



2012-06-13

The Pro-Inflammatory Contributions of Receptors for Advanced Glycation End-Products (RAGE) in Alveolar Macrophages Following Cigarette Smoke Exposure

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The Pro-Inflammatory Contributions of Receptors for Advanced Glycation
End-Products (RAGE) in Alveolar Macrophages Following
Cigarette Smoke Exposure

Adam B. Robinson

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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August 2012

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ABSTRACT

The Pro-Inflammatory Contributions of Receptors for Advanced Glycation End-Products (RAGE) in Alveolar Macrophages Following Cigarette Smoke Exposure

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Receptors for advanced glycation end-products (RAGE) are multi-ligand cell surface receptors of the immunoglobulin family expressed by epithelium and macrophages. RAGE expression increases following ligand binding and when diverse cells are exposed to a variety of insults including cigarette smoke extract (CSE). The current research sought to characterize the pro-inflammatory contributions of RAGE expressed by alveolar macrophages (AMs) following CSE exposure. Acute exposure of mice to CSE via nasal instillation revealed diminished bronchoalveolar lavage (BAL) cellularity and fewer AMs in RAGE null mice compared to controls. Primary AMs were obtained from BAL, exposed to CSE *in vitro*, and RNA, DNA, and protein were analyzed. CSE significantly increased RAGE expression by wild type AMs. Employing ELISAs, wild type AMs exposed to CSE had increased levels of active Ras, a small GTPase that perpetuates pro-inflammatory signaling. Conversely, RAGE null AMs had less Ras activation compared to wild type AMs after exposure to CSE. In RAGE null AMs, assessment of p38 MAPK and NF- κ B, important intracellular signaling intermediates induced during an inflammatory response, revealed CSE-induced inflammation occurs at least in part via RAGE signaling. For example, activated p38 was diminished in RAGE null AMs compared to controls and assessment of phosphorylated NF- κ B in CSE exposed RAGE null AMs suggest lessened nuclear translocation of NF- κ B compared to wild type AMs exposed to CSE. Importantly, quantitative RT-PCR revealed that mRNA expression of pro-inflammatory cytokines including TNF- α and IL-1 β were detectably decreased and analysis of secreted proteins by ELISA displayed diminished IL-1 β in RAGE null AMs exposed to CSE compared to CSE-exposed wild type AMs. These results reveal that primary AMs orchestrate CSE-induced inflammation, at least in part, via RAGE-mediated mechanisms.

Keywords: RAGE, lung, macrophages, tobacco smoke

ACKNOWLEDGMENTS

I wish to convey my most sincere thanks to my advisor and mentor, Dr. Paul Reynolds. His standard of knowledge, work ethic, and sincere capacity to aid and guide me will ever be an example to me. This work could not have been completed without his encouragement, patience, and devotion. I wish to thank him for his personal attention and in offering me unique opportunities to move forward in learning and skill. I also wish to thank Dr. Sudweeks and Dr. Poole, for their continued support and insight, and the Department of Physiology and Developmental Biology at Brigham Young University, for providing the opportunity for me to learn and do research at this great facility. Members of the Reynolds' lab have also assisted and supported my research, as well as create an enjoyable and cooperative environment. I appreciate my parents, for their continual support, love, and encouragement. Their diligence, sacrifice, and righteousness will always be guiding principles in my life. Finally, my greatest thanks are for my wonderful wife, Megan, who has supported and sustained me throughout my time at Brigham Young University. I wish to thank her for her love, patience, and abilities as an incredible wife and mother.

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CHAPTER 1: INTRODUCTION

During the sixteenth and seventeenth centuries, tobacco was recommended to treat many medical complaints including airway diseases until epinephrine was introduced in the early twentieth century (46). Decades later, increasing evidence revealed the harmful effects of tobacco smoke, specifically in asthmatics (7). Today, tobacco use is the second major cause of death in the world and by 2030, if trends continue, smoking is estimated to kill more than nine million people annually (50). The number one cause for death related to smoking is myocardial infarction; however smoking significantly increases the risk of death from peripheral vascular disease, cerebrovascular disease, chronic obstructive pulmonary disease, and a host of malignancies including those that affect the lung, breast, pancreas, and prostate (6, 21, 44).

Tobacco smoking is the main risk factor for the hastened degeneration of lung function observed in patients diagnosed with Chronic Obstructive Pulmonary Disease (COPD) (33). COPD is currently the fourth leading cause of death in the world and is projected to increase in the next decade. The indirect yearly cost of COPD has been estimated to be upwards of twenty three billion dollars (10). COPD is characterized by airflow limitations stemming from inflammation of the airways, respiratory parenchyma, and pulmonary vasculature (31). Interestingly, even with smoking cessation, leukocyte-mediated inflammation does not resolve and patients continue to progress (18). Persistent inflammation, a hallmark of COPD, also leads to adverse airway remodeling, culminating in the destruction of lung parenchyma associated with emphysema. Eliciting abnormal enlargement of the distal airspaces, emphysema mechanistically involves inflammation-mediated disruption of elastic fibers and tissue loss that culminate in diminished alveolar septa (16, 20). Despite the pervasiveness of COPD, its precise

pathophysiological mechanisms are not well known. Furthermore, a clearer understanding of causative mechanisms may provide avenues for valuable research into lessening the debilitating effects of disease progression

Cigarette smoke is postulated to consist of over 4700 components (51) and chemical assessment reveals that it contains high levels of reactive oxygen species that activate nuclear factor kappa B (NF- κ B). Cigarette smoke contains approximately 10^{17} oxidants per puff (32), several carcinogens including nitrosamines, polycyclic aromatic hydrocarbons, and aromatic amines (13), and thousands of other chemicals that have cytotoxic, mutagenic, carcinogenic, or antigenic properties (26). These compounds and cytokines stimulate and interact with nearly all airway cells, causing a variety of signaling cascades to occur. Such cascades are mediated by the sequential activation of intermediate signaling proteins including mitogen activating protein kinases (MAPK) such as ERK1/2, JNK, and p38, and transcription factors such as early growth response gene 1 (EGR-1) and NF- κ B, furthering the expression of additional cytokines and inflammatory mediators (28, 36, 41). Specifically, p38 kinases, a family of MAPKs detected in epithelial cells and macrophages, have been found to be involved in the inflammatory response following cigarette smoke exposure (8).

Inflammation involves interrelated processes that center on the activation of the immune system in order to defend against infection and/or repair tissue damage. The effects of even basic inflammatory responses become amplified and problematic in the lung due to the critical nature of its role in gas exchange. A significant outcome of inflammation in the lung includes the influx and accumulation of phagocytic and reactive immune cells at the site of injury, including monocytes and macrophages (11). Macrophages are not only key to

eradicating the body of pathogens, debris, apoptotic cells, and some tumor cells, they are also one of the most active secretory cells in the body (24, 25). At a site of acute inflammation, monocytes aggregate, often stimulated to differentiate into their progeny macrophages, leading to increased inflammatory activation (52). Activated macrophages simultaneously produce pro-inflammatory cytokines that amplify the immune response by up-regulating adhesion molecules required for neutrophil and monocyte chemotaxis. These same cytokines also help to induce monocyte differentiation into macrophages. Primary cytokines that have been found to be responsible for acute inflammation stemming from tobacco smoke exposure include tumor necrosis factor α (TNF), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) (47). In addition to pro-inflammatory cytokines, macrophages also secrete collagenases and elastases that destroy connective tissue contributing to emphysema (29). Interestingly, the current hypothesis describing mechanisms leading to COPD suggest cigarette smoke causes airway inflammation by activating immune cells including alveolar macrophages, which release pro-inflammatory mediators such as those previously described (18, 42).

Cytokines are small protein molecules involved in intercellular signaling, some of which can be found up-regulated most often during an immune response. Cytokines that perpetuate the immune response are termed “pro-inflammatory” cytokines such as IL-1 β , TNF- α , and others. Release of pro-inflammatory cytokines appears from nearly any nucleated cell and are known to locally stimulate leukocyte proliferation, cytotoxicity, release of proteolytic enzymes, and synthesis of prostaglandins and initiate a cascade of “secondary” cytokine synthesis and secretion (5). However, endothelium, epithelium, and macrophages are the most potent producers of IL-1 β , TNF- α , and IL-6 (5). IL-1 β signals through the IL-1 receptor, which is shown

to lead to downstream activation of NF- κ B, and leads to effects similar to TNF- α excluding inducing apoptosis (9, 12). TNF- α , one of the first cytokines to be discovered, was initially described as the principal mediator of endotoxin related inflammation and is now known to be a key player in the development of septic shock (1). TNF- α binds TNF receptors signaling activation of JNK/MAPK such as p38 MAPK, increasing translocation NF- κ B, and stimulating production of several pro-inflammatory cytokines, growth factors, and reactive oxygen species (25). TNF signaling cascades lead to possible cell-induced apoptosis, leukocyte migration, activation of phagocytic immune cells, and exacerbation of tissue injury (25, 49).

RAGE, the receptor for advanced glycation end-products, is a member of the immunoglobulin superfamily of cell surface receptors found in various cell types including smooth muscle cells, fibroblasts, macrophages, and epithelium (40). The receptor contains a V-region-like domain essential for ligand binding and two C-region like domains, a single-pass hydrophobic transmembrane domain and a short, 43 amino acid, highly charged cytoplasmic domain essential for intracellular signaling (4). RAGE is found to exist in several isoforms, namely full length RAGE, dominant negative RAGE (dn-RAGE), and soluble RAGE (sRAGE). The full length RAGE receptor contains all domains; however, splicing variants may give rise to the two other mentioned isoforms. Dn-RAGE is a membrane anchored splice variant of RAGE capable of ligand binding but lacking the intracellular domain necessary for signal transduction. RAGE has also been shown to exist in a soluble secreted form sRAGE. The sRAGE isoform yields the same V and C-regions of the full length-RAGE but lacks both the hydrophobic transmembrane and the intracellular domains (4). The altered variants of RAGE are thought to bind and mop up ligands without the consequences of activating signal transduction and

initiating gene transcription and have been proposed as possible therapeutic targets in the reduction of RAGE-mediated inflammatory responses in various tissues.

RAGE is a dynamic receptor capable of binding ligands of various types, being able to distinguish tertiary structures (30). RAGE is most abundant in the lung (3) with sparse expression in alveolar type II (ATII) cells and abundant expression on basolateral membranes of alveolar type I (ATI) differentiated cells (43). Recently, RAGE has been shown by our lab to mediate inflammatory signals following smoke exposure in lung epithelial cells (38, 39). Initially characterized and named for its ability to bind to non-enzymatically glycosylated macromolecules, advanced glycation end-products (AGEs), RAGE also binds a myriad of other molecules including pro-inflammatory cytokine-like mediators of the S100/calgranulin family (S100A12 and S100B), amyloid β -fibrils, high mobility group box 1 (HMGB1), Mac-1 (4, 43), and specific DNA and RNA structures (45).

Because RAGE binds a handful of ligands it is thus linked to several signaling pathways. These include PI3/Akt (48), RhoGTPases (19), Jak/STAT (22), and Src family kinases (34). Of notable interest are two pathways stimulated by RAGE activation that are in response to either damage-associated molecular patterns (DAMPs) (NF- κ B pathways) (2) or tobacco smoke-induced pulmonary inflammation (Ras pathway) (40). Key DAMP molecules are those of the S100/calgranulin family and HMGB1, both of which bind to RAGE among other receptors including toll-like receptor 4 (TLR4) (17, 39). These molecules, normally secreted following apoptotic or necrotic events, can serve as trophic factors in low concentrations or enhance the inflammatory/cell death response in high concentrations. Downstream gene products produced through RAGE signaling are thought to include NF- κ B, Cox-2, IL-1 β , and TNF- α (2).

Because the RAGE gene contains NF- κ B and SP-1 promoter binding sites (27) and is regulated by Egr-1 in cases of tobacco smoke-related disease (37), a possible auto-inflammatory loop may be triggered giving suggestion to RAGE involvement in chronic disease states.

Previous work in our lab demonstrated that RAGE, S100A12, and HMGB1 were up regulated in several immortalized cell lines including rat (R3/1) and human (A549) alveolar epithelial cells and macrophages (RAW264.7) following exposure to cigarette smoke extract (CSE). Additional published studies also reported that Ras was induced in R3/1 cells after exposure to CSE, resulting in up-regulation of NF- κ B, leading to increased secretion of pro-inflammatory cytokines such as TNF- α and IL-1 β (36, 40). Ras is an intracellular signaling molecule that alternates between an active guanosine triphosphate bound and inactive guanosine diphosphate bound state (14, 35). Furthermore, Ras is a key regulator of many features of normal cell growth and malignant transformation (15). Ras has also been associated with development, cellular proliferation, and differentiation, as a result of the signaling through MAPK kinase (35). When macrophage RAGE is stimulated by HMGB1, three MAP kinases, p38 MAPK, p44/42 MAPK, and SAPK/JNK were phosphorylated (23). Whether Ras mediates the activities of these signaling intermediates is untested. Furthermore, the direct role of Ras signaling following pulmonary macrophage stimulation by CSE and the subsequent release of cytokines requires further investigation. This and other research that considers acute lung exposure to cigarette smoke may yield important data related to alveolar macrophage activation. Furthermore, RAGE may be further implicated in the debilitating expression of pro-inflammatory cytokines and their functions in respiratory disease.

The following chapter (ch.2) is a copy of the published manuscript that includes the research completed during my tenure at BYU studying alveolar macrophages and their role in cigarette smoke-induced inflammation. The manuscript was published in the American Journal of Physiology, Lung Cellular and Molecular Physiology.

Chapter 2

Robinson AB, Johnson KD, Bennion BG, Reynolds PR. RAGE signaling by alveolar macrophages influences tobacco smoke-induced inflammation. *Am J Physiol Lung Cell Mol Physiol*. 2012 Apr 13. [Epub ahead of print]

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CHAPTER 2: RAGE SIGNALING BY ALVEOLAR MACROPHAGES INFLUENCES TOBACCO SMOKE-
INDUCED INFLAMMATION

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Running Title: RAGE signaling in smoke-exposed macrophages.

This work was supported by a grant from the Flight Attendant's Medical Research Institute (P.R.R.) and a Brigham Young University Mentoring Environment grant (P.R.R.).

Abstract

Receptors for advanced glycation end-products (RAGE) are multi-ligand cell surface receptors of the immunoglobulin family expressed by epithelium and macrophages and expression increases following exposure to cigarette smoke extract (CSE). The current study sought to characterize the pro-inflammatory contributions of RAGE expressed by alveolar macrophages (AMs) following CSE exposure. Acute exposure of mice to CSE via nasal instillation revealed diminished bronchoalveolar lavage (BAL) cellularity and fewer AMs in RAGE knock out (KO) mice compared to controls. Primary AMs were obtained from BAL, exposed to CSE *in vitro*, and analyzed. CSE significantly increased RAGE expression by wild type AMs. Employing ELISAs, wild type AMs exposed to CSE had increased levels of active Ras, a small GTPase that perpetuates pro-inflammatory signaling. Conversely, RAGE KO AMs had less Ras activation compared to wild type AMs after exposure to CSE. In RAGE KO AMs, assessment of p38 MAPK and NF- κ B, important intracellular signaling intermediates induced during an inflammatory response, revealed CSE-induced inflammation may occur in part via RAGE signaling. Lastly, quantitative RT-PCR revealed that the expression of pro-inflammatory cytokines including TNF- α and IL-1 β were detectably decreased in RAGE KO AMs exposed to CSE compared to CSE-exposed wild type AMs. These results reveal that primary AMs orchestrate CSE-induced inflammation, at least in part, via RAGE-mediated mechanisms.

Key words: RAGE, lung, macrophages, tobacco

Introduction

If trends continue, tobacco use in 2030 will kill an estimated nine million people annually (56). Smoking is the main risk factor for degeneration of lung function observed in patients with chronic obstructive pulmonary disease (COPD). COPD is currently the fourth leading cause of death worldwide and in 2010 the economic burden associated to COPD was projected to be \$49.9 billion, which includes \$29.5 billion in direct costs (8). COPD is characterized by airflow limitations stemming from inflammation of the airways, respiratory parenchyma, and pulmonary vasculature (32). Interestingly, even with smoking cessation, leukocyte-mediated inflammation does not resolve and patients continue to progress (18). Persistent inflammation also leads to adverse airway remodeling, culminating in the destruction of lung parenchyma observed in emphysema. Eliciting abnormal enlargement of the distal airspaces, emphysema mechanistically involves inflammation-mediated disruption of elastic fibers and tissue loss that culminate in diminished alveolar septa (13, 21). Despite the pervasiveness of COPD, its precise pathophysiological mechanisms are not well known. Furthermore, a clearer understanding of causative mechanisms may provide avenues for research into lessening the debilitating effects of disease progression.

Inflammation involves interrelated processes that center on the activation of the immune system in order to defend against infection and/or repair of damaged tissue. The effects of even basic inflammatory responses become amplified and problematic in the lung due to the critical nature of its role in gas exchange. A significant outcome of inflammation in the lung includes the influx and accumulation of phagocytic and reactive immune cells at the site of injury (16). Activated macrophages produce pro-inflammatory cytokines that amplify the

immune response via up-regulation of adhesion molecules required for leukocyte chemotaxis. Simultaneously, cytokines also help to induce monocyte differentiation into additional macrophages (15). Among others, the primary cytokines responsible for acute inflammation stemming from tobacco smoke exposure include tumor necrosis factor- α (TNF- α) and Interleukin-1 β (IL-1 β) (7, 48). In addition to pro-inflammatory cytokines, macrophages also secrete collagenases and elastases that destroy connective tissue in the lung.

The receptor for advanced glycation end-products, or RAGE, is a member of the immunoglobulin superfamily of cell surface receptors found in various cell types including smooth muscle cells, fibroblasts, macrophages, and epithelium (40). RAGE is most abundant in the lung (2) with sparse expression by alveolar type (AT) II cells and abundant expression on membranes of differentiated ATI cells (44). Although RAGE was first described as a progression factor in cellular responses induced by advanced glycation end-products (AGEs) that accumulate in hyperglycemia and oxidant stress, studies have also indicated that RAGE binds a myriad of other molecules (28) including pro-inflammatory cytokine-like mediators of the S100/calgranulin family (S100A12 and S100B), amyloid β -fibrils, high mobility group box 1 (HMGB1), Mac-1 (3, 44) and specific DNA and RNA structures (45).

RAGE expression increases as its ligands accumulate and RAGE-ligand interactions may contribute to pathological processes including diabetic complications, neurodegenerative disorders, atherosclerosis, and inflammation. We have recently demonstrated that RAGE influences CSE-induced inflammation mediated by pulmonary epithelial cells. Specifically CSE induces pulmonary epithelium to increase the expression of RAGE, its ligands, Egr-1 (a transcription factor abundantly expressed in the lungs COPD patients), pro-inflammatory

signaling intermediates, and various cytokines (37-39). The possibility that AMs cooperate with pulmonary epithelium in CSE-induced inflammation via up-regulation of RAGE and its signaling pathway has not yet been assessed.

As an intracellular signaling molecule that regulates the fate of target cells, Ras oscillates between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)- bound conformations (11). Ras signaling has been associated with development, cellular proliferation, and differentiation, as a result of signaling through Raf/MAP kinase, PI3K, JNK/p38, and Rho pathways (36). Furthermore, Ras is a key regulator of many features of normal cell growth and malignant transformation, as a result of the signaling through mitogen-activated protein kinases (MAPKs) (12). Published work by our lab provides evidence indicating that the role of Ras in inflammation appears to be downstream of RAGE in pulmonary epithelial cells. However, the role of Ras and other intermediate signaling molecules in AMs stimulated by CSE requires further investigation.

In the present study, we tested the hypothesis that AMs exposed to CSE induce RAGE and that signaling involving Ras, p38 MAPK, and pro-inflammatory cytokine secretion occurs via RAGE-mediated mechanisms. Although RAGE KO AMs are purported to be indistinguishable from AMs from wild type strains of mice (53), we demonstrate that RAGE KO AMs exposed to CSE experience reduced Ras and p38 MAPK activation, less NF- κ B translocation, and diminished production of TNF- α and IL-1 β when compared to CSE exposed wild type AMs. Collectively, these data offer new insights into the potential mechanisms whereby RAGE expressed by AMs participates in inflammation following tobacco smoke exposure. Further research may

demonstrate that RAGE, and its specific downstream effectors, are potential targets in the treatment or prevention of tobacco smoke related pulmonary complications.

Materials and Methods

Mice

Wild type C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). RAGE KO mice that lack RAGE were generated in a C57Bl/6 background (50). In line with *The Guide for the Care and Use of Laboratory Animals* and approved IACUC protocols at Brigham Young University, mice had free access to food and water and were housed in a dedicated animal facility that controlled for light, temperature, and humidity. Bronchoalveolar lavage fluid (BALF) was obtained from RAGE KO and C57Bl/6 control mice as outlined below. Additional mice were nasally instilled with either sterile PBS or 10% CSE (37) as outlined previously (35). 24 hours after nasal instillation, lungs (n = 6 mice per group) were inflated fixed for immunohistochemistry as already described (38) or used to determine total cell quantity and differential cell counts in BALF as already outlined (35).

Bronchoalveolar lavage fluid (BALF) and cell culture

To obtain BALF, mice were sedated and then exsanguinated to ensure euthanasia as outlined previously (40). BALF was specifically harvested through the instillation and recovery of seven 1 ml boluses of PBS attached to a catheter for a total of 7 ml. Each 7 ml sample of BALF was centrifuged at 1000 RPM for 10 minutes. The cell pellet was resuspended in warm DMEM and equal concentrations of approximately 50,000 cells were plated and allowed to adhere overnight before exposure to CSE (37) or fresh DMEM. Cells were exposed to 10% CSE for 30 minutes to 4 hours depending on the experiment. Following exposure to either CSE or fresh media, cells were washed with two changes of PBS before total RNA or protein was isolated.

Immunocytochemistry and quantitative RT-PCR

Cells obtained from BALF were washed and fixed with 4% paraformaldehyde and stained for Mac-3 (1:50, BD Biosciences, San Jose, CA), an antibody that recognizes the 110 kDa Mac-3 protein expressed by mononuclear phagocytes, and a goat anti-rat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as outlined previously (39). Sections of lung tissue (5 μ m each) were similarly immunostained with Mac-3 in order to qualitatively assess relative AM quantity. Total RNA was isolated from cells using the Qiagen RNeasy Plus Micro kit (QIAGEN, Valencia, CA,) in accordance with the provided instructions and reverse transcription of RNA was performed using the Invitrogen Superscript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) in order to obtain cDNA for PCR. The following primers were synthesized and HPLC purified by Invitrogen Life Technologies: RAGE (For-ACT ACC GAG TCC GAG TCT ACC and Rev-GTA GCT TCC CTC AGA CAC ACA), TNF- α (For-TGC CTA TGT CTC AGC CTC TTC and Rev-GAG GCC ATT TGG GAA CTT CT), IL-1 β (For-TGT AAT GAA AGA CGG CAC ACC and Rev-TCT TCT TTG GGT ATT GCT TGG), and GAPDH (For-TAT GTC GTG GAG TCT ACT GGT and Rev-GAG TTG TCA TAT TTC TCG TGG). cDNA amplification and data analysis were performed using Bio Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and a Bio Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Primers were used at a concentration of 75 nM each in 25- μ l reactions. Cycle parameters were as follows: 3 min at 95°C, and 40 cycles composed of 1 min at 95°C, 15 sec at 55°C and 15 s at 72°C. Control wells lacking template or RT were included to identify primer-dimer products and to exclude possible contaminants.

Ras activation ELISA

Ras Activation ELISA Kits (Millipore, Temecula, CA) were used to assess active and inactive Ras (39). Cell lysates were quantified by BCA assay then screened for Ras in 20 μ g aliquots of total cell lysates obtained by homogenizing cells in RIPA buffer with inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). *In vitro* experiments were repeated at least three times, each in triplicate.

p38 MAPK and NF- κ B Fast Activated Cell-Based ELISA (FACE)

Total and active p38 and NF- κ B levels were assessed through the use of colorimetric high-throughput FACE assays available from Active Motif (Carlsbad, CA). Briefly, freshly isolated AMs from RAGE KO and control mice were plated overnight as outlined and a time course of 10% CSE exposure that ranged from 30 minutes to 4 hours was conducted. Cells were then screened with antibodies specific to total and active phosphorylated proteins as outlined in the manufacturer's instructions.

Statistics

Mean values \pm S.D. from three experimental replicates were assessed by one and two-way analysis of variance (ANOVA). When ANOVA indicated significant differences, student t tests were used with Bonferroni correction for multiple comparisons. Results are representative and those with p values <0.05 were considered significant.

Results

AMs induce RAGE expression following CSE exposure

Wild type and RAGE KO mice were exposed to an acute bolus of PBS or CSE and BALF was assessed. Total cell quantity in BALF was significantly increased in wild type mice following CSE exposure compared to PBS-instilled controls (Figure 1A). Even though total cell quantity was also elevated in RAGE KO BALF, total BALF cells were significantly diminished in RAGE KO mice exposed to CSE compared to CSE-exposed wild type animals (Figure 1A). The percentage of AMs in WT and RAGE KO BALF following acute CSE exposure trended downward, but was not significantly different when compared to PBS-exposed mice (Figure 1B). Lung sections from RAGE KO and wild type animals were also immunostained for Mac-3 24 hours after a single exposure to PBS or CSE by nasal instillation. There was a detectable increase in Mac-3 positive cells in the wild type lung exposed to CSE (Figure 1D) compared to age-matched wild type littermates exposed to PBS vehicle only (Figure 1C). In addition, qualitative assessment of AMs in CSE-exposed RAGE KO mice by immunostaining (Figure 1E) revealed elevated Mac-3 cells, but most histological views appeared similar to wild type PBS-exposed mice (Figure 1C). In order to dissect RAGE-mediated signaling in AMs, total BAL cells from wild type and RAGE KO mice were harvested and cultured as outlined in Materials and Methods. So that a stable population of AMs could be identified, immunohistochemistry was performed using a Mac-3 antibody and significant immunoreactivity was observed in adherent cells (Figure 1F, G).

In order to assess whether RAGE signaling was involved in primary AMs exposed to CSE, we evaluated the degree to which RAGE was expressed at baseline and whether RAGE expression increases following exposure to CSE. Through experiments involving quantitative

RT-PCR, we discovered that there was a significant increase in RAGE expression by primary AMs isolated from wild type mice following CSE exposure compared to wild type AMs exposed to fresh media (Figure 2). RAGE KO AMs were similarly evaluated and as anticipated, no RAGE expression was detected in both the CSE or media exposed cell populations (Figure 2).

RAGE KO AMs have significantly diminished CSE-induced Ras activation

Active Ras, an intracellular molecular switch involved in many signal transduction pathways, was recently assessed in alveolar epithelium exposed to tobacco smoke (39). Our research revealed RAGE-mediated mechanisms of elevated Ras activity that precedes inflammatory molecule elaboration in response to smoke exposure. In order to test the hypothesis that Ras is also activated in primary AMs exposed to CSE, we exposed AMs isolated from wild type and RAGE KO mice to 10% CSE over the course of 1, 2, and 4 hours. Using techniques that immunologically assess active Ras, we discovered a significant increase of active Ras expression in wild type primary AMs after only one hour of CSE exposure (Figure 3). The data related to the time course revealed consistent increases in Ras activity as the duration of CSE exposure progressed. Ras activity was also increased in primary AMs obtained from RAGE KO mice; however, the amount of active Ras was consistently and significantly decreased at each time point assessed (Figure 3).

RAGE KO AMs have significantly diminished CSE-induced p38 MAPK and NF- κ B activation

Because RAGE is elevated in AMs that encounter tobacco smoke and Ras, an important cytosolic intermediate that perpetuates inflammation, is activated in part by RAGE, it was important to dissect additional RAGE-mediated signaling targets. A series of experiments was performed in order to analyze the activity of p38, an important mitogen-activated protein

kinase (MAPK) involved in inflammatory cell signaling (17). Total p38 MAPK was not significantly different when comparing AMs from RAGE KO or wild type mice (Figure 4A). However, total p38 MAPK was decreased in both AM populations after 30 minutes of CSE exposure (Figure 4A). Data revealed that CSE exposure for 30 minutes resulted in increased phosphorylation and subsequent activation of p38 MAPK in both wild type and RAGE KO AMs (Figure 4B). Importantly, p38 MAPK activation in RAGE KO AMs after 60 minutes of CSE exposure was significantly diminished when compared to levels observed in CSE-exposed wild type AMs (Figure 4B).

We next assayed the state of NF- κ B activation in cells exposed to CSE. As a downstream target of Ras and MAPKs, including p38, NF- κ B is phosphorylated and liberated from cytosolic sequestration during an inflammatory response and it functions as a potent nuclear transcription factor. Total NF- κ B was not significantly different when comparing AMs from RAGE KO and wild type mice (Figure 5A). Results revealed that the expression of active NF- κ B in CSE-exposed RAGE KO AMs was significantly reduced when compared to CSE-exposed AMs from wild type controls (Figure 5B). Combined, these data suggest that CSE induces the activation of p38 MAPK and NF- κ B in AMs at least partially through RAGE signaling pathways.

CSE-induced pro-inflammatory cytokine secretion is diminished in RAGE KO AMs

In order to test the hypothesis that CSE-induced RAGE expression leads to pro-inflammatory cytokine production, we analyzed the levels of TNF- α and IL-1 β in AMs with and without CSE exposure. Following exposure to CSE for two hours, quantitative RT-PCR revealed a significant increase in the expression of both cytokines by primary AMs from wild type mice (Figure 6). Production of TNF- α (Figure 6A) and IL-1 β (Figure 6B) mRNA was significantly

decreased in primary AMs from RAGE KO mice exposed to CSE compared to CSE-exposed AMs from wild type controls. Although AMs from both wild type and RAGE KO mice increased cytokine mRNA expression following CSE exposure, primary wild type AMs exposed to CSE had significantly more cytokine expression when compared to CSE-exposed RAGE KO AMs. Differential expression of TNF- α and IL-1 β after two hours of CSE exposure (Figure 6) was also observed in experiments that involved four hours of CSE exposure (not shown). An assessment of secreted cytokines after four hours revealed that IL-1 β production was similar to the mRNA profile in that a significant decrease was observed in media obtained from CSE-exposed KO AMs compared to CSE-exposed WT AMs (Table 1). Interestingly, acute TNF- α secretion after four hours was not different in any of the groups regardless of CSE exposure (Table 1).

Discussion

AMs and tobacco smoke-induced RAGE expression

Macrophages are dynamic cells capable of remarkably diverse gene expression patterns following signal recognition of tissue-specific events. In particular, phagocytosis and enhancement of the inflammatory response are defining macrophage characteristics in programmed responses to acute and chronic pathological conditions (9). In fact, macrophages are significant contributors to the organism's immune system, with functions that include phagocytic clearance of pathogens and other debris and service as a reservoir for a variety of mediators that regulate inflammation, adaptive immunity, and homeostasis (25). Such modulators of inflammation and homeostasis are normally released to target invading pathogens and enhance wound repair (24). However, regulatory mechanisms that control inflammatory mediators can also become dysfunctional when external stimulation, including exposure to tobacco smoke, persists. Such chronic stimulation of macrophage-mediated immune responses may culminate in irreversible remodeling of resident tissues.

Originally characterized for its ability to bind AGEs, abundant data implicate RAGE as a potent feed-forward receptor involved in inflammation (46). AGEs arise *in vivo* from the non-enzymatic addition of reducing sugars to amino groups (30). While AGEs such as carboxymethyllysine (CML) are formed more abundantly at sites of inflammation in hyperglycemic renal failure and in areas of localized oxidative stress (52), tobacco-derived AGEs formed via Maillard chemistry also provide abundant ligand for RAGE signaling (29). Importantly, RAGE is a pattern recognition receptor, so there is a clear likelihood that diverse,

yet related AGEs derived by a burning cigarette combine to induce pro-inflammatory RAGE signaling (5).

Our previous research demonstrated that exposure of pulmonary epithelial cells to CSE led to increased expression of RAGE and its ligands, suggesting that epithelial cells respond acutely to CSE by stimulating molecules required in the initial stages of RAGE signaling (38). Importantly, RAW264.7 cells, a murine macrophage cell line, up-regulated RAGE expression following exposure to CSE (38). Despite these data, the literature was silent in terms of RAGE biology in murine primary AMs that encounter tobacco smoke. Although not as robustly characterized in macrophages, RAGE has been shown to be the receptor responsible for HMGB1-induced inflammation in rodent macrophages (22). Studies document that low levels of RAGE are expressed by macrophages in normal conditions and that RAGE overexpression is observed in macrophages that elicit inflammation and cause lung damage (28). Morbini et al. confirmed the presence of AGEs in macrophages and observed AGE and RAGE are both coexpressed by these immune cells (28). Our discovery that RAGE was markedly increased by primary AMs exposed to tobacco smoke led to the hypothesis that RAGE signaling influences acute responses by macrophages in smoke environs.

RAGE signaling during tobacco smoke exposure

Activation of RAGE is dependent upon a variety of ligands and RAGE/ligand interaction leads to the activation of diverse signal transduction pathways, including Ras-extracellular signal regulated kinase 1/2 (ERK1/2), Cdc42/Rac, stress-activated protein kinase/c-Jun-NH2-terminal kinase (SAPK/JNK) and p38 MAPK pathways (14). These signaling events can culminate in the activation of transcription factors including NF- κ B or CREB (20, 55). Our lab has

previously published data that proposes the likelihood of a positive feedback loop in which ligand binding led to intracellular signaling that eventually enhanced additional RAGE expression (38). Furthermore, RAGE in CSE-exposed lung epithelial cells has been shown to activate Ras in the initiation of smoke-induced pro-inflammatory signaling (39). Our data detailing Ras activation in concert with increased RAGE availability links this important signaling cascade in AMs with similar effects observed in pulmonary epithelial cells. Because anomalous Ras expression has been implicated as a biomarker for COPD (26), our research suggests plausible cooperation between pulmonary epithelial cells and resident macrophages in advanced stages of inflammatory disease.

Our data further demonstrated that AMs exposed to CSE trigger an intracellular signaling pathway mediated by MAPKs and NF- κ B. Specifically, p38 MAPK activity peaked in AMs from wild type mice after just 30 minutes of CSE exposure (Figure 4). While p38 MAPK activation was also observed in RAGE KO AMs, there was a significant decrease in p38 MAPK activity after 60 minutes of CSE exposure compared to wild type AMs (Figure 4). We also demonstrated that after four hours, nuclear translocation of active NF- κ B was significantly diminished in smoke-exposed RAGE KO AMs compared to smoke-exposed AMs from control mice (Figure 5). While RAGE signaling via p38 MAPK and NF- κ B has recently been proposed in diverse tissue types under various conditions (19, 41, 58), such signaling paradigms have not been elucidated in AMs exposed to tobacco smoke. Importantly, the signaling kinetics presented in the current research are similar to those presented by Zhang et al. in that p38 MAPK activation peaked at 30 minutes and NF- κ B activation peaked at four hours following exposure of RAW234.7 to AGEs (57).

RAGE-mediated inflammatory cytokine secretion by AMs

The current investigation identified significant up-regulation of TNF- α and IL-1 β , two pro-inflammatory cytokines related to COPD pathogenesis (6), by wild type AMs. A substantial element of our research revealed that these important pro-inflammatory effector molecules were significantly decreased in CSE-exposed AMs that lacked the capacity to express RAGE. The current work is a natural extension of research by others that suggest an overall theme of RAGE-mediated responses to inflammatory insults including lipopolysaccharide (LPS), *Escherichia coli*, or HMGB-1 (22, 34, 47, 54). An intriguing discovery was that differential cytokine expression by RAGE KO AMs as early as two hours after CSE exposure occurred. Importantly, Wesselborg and colleagues outlined separate parallel pathways involving MAPK/NF- κ B in the orchestration of inflammation triggered by a variety of cellular stresses (51). While our data demonstrate the plausibility of linear signaling that involves Ras, p38, and NF- κ B as intracellular intermediates, the modulation of inflammatory cytokines by RAGE after only two hours of CSE exposure (Figure 6) suggests the likelihood of alternative parallel pathways.

Research by others confirmed that macrophage numbers are significantly increased in the airways, parenchyma, BALF, and sputum of patients with COPD (1) and that activated alveolar macrophages secrete various cytokines and chemokines following cigarette smoke exposure (4). Additionally, specific analysis of peripheral blood monocytes from active smokers further showed that enhanced NF- κ B activation (49) coincided with altered levels of TNF- α , IL-1 β , IL-6, and IL-8 in BALF (27, 42). Primarily expressed by macrophages, TNF- α stimulates the release of diverse pro-inflammatory and cytotoxic mediators including IL-1 β , IL-6, platelet activating factor (PAF), eicosanoids, and reactive oxygen species (25). TNF- α has been shown to

regulate cellular proliferation (25) and the expression of ICAM-1, an endothelial adhesion molecule involved in leukocyte transmigration (23, 27). AM secretion of TNF- α is increased in experimental models of tissue injury (33) and when levels of TNF- α increase, emphysematous damage to lung parenchyma mediated by the release of matrix metalloproteinases (MMPs) occurs (31, 48). IL-1 β is another potent cytokine released by macrophages known to stimulate additional chemokine production, recruit leukocytes to sites of injury, and feedback to induce elevated production of TNF- α (17). Like TNF- α , IL-1 β can also trigger MMP secretion, particularly MMP-9 (43), increase the proliferation of fibroblasts (10) and enhance collagen synthesis (48). Sputum and lavage fluid from smokers have increased levels of IL-1 β , and it is proposed that high levels of IL-1 β are inversely related to pulmonary function (7). It is possible that while additional mediators may lead to the amplification of inflammatory events coordinated by TNF- α and IL-1 β , significantly diminished levels of these molecules in RAGE KO AMs exposed to smoke reveal distinct RAGE-mediated pathways.

Conclusions

The current study provides support for a model of smoke-exposed AMs wherein RAGE-mediated signal transduction pathways influence inflammation. Therefore, our findings that smoke increases RAGE expression, activates known intracellular pathways, and causes NF- κ B-mediated cytokine elaboration have important implications for elucidating the mechanisms of progressive lung inflammation experienced by both former smokers and those unable or unwilling to quit. Clarifying RAGE signaling in the context of possible parallel pathways remains an important consideration, however. As such, additional research that centers on the

important role of RAGE signaling and the resulting inflammatory response should remain a priority.

Acknowledgments

The authors appreciate greatly the advice and support of Dr. Sterling N. Sudweeks (Brigham Young University) for invaluable assistance with quantitative RT-PCR experiments. The authors also wish to thank Jeffrey A. Stogsdill, Ali C. Fredrickson, and Tayler L. Sefcik (Brigham Young University) for insightful discussion and valuable suggestions during the course of the studies.

Author Contributions

ABR managed the research study and participated in all aspects of the various experiments. KDJ and BGB assisted with the procurement of AMs and quantitative RT-PCR. PRR conceived of the study and supervised in its implementation, interpretation, and writing. All authors participated in the preparation of the manuscript and each approved of the final version.

Figures and Tables

Figure 1

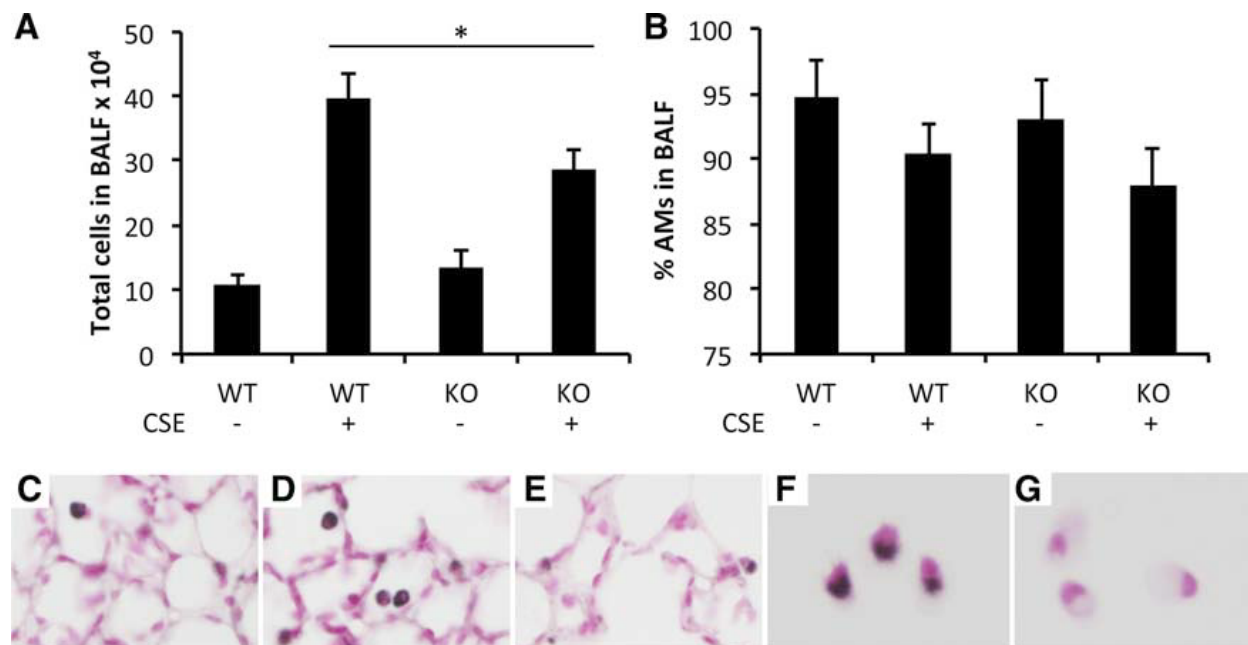


Figure 1 Bronchoalveolar lavage fluid was procured from RAGE KO and wild type mice acutely exposed to CSE and assessed. Total BALF cells and the percentage of AMs were significantly diminished in RAGE KO animals exposed to CSE compared to CSE-exposed controls (A, B). Twenty four hours after a single nasal instillation of PBS (C) or 10% CSE (D), wild type mice stained for Mac-3 revealed a marked increase in Mac-3 positive AMs following CSE exposure. RAGE KO mice were similarly administered 10% CSE and there was a detectible decrease in the number of Mac-3 positive AMs following qualitative immunohistochemical assessment (E). BALF cells were procured, plated and immunostained with Mac-3 in order to identify a homogenous AM population. All adherent cells expressed Mac-3 (A) and immunostaining in the absence of primary antibody revealed no immunoreactivity (B). BALF

analyses were performed in triplicate and $*p \leq 0.05$ (ANOVA). Representative images (400X original magnification) of $n = 3$ mice in each group are shown.

Figure 2

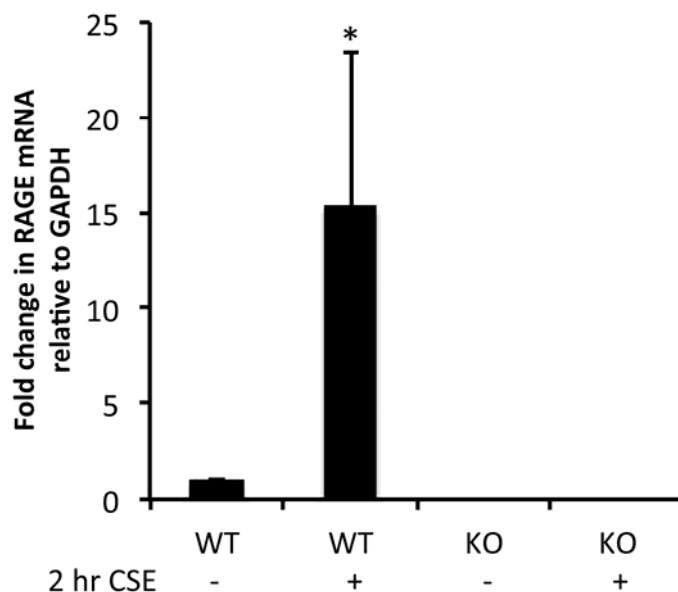


Figure 2 Primary AMs from wild type or RAGE KO mice were plated overnight, exposed to fresh media or 10% CSE for two hours, then screened by quantitative RT-PCR. There was a significant increase in the expression of RAGE by wild type AMs exposed to CSE and RAGE was not detected in RAGE KO AMs with or without CSE exposure. RAGE transcripts were normalized to GAPDH and representative data of experiments performed in triplicate are shown. $*p \leq 0.05$ (Student's t tests were conducted between both wild type groups and no tests were conducted in KO samples due to failure to detect RAGE).

Figure 3

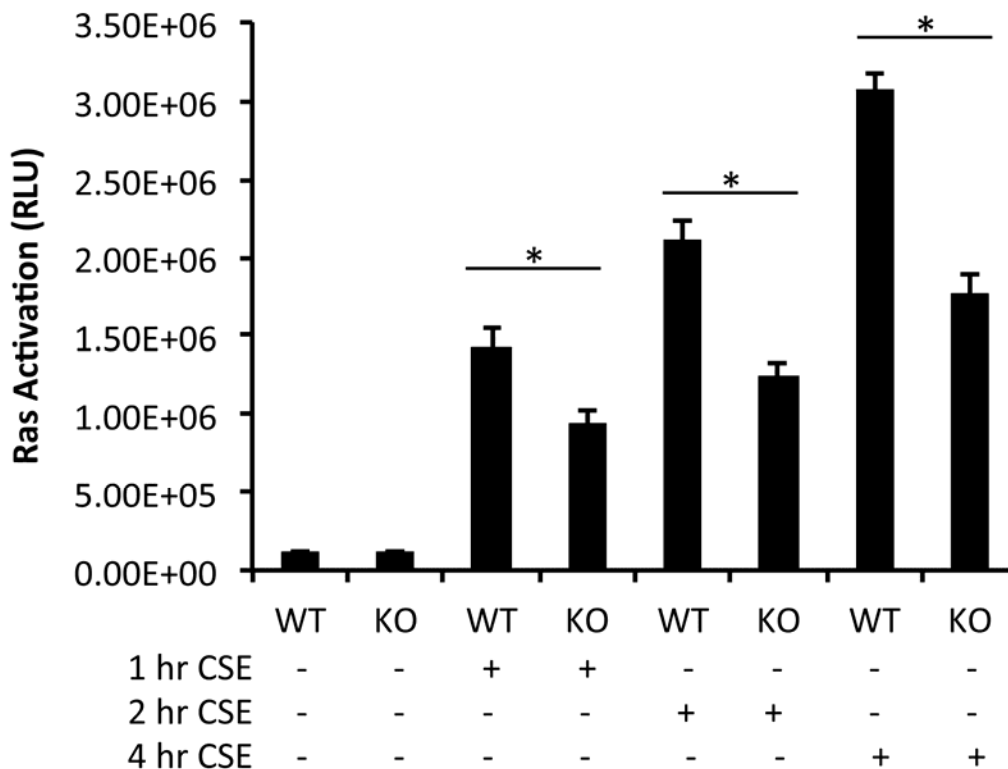


Figure 3 Primary AMs from wild type or RAGE KO mice were plated overnight, exposed to fresh media or 10% CSE for one, two, or four hours, then screened for active Ras. There was a significant increase in active Ras after exposure of each AM population to 10% CSE. Importantly, the quantity of active Ras was significantly decreased in RAGE KO AMs compared to wild type AMs at each time point. Data are representative of experiments performed in triplicate and $*p \leq 0.05$ (ANOVA).

Figure 4

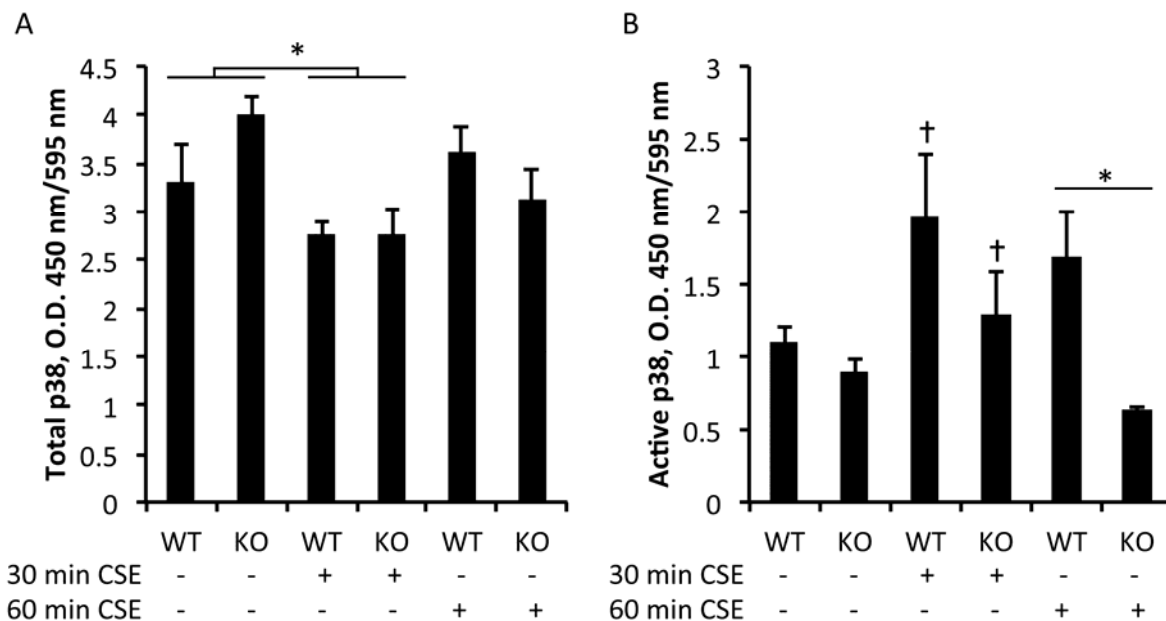


Figure 4 Primary AMs from wild type or RAGE KO mice were plated overnight, exposed to fresh media or 10% CSE for 30 or 60 minutes, then screened for total and active p38 MAPK. Total p38 MAPK levels were not different between the two groups of cells at each time point, although the total amount of p38 MAPK was diminished after 30 minutes of CSE exposure (A). There was a significant increase in phosphorylated active p38 MAPK after exposure of each AM population to 10% CSE for 30 minutes (B, † $p \leq 0.05$). Importantly, the quantity of active p38 MAPK was significantly decreased in RAGE KO AMs compared to wild type AMs at 60 minutes of exposure. Data are representative of experiments performed in triplicate and * $p \leq 0.05$ (ANOVA).

Figure 5

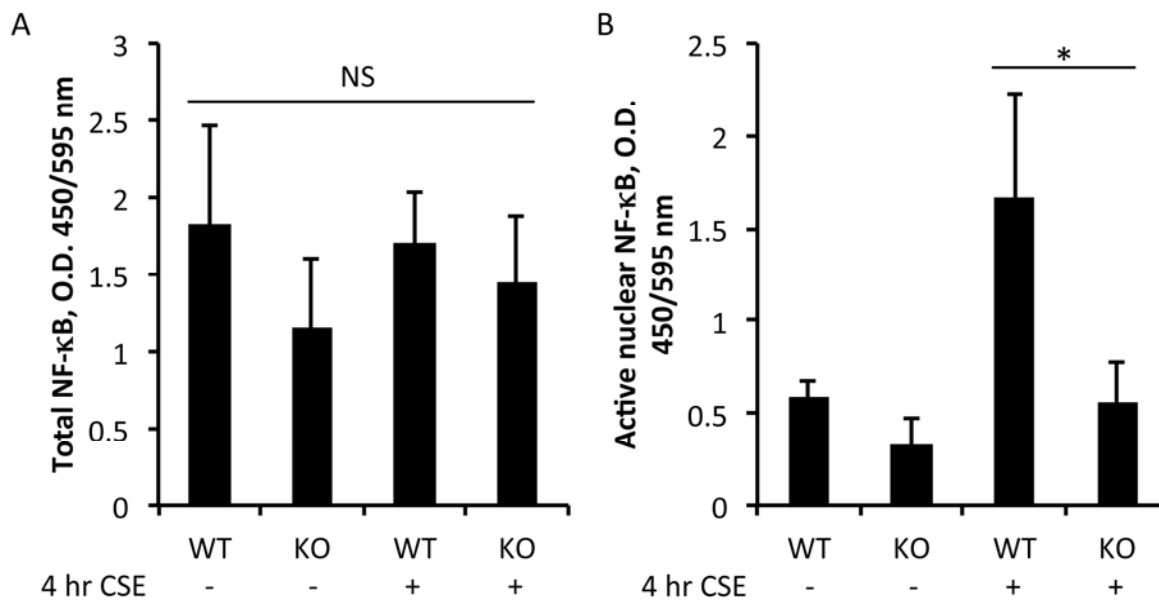


Figure 5 Primary AMs from wild type or RAGE KO mice were plated overnight, exposed to fresh media or 10% CSE for four hours, then screened for total and active p65 NF-κB. Total NF-κB was not different in any of the AM populations (A). There was a significant increase in phosphorylated active NF-κB after exposure of wild type AMs to CSE (B). Furthermore, RAGE KO AMs exposed to CSE were not different from wild type AMs in the absence of CSE exposure. Data are representative of experiments performed in triplicate and $*p \leq 0.05$ (ANOVA).

Figure 6

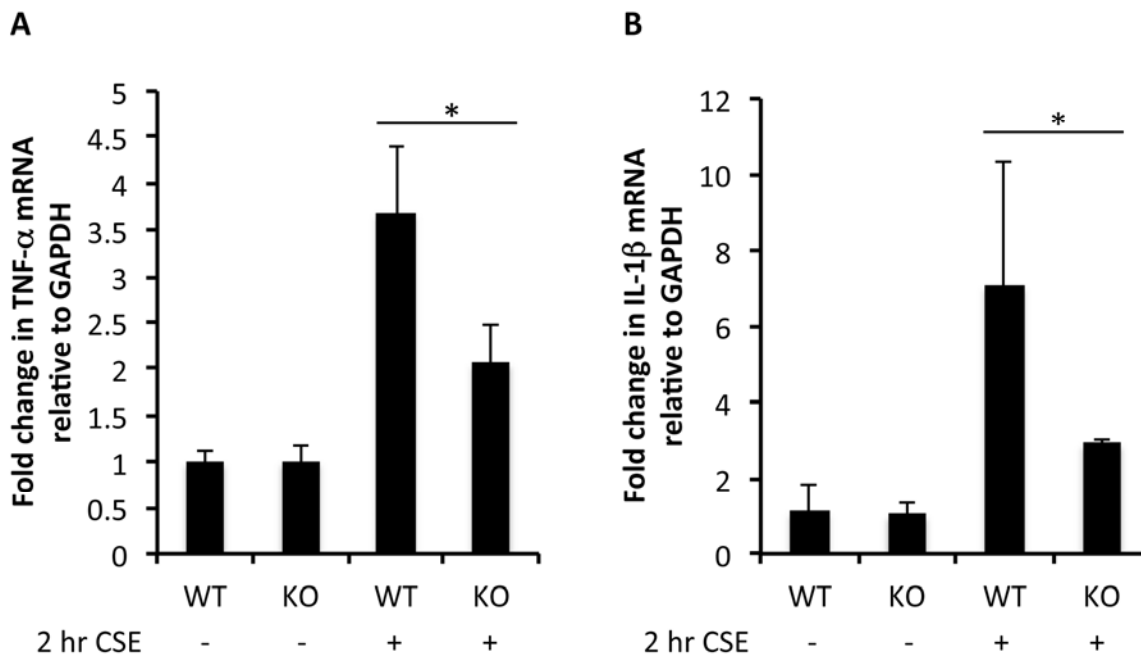


Figure 6 Primary AMs from wild type or RAGE KO mice were plated overnight, exposed to fresh media or 10% CSE for two or four hours, then screened for cytokine mRNA expression. Utilization of molecule-specific quantitative RT-PCR revealed that RAGE KO AMs exposed to CSE expressed less TNF- α and IL-1 β compared to wild type AMs exposed to CSE for two hours. The pattern of differential cytokine mRNA expression was similar after four hours of exposure (not shown). Data are representative of experiments performed in triplicate and $*p \leq 0.05$ (ANOVA).

Table 1**Table 1: Production of IL-1 β and TNF- α by AMs +/- CSE exposure**

	WT AMs	WT AMs + CSE	KO AMs	KO AMs + CSE
IL-1 β	28.9 \pm 9.9	85.86 \pm 9.3*	25.38 \pm 4.3	61.44 \pm 2.9* [†]
TNF- α	215 \pm 5.9	197 \pm 19.2	226 \pm 18.2	211 \pm 21.2

Data are expressed as average pg/mL \pm SD from at least two experiments performed in triplicate. *Significant differences between no CSE vs. CSE exposed WT or KO cells and [†]significant differences between WT AMs + CSE vs. KO AMs + CSE ($p \leq 0.05$, AVOVA).

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CHAPTER 3: DISCUSSION AND FUTURE DIRECTIONS

The conserved pro-inflammatory contributions of RAGE have recently become subjects of intense investigation due to their implication in numerous pathological states including diabetes, atherosclerosis, rheumatological disorders, Alzheimer disease, and COPD (13, 26). However, the involvement of explicit immune cells and the signaling events associated with perpetuation of these debilitating diseases in the context of RAGE remains unclear. The current study provides support for a model of smoke-exposed AMs wherein RAGE-mediated signal transduction pathways influence inflammation. These findings revealed that smoke induced AMs increased RAGE expression, activated known intracellular pathways such as Ras and p38 MAPK, and caused NF- κ B-mediated cytokine elaboration facilitated by RAGE mediated mechanisms. These conclusions have important implications for elucidating the mechanisms of progressive lung inflammation experienced by both former smokers and those unable or unwilling to quit. Clarifying RAGE signaling in the context of possible parallel pathways remains an important consideration, however. As such, additional research that centers on the important role of RAGE signaling and the resulting inflammatory response should remain a priority.

Although the data presented here clarifies a linear RAGE-mediated signal transduction pathway activated by inflammation, further research may require investigating redundant cell surface receptors that may also contribute to the overall inflammatory status of AMs following CSE exposure. Current research has identified some of these contributing receptors including IL-17RA (4), VEGFR2 (6), IL-1R (5), and Toll-Like Receptors (TLRs) (29). TLRs are pattern recognition receptors closely associated with RAGE mediated mechanisms involved in

inflammation and may contribute to the increased cytokine production and activated signaling intermediates seen following CSE exposure in macrophages (19, 29). These data show that even when RAGE is abolished in knock out AMs, cytokine production and activated intermediates still significantly increase, although the increase is significantly diminished when compared to wild type controls. Further research is needed to explore the hypothesis that TLRs in conjunction with RAGE may be responsible in initiating the inflammatory response following CSE exposure in AMs.

While RAGE signaling via p38 MAPK and NF- κ B has recently been proposed in diverse tissue types under various conditions (9, 18, 30), such signaling paradigms have not been elucidated in AMs exposed to tobacco smoke. The data in the current thesis demonstrate that p38 and NF- κ B activation occur, in part, through RAGE signaling. The data presented herein also support signaling kinetics published by Zhang et al. in that p38 MAPK activation peaked at 30 minutes and NF- κ B activation peaked at four hours following exposure of RAW264.7 to AGEs (28). However, whether p38 and NF- κ B activation occur in a sequential signaling pattern remains unclear. Additionally, downstream signaling intermediates require analysis such as Src kinase, which is implicated as perpetuating the intracellular signal following RAGE activation, leading to activation of MAPKs such as p38, ERK1/2, and transcription factors such as NF- κ B (15).

Macrophages are dynamic cells capable of remarkably diverse gene expression patterns, as well as abundant secretion of inflammatory mediators, following signal recognition of tissue-specific events. Although macrophages are well known for their phagocytic activity, lung macrophages develop the capacity to release large quantities of mediators that regulate

inflammation and homeostasis including cytokines, growth factors, oxidants, and enzymes (11). The data presented in the current work indicate that AMs up regulate pro-inflammatory cytokines TNF- α and IL-1 β via RAGE after CSE exposure, but does not evaluate the entire gamut of AM secretory products. MMPs, matrix metalloproteinases, are enzymes secreted by macrophages that may contribute to perpetuating inflammation and induce lung remodeling as seen in patients with COPD. In particular, MMP-12 is increased in patients with COPD (12), and MMP-9 is implicated in causing cigarette smoke-induced emphysema (1). Work by our lab has shown that RAGE over expression in alveolar epithelium leads to increased MMP-9 activity and results in destabilization of the basement membrane. Additional studies are needed to complete a thorough examination of MMP and cytokine secretion activity by AMs and determine whether RAGE mediates their activities.

HMGB1, high mobility group box 1, has a high affinity for binding RAGE and is released by damaged or necrotic cells, and by immune cells such as macrophages (7, 10). Kokkola et al found that HMGB1 induces a pro-inflammatory phenotype through RAGE in rodent macrophages (10) and previous work in our lab has shown that RAW264.7, a macrophage cell line, exposed to CSE increases HMGB1 expression, in addition to various cytokines (16). HMGB1 has also been shown to induce macrophage production of TNF- α (20) and IL-1 β (14) as well as chemotactic and cytotoxic factors (22). In patients with COPD, HMGB1 expression is increased, and it is suggested that binding of HMGB1 to RAGE amplifies the inflammatory and remodeling signals that contribute to COPD pathogenesis (7). The data presented in the current research lays the groundwork for additional studies because of the suggestion that

RAGE signaling triggers pro-inflammatory effects in AMs following exposure to CSE, but further work exploring the interactions HMGB1 and RAGE due to CSE exposure may prove beneficial.

Hogg et al states that a more complete understanding of the cytokine pathways that control disease progression may lead to effective treatments and mechanisms wherein therapies can improve (8). The data currently presented indicate that AMs up regulate pro-inflammatory cytokines TNF- α and IL-1 β via RAGE after CSE exposure. Several other cytokines in the current literature such as IL-8 (3, 27), TGF- β 1 (29), IL-6 (25), and MCP-1 and MIP (24) increased following cigarette smoke exposure in macrophages. In addition, cigarette smoke induced oxidative stress due to components in tobacco smoke or reactive oxygen species generated by macrophages may potentiate the inflammatory and irreversible remodeling process in the lung (19, 21). Since RAGE has been shown to be involved in propagating oxidative stress mechanisms leading to inflammation, studying the participation of RAGE in AM's ability to generate and produce oxidants will be critical in understanding RAGE contributions (2, 17).

The current study provides support for a model of smoke-exposed AMs wherein RAGE-mediated signal transduction pathways influence inflammation. This research provides the basis for possible therapeutic strategies that could be formulated for the prevention of smoke-induced pro-inflammatory effects. Furthermore, therapeutic intervention may assist in the alleviation of lung disease exacerbations in individuals with respiratory susceptibilities to a host of other particulates as well. Finally, the data presented here not only clarify RAGE biology in the context of smoke-exposed alveolar macrophages, but also provide evidence suggesting

involvement of inadequately characterized redundant receptors and signaling pathways that function in tobacco smoke-induced pulmonary inflammation.

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Submitted manuscripts

Robinson AB, Stogsdill JA, Lewis JP, Wood TA, and Reynolds PR. RAGE biology and Second hand smoke: Insights in Chronic Obstructive Pulmonary Disease. *In preparation*.

ABSTRACTS:

Robinson AB, Johnson KD, Bennion BG, and Reynolds PR (2012). RAGE signaling influences tobacco smoke-induced inflammation by pulmonary macrophages. *FASEB*.

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