CIGARETTE SMOKE AND DNA CLEAVAGE PROMOTE LUNG INFLAMMATION AND EMPHYSEMA

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ABSTRACT

Smoking-related lung diseases are among the most preventable and incurable ailments in the world. Smokers are at increased risk of developing chronic obstructive pulmonary disease that can be further complicated by emphysema and lung cancer. A subset of former smokers shows persistent lung inflammation and progressive loss of lung function, indicating a role for activation of acquired immunity in smoking-induced lung diseases. In addition to the well-established noxious effects of volatile compounds in cigarette smoke, incomplete combustion of tobacco generates nano-sized carbon black (nCB) that accumulate in lung myeloid dendritic cells and macrophages. Experimentally, intra-nasal instillation nCB can cause airway inflammation and emphysema in mice, underscoring their pathogenic role in inflammatory lung diseases. High throughput analyses of macrophages that have engulfed nCB reveal *de novo* activation of DNA repair enzymes, and histological studies provide evidence for DNA double-stranded breaks. Emphysematous lung myeloid dendritic cells that contain nCB express pro-inflammatory cytokines, and can efficiently differentiate naive CD4 T cells to interferon-γ–secreting T helper 1 and interleukin 17A expressing cell subsets. Together these findings indicate that nCB accumulation in lung innate immune cells can initiate and sustain lung inflammation and promote emphysema development.

INTRODUCTION

Over the past 50 years, the US government and several private foundations have disseminated information highlighting cigarette smoking hazards and have sponsored awareness programs in schools to educate children and reduce tobacco consumption in young adults (1). Although these efforts have been successful and have reduced smoking prevalence in the United States, globally, chronic obstructive pulmonary

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disease (COPD) morbidity and mortality rates attributed to tobacco continue to rise (2,3). China and India, two of the most populous countries in the world, lack educational programs and disproportionately produce and consume tobacco products (4). The continual rise in smoking prevalence in these developing countries is alarming because, if this trend continues, tobacco smoking is projected to cause approximately one billion deaths by the end of the 21st century (4).

Chronic exposure to first- and second-hand smoke is associated with an expanding list of malignancies (5), respiratory diseases (e.g., COPD, chronic bronchitis, emphysema) (6,7), and cardiovascular-related injuries including stroke and heart diseases (8–10). Other less frequent tobacco-associated conditions include infection of the lower respiratory tract (e.g., tuberculosis, pneumonia, etc.), chronic inflammatory diseases such as diabetes, and arthritis, as well as gastrointestinal ailments (Crohn's disease and colitis) (4). Initial studies using autopsy specimens reported an intense recruitment of innate immune cells (e.g., neutrophils and macrophages) in the lungs of asymptomatic smokers (11). However, in the past decade, several laboratories have identified a causative role for activated B and T lymphocytes (adaptive immunity), and myeloid dendritic cells (mDCs) in COPD pathogenesis (12–15). Notably, we have shown that lung mDCs from human smokers show increased expression of SPP1, and reduced expression of peroxisome proliferator activated receptor gamma (PPAR-γ), that parallels findings in an experimental model of emphysema (16).

One of the hallmarks of long-term smoking is a blackening of the lung tissue that persists despite smoking cessation. Previously little was known about the composition of the substance that causes this blackening, or its significance in emphysema development. By studying lung tissue obtained from smokers with emphysema, we have shown that the black substance is composed of nano-sized particles of elemental carbon (17). Incomplete combustion of tobacco generates nanoparticle carbon black (nCB) that accumulate in mDCs, and can cause emphysema in mice (17,18). Closer examination of the lung damage caused by nCB revealed that they trigger DNA double-stranded breakages (DSBs), which lead to lung inflammation (17). Lung macrophages and mDCs phagocytose nCB in the lungs and carry them to lung draining lymph nodes. However, nCB accumulation in the lungs can cause repeated cycles of phagocytosis and cell necrosis that perpetuate sterile lung inflammation, and induce cellular damage. These paradigm-shifting findings indicate that nCB, and by extension anthracotic pigments, cause sterile inflammation in the lungs, and could explain adverse health outcomes seen in former smokers.

DEGRADATION OF LUNG ELASTIN IN SMOKERS

Elastin fibers absorb mechanical stress and provide the elasticity required for lung and blood vessel integrity (19). This critical function is lost when alveoli and surrounding capillary beds are destroyed in response to smoking induced lung inflammation (20–22). The pathophysiology of emphysema in the setting of tobacco smoking and deficiency of the elastase inhibitor, alpha 1 anti-trypsin is related to excess of elastolytic proteinases (23–25). Nonetheless, the heterogeneous nature of emphysema in active or former (ever)–smokers that spans the spectrum of no disease to the most severe and debilitating forms of dynamic hyperinflation despite smoking cessation, remains poorly understood (26–28). There is growing interest in understanding the factors that initiate and perpetuate lung destruction in response to chronic smoke. Our team has dissected the pathophysiology of smoke-induced acquired immune responses against elastin molecules in smokers with emphysema (13,29–33). Specifically, we have shown that smokers with emphysema harbor activated T helper subsets in their lungs and persistence of autoreactive T cells is associated with increased rate of physiological decline (30,32,34).

PRE-CLINICAL MODELS OF EMPHYSEMA

Several important pathogenic mechanisms of smoke-induced lung disease have been addressed in animal models of emphysema that share many characteristics with human disease (35). Genetic susceptibility to cigarette smoke was studied in five different strains of inbred mice over 6 months. Three of five strains (C57BL6/J, A/J, and SJ/L) that were tested showed changes in mean linear intercepts (an indicator of emphysema), but the inflammatory cell and cytokine profiles, critical features of cigarette smoke–induced emphysema, varied unpredictably among these strains (36). Transgenic models of emphysema have further been instrumental to our current understanding of its pathophysiology: deletion of macrophage metalloproteinase (MMP) 12 rendered animals resistant to cigarette smoke–induced emphysema, showing the potential importance of MMP12 in emphysema (37,38). Overexpression of interferon gamma (IFN-γ) targeted to the lung further results in spontaneous development of emphysema and accumulation of innate inflammatory cells in the lung (39). Animal models of acute emphysema using purified papain or elastase showed that elastic fiber repair is associated with formation of secondary inflammation that leads to secretory cell metaplasia comparable to

chronic bronchitis (40,41). Although a role for $CD8^+$ T cells in a cigarette-smoke–exposed model of emphysema has been proposed (42), $CD4^+$ T helper responses have recently been shown to play a role in animal models of emphysema (43). Although interleukin 17 (IL17) T helper (Th17) cells have been associated with many human diseases (44,45), their involvement in human emphysema, and mouse model of emphysema was shown (46). Increased levels of IL-17A have been detected in lung tissue and peripheral blood mononuclear cells of emphysema patients consistent with chronic autoreactive inflammatory disease that target the lung parenchyma (30,47). IL-17A was also found in sputum of COPD patients, but to a lesser extent than found in asthma patients (48). Mice exposed to tobacco smoke also had increased IL-17A in the lungs, and mice deficient in *Il-17a* or its receptor showed attenuated emphysema, indicating its critical requirement emphysema development (46,49,50). These findings indicate there is strong association between Th17-related cytokines with emphysema, but the full extent of mediators involved in induction of Th17 in response to cigarette smoke is now emerging.

INNATE AND ADAPTIVE IMMUNITY IN SMOKERS WITH EMPHYSEMA: AUTOIMMUNITY AND EMPHYSEMA

T cells that home to the lung secrete distinct repertoires of cytokines that distinguish them classically into T helper 1(Th1) (secreting IL-2 and IFN-γ), Th2 (secreting IL-4, IL-5 and IL-13) (51), Th17 (secreting IL-17A, IL-17F, IL-22, IL-23) (52), and T regulatory (secreting IL-10, transforming growth factor beta) (53) subsets. Several published systematic studies of peripheral lung T effector cells in smoking subjects have emphasized the over-riding importance of CD4 T cells to the development of emphysema (13,54,55). Isolated peripheral blood $CD4^+$ T cells from emphysema but not control subjects released IFN-γ and IL-10, but not IL-13, when stimulated with lung elastin peptides and this correlated positively with the severity of emphysema (29). Further, blocking major histocompatibility complex class II molecules attenuated T cell responses to elastin peptides, confirming the specific nature of CD4 T cell activation that required presentation of antigen in the context of major histocompatibility complex class II molecules (29). These findings have shown a strong association between memory T cell responses to a self-antigen (lung elastin peptides) and severity of emphysema. Mechanistically, Th1-related chemokines [e.g., interferon gamma inducible protein-10 (IP-10) or C-X-C motif chemokine ligand 10 (CXCL10)] control the secretion of elastolytic MMPs, further providing a link between the

autoimmune Th1 response and the degradation of elastin (13). However, rather than a bystander target of an otherwise unrelated Th1 response, elastin is specifically targeted by a powerful type 1 immune response. We further showed that Th17 cells are also present in the peripheral lung of smokers with emphysema and, similar to Th1 cells, are auto-reactive against elastin and promote elastolysis by innate immune cells through IL-17A and C-C motif chemokine ligand 20 (CCL20) (30).

Thus, although chronic lung destruction in some smokers appears to be perpetuated by auto-reactive Th1 and Th17 cells, the mechanism by which these cells, and no other T cell subsets such as T regulatory cells and Th2 cells, are selectively activated in emphysema remain obscure. Because airway myeloid dendritic cells (mDCs), also known as antigen-presenting cells, are among the first cells to encounter environmental irritants such as cigarette smoke and are essential initiators of T helper cell differentiation, we examined the function of mDCs in T cell differentiation that favors Th1 and Th17 responses (46).

SPP1 gene encoding osteopontin, a pleiotropic cytokine that is implicated in autoimmune responses, is highly expressed in mDCs isolated from lung tissue of former smokers with emphysema (16). We showed that PPAR-γ expression was downregulated in mDCs of smokers with emphysema and mice exposed to chronic smoke (16). Conditional knockout of *Ppar*-γ in dendritic cells (DCs) ($CD11c^+$) in mice resulted in increased recruitment of activated mDCs, whereas loss of osteopontin (encoded by *SPP1*) in these mice prevented inflammation and emphysema development (16). These findings suggest a homeostatic anti-inflammatory role for PPAR-γ whereby it negatively regulates *SPP1* expression in the lung. In support of this, 2 months of PPAR-γ agonist treatment in wild-type mice reversed emphysema despite continuous smoke exposure, and endogenous PPAR-γ agonists were found to be reduced in the plasma of smokers with emphysema (16).

These findings revealed a pro-inflammatory pathway in which reduced PPAR-γ activity promotes emphysema pathogenesis and using agonists; this pathway can be readily targeted in smokers to prevent and or reverse emphysema. In addition to discovering the molecular pathways involved in emphysema pathogenesis, we also focused on the physical appearance of the mDCs in smokers with emphysema. We observed that mDCs isolated from emphysematous lung tissue are discolored and appear darker in color when compared to the same cell population isolated from the lungs of non-smokers or light smokers without emphysema. Therefore, next we examined the nature and physical property of the particulate matter present in mDCs isolated from the lungs of former heavy smokers.

IDENTIFICATION OF NANO-SIZED CARBON BLACK IN LUNG MDCS

Histopathological analysis of the lungs of heavy smokers invariably reveals dark-staining (anthracotic) pigment, often attributed to poorly soluble material found in tobacco smoke (56). Anthracotic pigment is also found in the lymph nodes of smokers (57), but because its presence in lung tissue and lymph nodes had been considered benign (56), its chemical composition and potential contribution to smoking-related diseases had not been explored. We isolated the insoluble particulate (e.g., anthracotic) material after complete proteolytic digestion of human lung, and identified the residual black material as aggregates of 20 nm to 50 nm spheroids that were composed of nCB. Raman spectroscopy, hyperspectral imaging, and high-resolution transmission electron microscopy confirmed that nCB accumulates specifically in $CD1a^+$ lung mDCs and alveolar macrophages (17).

Experimentally, mice exposed to smoke also accumulate nCB in the lung macrophages and mDCs. Moreover, delivered by chronic exposure to cigarette smoke or purified preparation intranasally, nCB persist indefinitely in mouse lung, can activate lung mDCs, and promote Th17 cell differentiation mediated by the inflammasome assembly in phagocytes (17,18). Notably, we found that highly hydrophobic form of nCB found in smoker's lungs can cause DNA DSBs, the most ominous form of DNA damage in eukaryotic cells, and activate inappropriate DNA repair responses that have been linked to induction of genomic instability, and accelerated cellular aging (17,58,59).

Increasing the polarity or size of nCB mitigated many adverse effects because larger and less hydrophobic particles abrogated phosphorylation of histone 2AX (γH2AX), an indicator of cellular DSBs. We have also used high-resolution imaging techniques to examine the effect of nCB size and polarity on cellular uptake and localization within lung mDCs. In addition to changes in polarity of nCB using polyethylene glycol formulation (17), we have used several sizes (50 nm to 500 nm) of nCB in isolated lung mDCs and in animal models to examine their proinflammatory function in the lungs (17). These studies have confirmed that small (50-nm to 70-nm) hydrophobic nCB can be detected within cellular vesicles and the nucleus and these properties are critical for induction of sterile inflammation in the lungs (Figures 1 and 2).

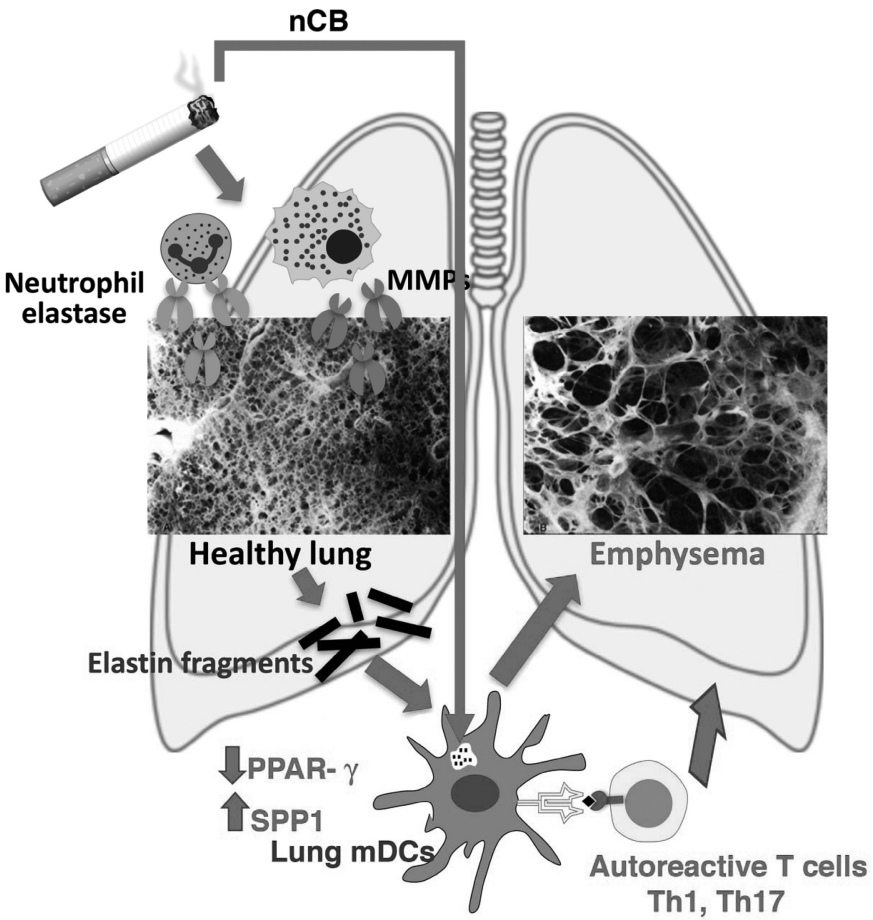

FIG. 1. Schematic diagram of cigarette smoke–induced innate and adaptive immune mediated lung destruction. Cigarette smoke can initiate recruitment of activated innate immune cells such as neutrophils and macrophages into the lungs. Secretion of tissue degrading enzymes, such as neutrophil elastase and matrix metalloproteinases, can digest lung parenchyma and release fragments (e.g., elastin) that can act as self-antigens. Lung myeloid dendritic cells (mDCs) process self-antigens and present them in context of major histocompatibility class II molecules to self-reactive $CD4^+$ T cells. In response to cognate antigen, T cells proliferate and secrete cytokines and chemokines that promote emphysema development. In addition, particulate matter such as nanoparticle carbon black (nCB) generated by incomplete combustion of tobacco can accumulate in the lungs and promote sterile inflammation which can accelerate T helper 1 (Th1) and Th17 cell differentiation. Abbreviations: MMP, metalloproteinase; PPARγ, peroxisome proliferator activated receptor gamma.

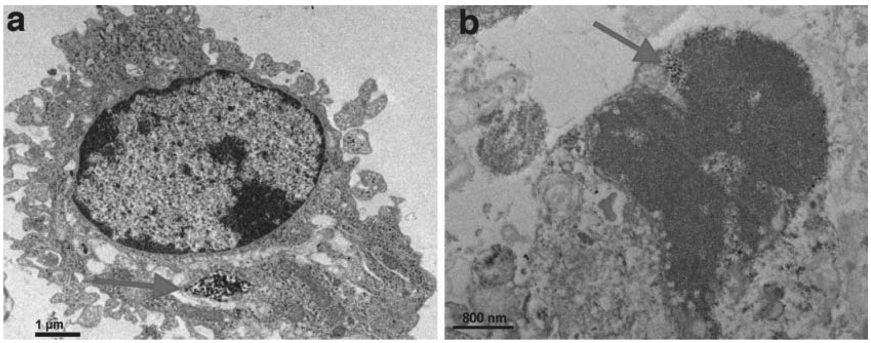

FIG. 2. Transmission election microscopy detects nanoparticle carbon black (nCB) within cellular organelles in lung myeloid dendritic cells (mDCs). Lung mDCs from nCB-treated mice and 60-nm sections of cells displayed accumulation of nCB (A) inside cellular vesicles and (B) inside the unstained nuclear capsule; original magnification ×2000 and ×8000, respectively. *Red arrows* point to nCB inside lung mDCs.

CONCLUDING REMARKS

Previously considered benign, anthracotic pigment generated in response to chronic smoking are composed of hydrophobic nCB particles which can induce cell damage. Accumulation of nCB in lung phagocytic cells activates the inflammasome pathway and can perpetuate lung inflammation. It remains unclear whether nCB can directly activate the inflammasome pathways and mediators that promote lung cellular damage. Studies using high-resolution transmission electron microscopy could provide an ideal approach in determining whether changes in nCB size and polarity could impact their intracellular localization and alter mDC activation in the lungs. The functional significance of nCB physical and biochemical properties, and induction of DSB (detected via γH2AX) in mDCs could further provide new insight in the mechanism of sterile inflammation and emphysema pathogenesis. Future studies are needed to understand how nCB can induce sterile inflammatory responses in the lungs of smokers with emphysema.

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DISCUSSION

Zeidel, Boston: Very nice paper. As a nephrologist listening to this I am reminded of the proximal tubule cell, getting material in it that it can't process in its lysosomes. So, you wonder if this carbon is getting to the lysosomes that can't be degraded and then you get into the kinds of damaged pathways that you see in acute renal failure settings with contrast to hemoglobin and in the kidney. I wonder if that may be because you use some

of these same pathways activated in that setting, that you are describing, and maybe the start of it is that the cell fills up with the stuff that it can't degrade and then you begin to go down that pathway.

Kheradmand, Houston: So, absolutely, and I am sorry I didn't have the full time to share with you but this work is published. Inflammasome pathways are very much important for not only activation of the immune system but also DNA damage.

Higgenbotham, Philadelphia: Thank you for your very nice paper. When I am not dancing, I am actually on the Defense Health Board where we are considering what happens to our military when they are exposed to smoke pits, and so recently, within the last couple of years, the question of lung biopsies has come up. So, my question back to you: is there something that is not invasive that we could use to identify those military, particularly with the repeated deployments so that we can actually tease out those individuals who may down the line have this peripheral damage?

Kheradmand, Houston: Those are all absolutely amazing questions... and I think that maybe sputum cytology would be a very good way to go after that.

Gravallese, Worcester: So, I just wanted to mention a very interesting animal model where researchers knocked out a DNA enzyme that degrades DNA, DNAase-2, and DNA accumulated in the lysosome, leached into the cytosol and activated these cytosolic DNA sensor pathways, one of which is an inflammasome: called the AIM-2 inflammasome. It was subsequently shown that endogenous DNA can also activate these pathways, and I am wondering what might be happening here is the activation of these cytosolic pathways staying in AIM-2; Have you thought about that?

Kheradmand, Houston: Yes, and in fact some of these experiments were published in eLIFE [Nanoparticulate carbon black in cigarette smoke induces DNA cleavage and Th17-mediated emphysema. *Elife* 2015;4:e09623], so I'd be happy to talk to you in person, yes it is.