer, and

pneumonia based on age, gender and smoking status (193). CS exposure is associated

with multiple illnesses including lung cancer, emphysema, COPD, cardiovascular

disease, and stroke. CS exposure is associated with an increased risk of viral infections,

such as influenza (43-45) and by using *ex vivo* and *in vivo* models of influenza infection,

we suggest that smokers have suppressed antiviral responses of NEC, DCs, and NK cells.

This mechanism of antiviral suppression could be extended to other pollutants such as

DE or SHS that have relevant affects of respiratory health.

Advantages and Disadvantages of Human Exposure Models

Conducting clinical research is both rewarding and challenging. In these studies, I

was fortunate to use both *in vivo* and *ex vivo* models of human influenza infection to

determine differences in viral susceptibility between normal and pollutant-exposed

populations. By using an FDA-approved vaccine, our study had the added benefit of

protecting our subjects from influenza infections. Using all human-based models

increased the significance and relevance of my findings. However, there are challenges in

conducting research using human subjects. In clinical research, the human subject is the

top priority. Unlike when performing animal or strictly *in vitro* studies, researchers must rely on the relationships they develop with their subjects to encourage study participation and limit factors such as subject dropout and study noncompliance. The inability to control certain confounding factors (i.e. air pollutant exposures or respiratory infections that are exogenous to the study) necessitates complex data analyses highlight potentially important but subtle findings in our subject populations.

Other challenges of working within human exposure models are the technical limitations. In rodent models of influenza infection, one can extract the entire lung from the animal and measure immune responses at the cellular, tissue, and organ level. One can perform mechanistic studies by either over or under-expressing molecules important in the immune response. We are limited in these respects when using human models. The challenge in garnering mechanistic data from human studies could be addressed twofold: 1) develop complex assays using sensitive and specific techniques like flow cyometry to assess immune response in tissues acquired using noninvasive methods and 2) use human-derived cells to "build" organotypic culture conditions that both mimic *in vivo* tissues and can be manipulated to test hypotheses. To expand upon the co-culture model presented in this project, our laboratory is currently developing a triple cell co-culture model of the nasal mucosa to examine interactions between NK cells, NEC, and DCs using cells from either normal populations or diseased populations such as atopic asthmatics. I believe the work described in this dissertation will provide a foundation to increase the usefulness and sophistication of *in vitro* cell culture models while preserving *in vivo* relevance. Hopefully, elegant and innovative assays will supply researchers with tools to manage the technical limitations that accompany human research.

How Does Cigarette Smoke Modify Antiviral Defenses?

Our lab has shown that smokers (3) and NEC obtained from smokers (53) have increased markers of influenza viral infection with decreased activation of antiviral defenses in the epithelium. I have demonstrated that in these influenza infection models, specific activities of cytotoxic NK cells, DCs, and NEC are suppressed in smokers. However, γδT cell function in the nasal mucosa remains similar between nonsmokers and smokers, perhaps because γδT cells in the submucosa are not directly exposed to CS in the airways. Therefore, suppression of some innate but perhaps not adaptive immune cells in smokers during viral infections may be contributing to smokers' enhanced viral susceptibility.

Smokers Have Suppressed Type I IFN Responses to Influenza Virus

The exact mechanism that causes innate immune suppression in smokers is still unknown. Because NK cells, DCs, and NEC communicate along multiple axes including through "hardwired" receptor interactions and also "wireless" cytokine or exovesicle secretion, it is difficult to determine if, for example, suppressed NEC activity causes suppressed NK cell activity, or vice versa, or both. One common pathway that is suppressed in both NEC and DCs in our *in vitro* smoker co-culture model is the type I IFN pathway (53; 194). In this case, it is clear that the lack of IFNα secreted by NEC from smokers is likely responsible for depressed DC activation following an influenza infection. Suppressed type I IFN could also suppress NK cell activity in the local mucosa. As our lab has previously shown, the methylation of IRF7 genes in smokers (53) could be a mechanism for this IFN-dependent suppression. We have also demonstrated that nasal biopsies from smokers have increased DNA (cytosine-5)-methyltransferase 1 (DNMT1),

an enzyme that maintains DNA methylation patterns during DNA replication (53).

Therefore, once exposed to DNA methylating agents, NEC in chronic smokers may be more likely to perpetuate these epigenetic changes. This hypothesis could be addressed by treating healthy NEC from nonsmokers with DNA methylating agents *ex vivo* to create a "smoker" phenotype followed by influenza virus challenge. Silencing of the type I IFN pathway through epigenetic modification could be responsible for the suppressed antiviral to influenza responses seen in smokers.

Epigenetic Gene Modification in Smokers

Carcinogenic compounds in CS are likely responsible for gene methylation in smokers. Because smoking increases the risk of lung cancer (193), most of what we know about epigenetic effects of smoking in the lung is from tracking aberrant gene methylation patterns found in lung tumors. Methylation of CpG islands in promoters silences genes and enables tumor cell proliferation (195). Many genes important in tumor progression like p16, retinoic acid receptor β (RAR-β), RASSF1A, death-associated protein kinase (DAPK), and O6-methylguanine-DNA methyltransferase (MGMT) are methylated in non small-cell lung cancer (NSCLC) (195). There are many carcinogenic agents in CS including *N*-nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine, PAHs, aromatic amines, formaldehyde, volatile hydrocarbons, organic compounds, and heavy metals (196) that could contribute to these methylation changes. For instance, benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE) methylated the RAR-β2 promoter in both murine lung cancer and esophageal epithelial and cancer cells (196). The functional consequences of methylation of the RAR-β2 promoter included enhanced expression of epidermal growth factor receptor (EGFR), ERK1/2, activator

protein 1 (AP-1), and cyclooxygenase-2 (COX-2) (196). Nicotine in CS methylated the promoter of the tumor suppressor gene fragile histidine triad (FHIT) in esophageal squamous cell carcinoma cell lines (196). Nicotine also enhanced the expression of DNA methyltransferase 3a (DNMT3a), an enzyme that is responsible for directly methylating promoter sites (196). Carcinogenic agents in CS also induced methylation of genes such as HtrA serine peptidase 3 (HtrA3) (197) and runt-related transcription factor 3 (RUNX3) (198). CS exposure can also induce gene methylation indirectly by exposing individuals to radiation. Tobacco is grown in soil containing polonium-210 (^{210}Po), a source of ionizing α-radiation (199). Consequently, radioactive (^{210}Po) as well as radioactive lead-210 (^{210}Pb) are components of CS (199). In fact, two-pack-a-day smokers have approximately seven times the background level of radiation of inhaled ^{210}Po in the bronchial epithelium (199). This increase in α-radiation is associated with increased lung tumor incidence and methylation of the tumor suppressor gene p16 (199). Therefore, DNA methylating agents in CS are likely responsible for modifying many genes important in lung tumor progression as well as antiviral defense such as IRF7. A genome-wide analysis of DNA methylation patterns in NEC from smokers and non-smokers has identified additional genes potentially involved in antiviral defense responses that are differentially methylated in these two populations. Ongoing and future studies conducted in our lab will further examine their role in the suppressed antiviral defense responses seen in the nasal mucosa from smokers.

Reversibility of Epigenetic Modification in Smokers

Are these methylation changes reversible following smoking cessation programs? Studies have shown that in former smokers, smoking induced changes in gene

methylation patterns persist. For example, in patients with squamous cell lung carcinoma, both current and ex smokers were more likely to have positive methylation of both the p16 and FHIT genes compared to never smokers with carcinoma (200). Smoking can also cause reductions in gene methylation through modification of demethylating enzymes and therefore increase gene expression (201). For example, the promoter of monoamine-oxidase, a catabolic enzyme important in serotonin release, was hypomethylated in both smokers and former smokers compared to never smokers (201). The question of the reversibility of CS induced gene expression changes was addressed by analyzing bronchoscopy tissue from never smokers, former smokers, and current smokers for differential gene expression using microarray mRNA analysis (202). Linear model analysis identified 175 genes that were distinctly expressed between the 3 groups and were classified as rapidly reversible, slowly reversible, or irreversible (202). Genes that were slowly reversible or irreversible include those encoding metallothioneins, proteins that bind heavy metals, and the T cell chemokine CX3CL1 or fractalkine (202). Rapidly reversible genes included those involved in carcinogenic compound metabolism, especially those that regulate oxidoreductase activity (202). However, for genes such as IRF7 that are important in antiviral defense, it is unknown if methylation is reversible. Our laboratory is currently collecting nasal biopsies from smokers before and after the start of a smoking cessation program to determine if methylation of IRF7 in smokers is transient or permanent. Understanding if former smokers compared to current smokers have similar methylation changes in genes that regulate antiviral responses would highlight the effects of smoking cessation on respiratory antiviral immunity.

Can We Rescue Antiviral Defense in Smokers Using Antioxidants?

Understanding the mechanism by which CS leads to decreased influenza induced immune responses could allow us to devise smoker-specific therapeutics. This would decrease both the adverse health effects suffered by smokers in North Carolina and the economic costs to our state that occur during a pandemic outbreak. Because CS is an oxidant air pollutant, one "therapeutic" that might improve antiviral responses in smokers is nutritional supplementation with antioxidants. CS contains ROS like superoxide anion, nitric oxides, organic hydroxyl radicals, and hydrogen peroxides (203). Through reactions with endogenous iron, these ROS can produce endogenous hydroxyl radicals within tissues (203). By increasing ROS in lung epithelial cells, CS alters the redox balance and depletes endogenous cellular antioxidants like reduced (GSH) and oxidized (GSSG) glutathione (203). These antioxidant molecules neutralize ROS to prevent further oxidation. Pretreatment of cells with GSH prior to CS reduced H_2O_2 production and EGFR phosphorylation (204). Therefore, antioxidant supplementation has the potential to reduce CS induced oxidative stress.

Antioxidant Supplementation in Influenza Infection

Influenza infection induced oxidative stress responses *in vivo* (205; 206). Several studies suggested that supplementation with antioxidants like GSH (207) or quercetin, a flavonol found in fruits and vegetables, (208) was beneficial for influenza infection through neutralization of ROS and even suppression of viral replication. Resveratrol, a polyphenol antioxidant present in grapes, berries and peanuts has anti-inflammatory effects and inhibited influenza replication by preventing expression of viral proteins and increased survival in BALB/c mice (209). Polyphenolic catechins like epigallocatechin gallate (EGCG) found in green tea inhibited influenza infection in MDCK cells by

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suppressing hemagglutination, neuraminidase activity, and viral RNA synthesis (210). Selenium deficiency in differentiated human bronchial epithelial cells altered antiviral responses by enhancing IL-6 production, decreasing IP-10 secretion, and increasing influenza-induced apoptosis (188). Prophylactic use of selenium, vitamin E, glutathione, resveratrol, and quercetin was suggested as a regimen to protect against H5N1 avian influenza (211) although this has not been evaluated in humans. However, vitamin A, C, and E supplementation had no effect on risk of community-acquired pneumonia in women (212). Therefore, antioxidant supplementation may be beneficial in experimental influenza infections, but these findings have not yet been replicated in humans.

Antioxidant Supplementation in Smokers

Will supplementation with antioxidants in smokers reduce oxidative stress or inhibit influenza-specific virology? Smokers had decreased plasma levels of vitamin C, E, and beta-carotene although these deficiencies were not always seen in the airways (209). Alveolar macrophages from smokers and people with COPD treated with resveratrol had suppressed IL-8 and GM-CSF release via suppression of NF-kb, AP-1, Cox1, and Cox2 activation (209). However, it is unclear if supplementation with antioxidants will ameliorate CS induced suppression of antiviral defenses. Nrf2 is a transcription factor that regulates expression of antioxidant molecules and is protective against both CS and RSV induced airway damage (213). Nutritional supplements such as EGCG and sulforaphane are potent inducers of Nrf2 (214). Our laboratory is currently conducting a study that supplements nonsmokers and smokers with sulforaphane and measures *in vivo* responses to influenza. Antioxidant supplementation may be a convenient way to inhibit the effects of CS exposure on influenza infection.

Role of Cigarette Smoke Exposure and Respiratory Viruses in Other Airway Diseases

Asthma

Cigarette Smoke Exposure Increases Risk and Severity of Asthma

McLeish et al provide a systematical review examining the association between CS exposure and asthma (215). Although individual studies were conflicting, overall the authors determined that smokers were more likely to have asthma than nonsmokers and that smoking increased the risk for developing asthma (215). This association was overall more common in female smokers vs male smokers (215). Smoking also increased the risk of asthma exacerbations, the severity of asthma symptoms, and decline in lung function (215). Smoking decreased responses to inhaled corticosteroids, indicating that smokers may have more difficulty controlling their asthma (215). However, the authors determined that risk of asthma was not enough to deter smoking habits (215). Although smoking is a personal choice, it is alarming that SHS also contributes to asthma pathogenesis especially in young children. A recent review examines the role of indoor air pollution on asthma incidence in children because Americans spend the majority (90%) of their time indoors (216). SHS, a significant contributor to indoor air pollution, increased the risk, severity, and mortality of asthma in children (216). This susceptibility begins early. *In utero* SHS exposure from the mother decreased lung function, increased asthma symptoms, and even enhanced the likelihood that a child will develop corticosteroid resistance (216). Overall, CS exposure both increases the risk for and enhances the severity of asthma, especially in children.

Role of Respiratory Viruses in Asthma

In a recent review, Busse et al discuss current knowledge of the role of respiratory viruses in both asthma onset and asthma exacerbations (217). The "hygiene hypothesis", based on epidemiological studies of children who are in daycare or who have older siblings, suggests that frequent viral infections early in life can be protective against developing allergic disease or asthma (217). However, not all respiratory infections in early life appear beneficial for allergic airway diseases. In fact, one cohort study demonstrated that among children who wheeze in the first 3 years of life, detection of RSV and HRV by PCR in nasal wash cells increased the risk of asthma development by age 6, especially when combined with allergic sensitization (217). However, to explain this phenomenon, some have suggested there are common genetic predispositions to both respiratory viruses and asthma development. PCR analysis of samples from children undergoing asthma exacerbations revealed that respiratory viruses were associated with an overwhelming 85% of all asthma exacerbations with HRV responsible for two-thirds of all virus induced exacerbations. Thus, like CS exposure, respiratory viruses are involved in both the pathogenesis and exacerbation of asthma and allergic airway diseases. Potential mechanisms for this relationship include the ability of both viruses and allergens to induce immune responses that damage the airway epithelium and thereby increase the potential of either viruses or allergens to create secondary airway inflammation (217). Also, either virus or allergen induced perturbations of the Th1/Th2 cytokine axis may play a role in this association. Upon exposures to pathogens, DCs traditionally direct a Th1 mediated T cell response that may be protective against a Th2 mediated allergic T cell response in a mechanism that is supportive of the "hygiene hypothesis". However, it has been demonstrated that both allergens and certain viruses

like HRV can induce the secretion of TSLP, a Th2 cytokine that drives allergic reactions (218). Therefore, a possible mechanism for the role of virus infections in asthma pathogenesis is that early infection with HRV could "train" the airway epithelium to respond in a Th2 manner and promote development of allergic sensitization and reactions later in life (217). Taken together, current data show that although the role of respiratory viruses in asthma onset is unclear, respiratory viruses play a significant part in asthma exacerbations. Therefore CS exposure can have a two-pronged deleterious effect on children by first increasing the risk of developing asthma and second by increasing the likelihood one will suffer virus-related asthma complications. Controlling both CS exposure and the spread or respiratory viruses could dramatically improve the health of asthmatic children.

Chronic Obstructive Pulmonary Disease

Cigarette Smoke and Air Pollutant Exposure Increases Risk of COPD

COPD is defined as chronic lung disease that may include emphysema and chronic bronchitis with symptoms such as irreversible airway obstruction and loss of lung function (219). Extensive research shows that personal smoking increases the risk of COPD, and in 1984 the surgeon general determined that smoking causes 80-90% of COPD cases in the United States (220). However, not all smokers develop COPD, and smoking does not account for all cases of COPD worldwide (220). Besides smoking, other factors are attributed to COPD incidence. In public statement by the American Thoracic Society, researchers determined that genetic susceptibility (like α1-antitrypsin deficiency) and occupational exposures in coal miners, rock miners, tunnel workers, and concrete workers were linked to COPD causation (220). Exposure to other exogenous

factors like SHS, biomass, and outdoor air pollution like automobile exhaust were also associated with COPD (220). Once COPD is established, CS exposure contributes to disease progression by creating "frustrated" lung phagocytic cells such as macrophages and neutrophils that cannot remove foreign particulates or antigens and therefore secrete excess metalloproteases, proinflammtory cytokines, and ROS that cause tissue damage (221). Therefore, both CS and other air pollutants can contribute to COPD pathogenesis and may share similar mechanisms.

Role of Respiratory Viruses in COPD

Respiratory viruses contribute to COPD disease. In particular, respiratory viruses like influenza, HRV, and RSV have been identified in COPD patients using PCR technology (222). Seemungal et al identified specific respiratory viruses in individuals with COPD both during exacerbations and during disease stability (223). They found that 40% of COPD exacerbations were associated with virus infections, and COPD patients with virus induced exacerbations had longer recovery times (223) Of those virus induced exacerbations, 58% were positive for HRV, 29% were positive for RSV, 16% were positive for influenza A or B, and 11% were positive for coronavirus (223). Another similar cohort study showed that respiratory viruses were identified in 56% of COPD patients with rhinovirus contributing the most at 36% of all virus induced exacerbations (224). Some COPD cases were positive for multiple respiratory viruses (223; 224). The role of influenza infection in COPD exacerbations appears to depend on vaccination status (222). In a cohort of patients with chronic lung disease, patients without influenza vaccinations were twice as likely to be hospitalized and were more likely to die from COPD (222). This mechanism of virus induced exacerbation in COPD could be due to

dysregulation of respiratory immune responses. Data shown in this dissertation demonstrated that overall, current smokers without COPD had suppressed nasal innate immune type I IFN antiviral defenses. However, in COPD patients, CS exposure may enhance inflammatory responses such as IL-6 and IL-8 to respiratory viruses (222). This enhanced inflammatory response has also been shown in mice, as chronic CS exposure increased type I IFN, IL-18, IL-12, and IFNγ responses to poly I:C (225). Respiratory viruses may also play a role in COPD pathogenesis itself. RSV was detected in 32.8% of sputum samples from COPD patients at baseline, and RSV infection correlated with increased inflammation and decreased forced expiratory volume in 1 minute (FEV1), a common indicator of lung function (226). Therefore, chronic respiratory virus infection may contribute to COPD progression and disease worsening beyond acute exacerbations. Although it is unclear if COPD status itself increases the risk of respiratory virus infections compared to normal individuals (222), respiratory viruses clearly play important roles in both COPD pathogenesis and exacerbations.

Public Health and Economic Implications of Cigarette Smoke and Indoor Air Pollution

Smoking is a Local and Global Problem

Despite its health consequences, smoking remains a serious problem worldwide and in the US. In some Asian countries, smokers exceed 60% of males over 15, and countries such as Mongolia, Romania, Yugoslavia, Yemen and Kenya have combined male and female smoking rates of 44% or higher (227). Although smoking rates are comparatively lower in the US, approximately one fifth of US adults smoke (228). The 2004 Surgeon General's Report details that 400,000 Americans will die each year from cigarette smoking with a decreased life expectancy of 12-13 years (228). Overall, the

CDC estimated between 2000 and 2004 in the US, smoking was responsible for approximately $96 billion in direct medical costs and $97 billion in lost work productivity (229). These combined economic costs were calculated at $10.47 per pack of cigarettes (229). Due to a longstanding history of tobacco farming, North Carolina has an adult smoking rate of 22.6% compared to the national average of 20.5% (data from 2005) (228). Even more striking are data suggesting North Carolina has high percentages of adult, youth, and pregnant smokers, which represents a huge public health burden. In addition to being the US leader in tobacco production, North Carolina has high numbers of military bases with elevated smoking rates at approximately 32.2% (data from 2005) (230) in part due to donations, sales, and promotions introduced by cigarette manufacturers that are directed at our armed services (230). As a result, the Defense Department spends more than $209 million dollars annually in increased medical costs due to tobacco (230). Therefore, there are both health and economic costs associated with CS exposure.

Second Hand Smoke Is a Worldwide Burden

We have demonstrated that responses to viral infections are suppressed in smokers, but these observations can be expanded beyond active smokers to individuals exposed to SHS. Our data show that both *in vivo* (3) and *ex vivo* (unpublished observations), nonsmokers routinely exposed to SHS also have suppressed antiviral defenses. Worldwide, billions of people are exposed to SHS. An impressive retrospective analysis using data from 192 countries revealed that overall 40% percent of children, 35% of female nonsmokers and 33% of male nonsmokers were exposed to SHS in 2004 (231). Adult exposures generally occurred at home or in the workplace and child

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exposures were attributed to smoking parents. SHS exposure in nonsmokers decreased disability-adjusted-life-years (DALYs), an estimate of disease burden that quantifies years of life lost to poor health, illness, and death, by 10.9 million (231), and 0.7% of DALYs total disease burden worldwide were due to SHS (231). In children under 5, increases in lower respiratory tract infections in children exposed to SHS contributed the most to disease burden (231). In the US, it is estimated that exposure to SHS causes over $10 billion in increased costs of mortality, morbidity, and medical care annually (232). The dual threat of North Carolina's high smoking rates combined with both seasonal and pandemic influenza infection impacts our state's health and economy. In April 2009, the local outbreak of the H1N1 influenza A strain (formerly known as the "swine flu") emphasized that our community needs to combat influenza infection aggressively. Ambitious vaccination programs and public health education to address hygienic practices may limit the spread of respiratory viruses in the community at large and in populations susceptible to respiratory virus infections, particularly those exposed to CS and SHS. Because SHS increases respiratory disease incidence both in North Carolina and worldwide, reducing SHS exposure through smoking cessation programs is an attractive goal.

Reducing Indoor Air Pollution

Smoking Bans and their Health Effects

For public health officials, this research underscores the importance of smoking cessation programs and smoking bans in protecting citizens not only from smoking associated diseases like lung cancer and emphysema but also from diseases that are exacerbated by smoking like influenza. Within the last decade, many countries and some

US states have implemented workplace smoking bans (233). North Carolina lawmakers recognize the threat of tobacco, and the North Carolina state senate passed a bill to ban smoking in bars and restaurants statewide effective January 2010 (234). On January 1st, 2008, UNC followed UNC Hospitals to institute a campus wide smoking ban to protect its students and employees from the deleterious effects of SHS. Do these smoking bans have health benefits? A review assessing the effects of smoking ban legislation determined that workers felt fewer respiratory symptoms following the ban, and some studies reported increased lung function (233). Although the effects of smoking bans on respiratory infections specifically have not yet been determined, it is very impressive that implementing smoke-free laws caused a drop in percentages of myocardial infarction in the general population (233) thereby demonstrating the effectiveness of smoking bans.

Reducing Biomass Exposure

Biomass exposure from burning coal, wood, dung, and crop remains is a significant component of air indoor air pollution, especially in rural areas, and represents a global threat to respiratory health (114). Efforts suggested to reduce solid fuel exposure during cooking include avoidance through cooking outdoors, cooking in shifts, keeping children away from fires, or switching to "cleaner burning" fuel sources such as petroleum or natural gas (235). Technological advances could render biomass fuel emissions less toxic. Retrofitting old stoves to include chimneys and hoods to draw smoke away from users could be beneficial (235). Bio-filters, packed filters that contain biofilms of pollutant-digesting microorganisms such as bacteria or fungi, have been used to successfully remove ammonia and volatile organic compounds from the emissions food composting plants (236). Biomass waste is also generated in developed countries

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through forestry management, as the burning of trees is necessary to clear excess growth (237). As an alternative to open burning, forestry waste was cut and transported to a biomass electricity generating facility in California for processing, and resulted in decreased PM, nitrogen oxides, non-methane volatile organics, carbon monoxide, and carbon dioxide equivalents compared with open burning (237). Therefore, sophisticated methods to process biomass could simultaneously increase energy output and decrease harmful emissions, although these technologies will certainly be limited in developing countries.

Conclusions and Future Directions

The data presented in this dissertation demonstrate how CS exposure suppresses innate immune responses of the respiratory epithelium. The effects of CS induced suppression of NEC, DC, and NK cell responses in influenza infection may extend to other respiratory infections as well. The mechanisms underlying CS induced susceptibility to influenza virus elucidated here could be applied to other respiratory conditions, such as exposure to airborne pollutants or to chronic airway inflammation. Future directions for this project include using novel triple cell co-culture assays to explore the mechanism underlying this suppression in specific immune cell types (NEC/DC/NK cells) and determining the effects of therapeutic antioxidants on respiratory virus infection in smokers.

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