Online supplement

Cigarette smoke and platelet activating factor receptor-dependent adhesion of *Streptococcus pneumoniae* to lower airway cells

Jonathan Grigg[1] , E. Haydn Walters [2], Sukhwinder Singh Sohal [2], Richard Wood-Baker [2], David Reid [2], Iain Dickson[1], Michael Kirwan[1], Cang-Bao Xu[3], Lars Edvinsson[3], Lee Koh[1], Naseem Mushtaq[1]

[1] Centre for Paediatrics, Blizard Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University London, London, E1 2AT, UK

[2] NHMRC National Centre for Research Excellence in Chronic Respiratory Disease, Menzies Research Institute, 17 Liverpool St, Hobart, 7000, Australia.

[3] Division of Experimental Vascular Research, Institute of Clinical Science in Lund, Lund University, Lund, Sweden

Methods

Assessment of platelet activating factor receptor expression by flow cytometry.

Platelet activating factor receptor (PAFR) expression on A549 cells was quantified using flow cytometry. Briefly, airway epithelial cells, at 2 x 10^5/mL were seeded into 24-well plates (Costar, Sigma Aldrich Poole, Dorset, UK). Cigarette smoke extract (CSE) was added to cell monolayers and incubated for 4 h at 37^{O}C. CSE was removed by washing 3 x with PBS (Lonza, UK). We observed that CSE increased epithelial cell autofluorescence. Autofluorescence induced by CSE was therefore attenuated by crystal violet (CV) quenching. Quenching was done using the protocol reported by Ni *et al* [1]. Briefly, a CV solution was made by dissolving 2mg/mL in 0.9% normal saline by heating at 50°C and stirring for 3 h. The solution was left overnight at RT, then filtered through a 0.2 μm filter. For quenching, cells were washed 3 x in PBS/0.1% NaN_3 and then 200ul of the CV stock solution was added to each well and left on ice for 5 min. Cells were then washed 3 x in PBS/0.1% NaN_3 . Cells were pelleted at 1350 rpm for 5 min, the supernatant discarded, cells vortexed and washed 3 x in PBS (5 min at 1350 rpm). Primary (mouse anti-human PAFR; Cayman chemicals 160600) and isotype (mouse IgG2a-Invitrogen MG2A00) antibody was diluted (1/100 in 10% BSA) and 200 μL added to each appropriate tubes for 30min at RT. Cells were then washed and centrifuged 3x in PBS (5 min at 1350 rpm) and incubated at RT for 30 min with 200 μL of the secondary antibody Alexa-Fluor 488 goat anti-mouse FAb fragment (1/500 μL in 10% BSA), after which the cells were

washed 3 x in PBS. Cells were then resuspended in 500ul of PBS and 10,000 events analysed for each sample by FACS (BD FACSCanto II fitted with FACSDivia software version 6.1.1.). Specific median florescence intensity (MFI) was determined by MFI [PAFR] - MFI [isotypic control]. Data were expressed as fold-change in PAFR over control.

Expression of PAFR in bronchial biopsies

Following removal of paraffin and hydration to water, immunostaining for PAFR was done using an anti-PAFR monoclonal antibody (11A4, clone 21, Catalogue No. 160600, 1/80 dilution, for 1 h at 20 ^{o}C with no heat retrieval). In each case, the primary antibody was replaced using a species-appropriate isotype-matched IgG_2 (Dakocytomation, Denmark X0931 clone DAK-GO1) at equivalent dilutions and conditions as negative controls. Endogenous peroxidase blocking was done with 3% hydrogen peroxide in milli-ro water for 15 min at 20 ^{o}C to eliminate nonspecific staining but no serum block was necessary. Bound antibodies were elaborated using Peroxidase-labeled Envision + (Dakocytomation, Denmark cat. no. K4001) and liquid DAB + (Dakocytomation, Denmark cat. no. K3468). PAFR expression was determined by computer-assisted image analysis was performed (Leica DM 2500 microscope, Microsystems, Germany), with a Spot insight 12 digital camera and Image Pro V5.1 (Media Cybernetics, USA) software.

Legend for Online Figures

Figure 1. BEAS2-B cells cultured in medium alone, cigarette smoke extract 1%, and urban particulate matter at 50 μg/mL before and after washing.

Airway cells (BEAS-2B) before- and after washing by medium prior to infection with *S. pneumoniae*. (**A**) Cells cultured in medium alone (control), and (**B**) after washing. (**C**) Cells cultured in cigarette smoke extract (CSE) 1%. Brown-stained particulate matter (PM) is present on cells. (**D**) CSE 1%-exposed cells after washing. Most PM is removed by washing. There are areas where cells have detached during washing. (**E**) Cells cultured in 50 μg/mL urban air PM less than 10 μm in aerodynamic diameter (PM_{10}) before washing, and (**F**) after washing. PM is carbonaceous and therefore black, and is at a concentration that stimulates pneumococcal adhesion to A549 and primary bronchial epithelial cells [2]. The density of PM is higher than cells exposed to CSE 1 %. Cells imaged by phase contrast microscopy (x20).

Figure 2.

Airway cells (A549) cultured in; (**A**) medium, and (**B**) cigarette smoke extract (CSE) 1%. Cells were washed, and cytocentrifuged onto a microscope slide, then stained. There is no black carbonaceous particulate matter on CSE-treated cells. CSE 1%- treated cells show some cytoplasmic vacuolation. Cells imaged by light microscopy (x100).

Figure 3.

Non-specific immunostaining of bronchial biopsy sections using the isotypic monoclonal mouse IgG_2 control for platelet activating factor receptor (PAFR). **A)** Section from an active-smoker and **B)** from a control who has never smoked. Scale bar = 50 μm. Sections were lightly counterstained with H&E stain. Non-specific PAFR staining was absent in bronchial biopsy specimens from both non-smokers and smokers.

References for Online supplement

1. Ni K, O'Neill HC. Improved FACS analysis confirms generation of immature dendritic cells in long-term stromal-dependent spleen cultures. *Immunol Cell Biol* 2000;78(3):196-204.
2. Mushtaq N, Ezzati M, Hall L, Dickson I, Kirwan M, Png KM, et al. Adhesion of Streptococcus pneumoniae to human airway epithelial cells exposed to urban particulate matter. *J Allergy Clin Immunol* 2011;127(5):1236-42 e2.