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A 7-month cigarette smoke inhalation study in C57BL/6 mice demonstrates reduced lung inflammation and emphysema following smoking cessation or aerosol exposure from a prototypic modified risk tobacco product

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ABSTRACT

Modified risk tobacco products (MRTP) are designed to reduce smoking-related health risks. A murine model of chronic obstructive pulmonary disease (COPD) was applied to investigate classical toxicology end points plus systems toxicology (transcriptomics and proteomics). C57BL/6 mice were exposed to conventional cigarette smoke (3R4F), fresh air (sham), or a prototypic MRTP (pMRTP) aerosol for up to 7 months, including a cessation group and a switching-to-pMRTP group (2 months of 3R4F exposure followed by fresh air or pMRTP for up to 5 months respectively). 3R4F smoke induced the typical adaptive changes in the airways, as well as inflammation in the lung, associated with emphysematous changes (impaired pulmonary function and alveolar damage). At nicotine-matched exposure concentrations of pMRTP aerosol, no signs of lung inflammation and emphysema were observed. Both the cessation and switching groups showed a similar reversal of inflammatory responses and no progression of initial emphysematous changes. A significant impact on biological processes, including COPD-related inflammation, apoptosis, and proliferation, was identified in 3R4F-exposed, but not in pMRTP-exposed lungs. Smoking cessation or switching reduced these perturbations to near sham-exposed levels. In conclusion, the mouse model indicated retarded disease progression upon cessation or switching to pMRTP which alone had no adverse effects.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality worldwide (Mathers and Loncar, 2006). Cigarette smoking and both indoor and outdoor air pollution are important risk factors contributing to the pathogenesis of this preventable, complex pulmonary disorder. It is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and lung to noxious particles or gases (GOLD, 2014). Airflow limitation is caused by two major mechanisms: (i) small airways disease comprising airway inflammation, airway fibrosis, luminal plugs, and increased airway resistance; and (ii) parenchymal destruction (emphysema) with loss of alveolar attachments and

Abbreviations: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care International; BALF, bronchoalveolar lavage fluid; BIF, biological impact factor; CO, carbon monoxide; COHb, carboxyhemoglobin; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; FDR, false discovery rate; FLC, free lung cell; GOLD, Global Initiative for Chronic Obstructive Lung Disease; GSD, geometric standard deviation; HPHC, harmful and potentially harmful substances; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MIP, macrophage inflammatory protein; MMAD, mass median aerodynamic diameter; MMP, matrix metalloproteinase; MS, mainstream smoke; NPA, network perturbation amplitude; PAI, plasminogen activator inhibitor; pMRTP, prototypic modified risk tobacco product; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; TPM, total particulate matter; VCAM, vascular cell adhesion molecule.

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decrease of elastic recoil (GOLD, 2014). Human disease severity is measured clinically as grades I–IV using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria. Grades I and II represent the earliest stages of COPD, while grades III and IV correspond to the more severe stages of the disease (Vestbo et al., 2013).

The identification of a suitable *in vivo* model is a prerequisite for mechanistic studies on cigarette smoke (CS)-induced COPD. Several mechanisms of human COPD pathogenesis can be observed in mice exposed to CS (Brusselle et al., 2006), including the activation of the innate and adaptive immune response cascade. This leads to abnormal inflammatory responses and enhanced protease/anti-protease imbalances in the lung tissue and eventual alveolar wall degradation, suggesting that murine models are able to replicate these features of human COPD (Yoshida and Tuder, 2007).

Of the various mouse models, the C57BL/6 strain is most appropriate to study CS-induced COPD (Churg et al., 2006a; John et al., 2014; March and Seagrave, 2007; Suzuki et al., 2008; Vecchio et al., 2010; Yao et al., 2008; Zhou et al., 2013). However, this model only mimics some aspects of early human COPD resembling GOLD grades I and II (Pauwels and Rabe, 2004; Rabe et al., 2007) by primarily recapitulating the initiation and development of emphysema (Churg and Wright, 2007; Churg et al., 2006b, 2008). C57BL/6 mice have a moderate deficiency in serum alpha 1-proteinase inhibitor, which is more pronounced in females (Bartalesi et al., 2005). The antioxidant response may therefore be weakened by reduced *Nfe2l2* promoter activity (Cho et al., 2002). This strain also develops goblet cell metaplasia as well as emphysema upon smoke exposure (Bartalesi et al., 2005). In the lungs of smoke-exposed C57BL/6 mice, gene expression of type I pro-collagen and the expression of pro-fibrotic cytokines, particularly those related to transforming growth factor-β signaling, was persistently up-regulated. Although this would be expected to contribute to airway remodeling (Churg et al., 2006b), airflow limitation from small airway obstruction associated with chronic bronchitis, typical of later COPD stages (Hogg, 2004), is not fully recapitulated in mouse models of the disease (Vlahos and Bozinovski, 2014).

Here, we report on the application of the C57BL/6 mouse model to investigate the COPD risk reduction potential of aerosols generated from a prototypic modified risk tobacco product (pMRTP) compared with mainstream smoke (MS) from the conventional reference cigarette 3R4F, including a switching scenario compared with the continued exposure to MS from a conventional cigarette. As defined by the US Family Smoking Prevention and Tobacco Control Act of 2009, MRTP means 'any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco-related disease associated with commercially marketed tobacco products' (Food and Drug Administration, 2012a). The US Food and Drug Administration published a Draft Guidance on "Modified Risk Tobacco Product Applications" (Food and Drug Administration, 2012b) stating that applications must provide scientific evidence to demonstrate that the product significantly reduces harm and the risk of tobacco-related disease to individual users and benefits the health of the population as a whole, taking into account both users and non-users of tobacco products (Food and Drug Administration, 2012a; 2012b). In this context, non-clinical studies play an integral role in the evaluation of MRTPs (Food and Drug Administration, 2012b).

The pMRTP investigated here is based on distillation technology that aims to heat rather than burn tobacco, thus lowering the extent of pyrolysis and quantity of combustion products (Coggins et al., 1989; Schorp et al., 2012; Werley et al., 2008). A fast-lighting carbon tip is used as a heat source, which differs from the previously described electrically heated cigarette smoking system (Schorp et al., 2012; Werley et al., 2008), and the aerosol is created by gentle and controlled heating of the tobacco. This yields a smoke aerosol composed primarily of water and an aerosol former such as glycerol with reduced concentrations of combustion-related constituents such as aldehydes and polycyclic aromatic hydrocarbons.

Following on from our previous 28-day repeated dose inhalation study in rats that demonstrated reduced exposure to harmful and potentially harmful substances (HPHC) and reduced irritative and inflammatory effects for the pMRTP using both classical toxicological end points and a systems toxicology approach (Kogel et al., 2014), the present study investigated the impact of pMRTP exposure in a C57BL/6 mouse model of smoking-related COPD. Additionally, a cessation and a switching arm were added to investigate the potential recovery after 2 months of exposure to 3R4F (Fig. 1). The cessation/switching time point was chosen to occur at the onset of disease-related changes, based on a previous observation that exposure to 3R4F for 2 months resulted in the earliest quantifiable emphysematous changes at the levels of lung function, histopathology, and pulmonary inflammation (data not shown). We also assessed transcriptomics and proteomics data from affected tissues to provide mechanistic insights into emphysema pathogenesis as well as cessation and switching effects in this murine COPD model. A number of studies have reported various molecular pathways that are perturbed in the human emphysematous lung (Ezzie et al., 2012; Ning et al., 2004; Spira et al., 2004), but, to the best of our knowledge, this is the first comprehensive systems toxicology study on smoke-exposed C57BL/6 mice to include investigations of the transcriptome, proteome (this paper), and – reported separately – lipidome (unpublished results).

The objectives of this 7-month inhalation study were: (i) to confirm that C57BL/6 mice are a suitable emphysema model for tobacco product testing and to understand the molecular mechanisms perturbed during pathogenesis; (ii) to determine the potential of pMRTP to induce less emphysematous and inflammatory changes related to COPD development than conventional cigarettes and to identify the biological processes that are specifically impacted by pMRTP exposure; (iii) to understand the biological changes that occur in the C57BL/6 mouse emphysema model upon switching from a conventional cigarette to a pMRTP, or upon cessation; (iv) to investigate the degree of similarity between switching to a pMRTP and complete smoking cessation; and (v) to understand those biological network perturbations caused by conventional CS that may not be reversed by smoking cessation and/or switching to a pMRTP.

2. Materials and methods

2.1. Mice and experimental design

All procedures involving animals were performed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) accredited, AVA-licensed (Agri-Food & Veterinary Authority of Singapore) facility with approval from an Institutional Animal Care and Use Committee, in compliance with the National Advisory Committee for Laboratory Animal Research Guidelines on the Care and Use of Animals for Scientific Purposes. Female C57BL/6 mice bred under specific pathogen-free conditions were obtained from Charles River, Wilmington, MA, USA and were 8–10 weeks old at exposure initiation.

The study design included five groups (51 mice per group and time-point) of female C57BL/6 mice: (i) sham (exposed to fresh air), (ii) 3R4F (exposed to MS from the reference cigarette 3R4F, 750 μg total particulate matter (TPM)/l equivalent to 34.4 μg nicotine/l), (iii) pMRTP (exposed to mainstream aerosol from a pMRTP in which the nicotine level was matched to that of 3R4F), (iv) smoking cessation, and (v) switching to pMRTP. The combined assessment of classical toxicological end points with additional transcriptomics, proteomics, and lipidomics investigations in one study that includes a comparably small extra cohort of animals allows for the simultaneous investigation of a multitude of mechanistic parameters, biomarkers etc. that can be directly correlated with the toxicological outcomes, thereby avoiding the need for separate mechanistic studies with a higher number of animals including those for bridging between studies, confirmation of biological outcomes, positive and negative controls, etc. The allocation of mice to the treatment groups and time-points is shown in Fig. 1A. Female mice were chosen because of a possible increased susceptibility to develop emphysema (Bartalesi et al., 2005).

Mice were whole body-exposed for 4 × 1 h per day, 5 days per week, with intermittent exposure to fresh filtered air for 30 min after the first hour of smoke