On line Supplementary text

Role of Transient Receptor Potential and Pannexin Channels in Cigarette Smoke triggered ATP release in the Lung

Matthew Baxter, Suffwan Eltom, Bilel Dekkak, Liang Yew-Booth, Eric D. Dubuis, Sarah A. Maher, Maria G. Belvisi, Mark A. Birrell

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were obtained from Harlan UK Limited. $Trpv1^{-/-}$ mice (backcrossed ten times onto C57BL/6 genetic background) were purchased from The Jackson Laboratory. $Trpv4^{-/-}$ (backcrossed onto C57BL/6 background) mice were purchased from the Riken Bioresource Centre, Japan. Pannexin-$1^{-/-}$ mice were kindly donated by Dr. Dixit, Genentech. $P2X_7^{-/-}$ mice were provided by Professor Jean Kanellopoulos, Universite Paris-Sud, France, details on the development of which can be found in Solle *et al*, 2001 [1]. Trpa1-/- mice on a mixed C57BL/6;B6129 genetic background were purchased from The Jackson Laboratory then backcrossed onto a pure C57BL/6 genetic background in-house. All mouse colonies were maintained in Central Biomedical Services at Imperial College London, with food and water supplied *ad libitum*. All protocols were approved by a local ethical review process and strictly adhered to the Animals (Scientific Procedures) Act 1986 UK Home Office guidelines.

LPS driven model of airway inflammation

The LPS driven model of murine airway inflammation was previously characterised and optimised to elicit a sub-maximal neutrophilic response in the BALF [2]. Mice were challenged with 1 mg/ml of aerosolised LPS (*Escherichia coli*, serotype 0111:B4, Sigma-Aldrich Ltd. Poole, UK), or endotoxin-free saline (Fresenius Kabi, Warrington, UK). Mice were terminally anaesthetised 6 hours after the final exposure, before collection of BALF for the measurement of neutrophilia, IL-1β, and ATP as described CS exposure model method section.

CS driven model of airway inflammation

The CS driven model of murine airway inflammation was previously characterised and published [2]. Briefly male mice (8-12 weeks old) were placed in air tight chambers and exposed to either CS from 3R4F research cigarettes (Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) or room air (control), using a negative pressure flow system. For the CS exposed animals, a pinch valve was used to set the ratio of CS to room air drawn into the exposure chambers, 2 seconds CS: 4 seconds room air. Control mice were exposed to room air only. For both groups the flow rate into the chamber was set at 1.5 L/min during the 50 minute exposure periods followed by 10 minutes of ventilation at maximum airflow. Exposures were conducted twice daily, separated by 4 hours, and for three consecutive days. Total smoke particulate measurements were used to ensure consistency between exposures.

Mice were terminally anaesthetised 24 hours after the final exposure and BALF was collected for the measurement of inflammatory markers. The number of neutrophils in BALF was calculated by differential cell counts as a percentage of total leukocyte counts. Cytokines were measured by ELISA (kits from R&D systems). ATP levels were measured using ATPlite® luminescence detection system (Perkin Elmer, Cambridge, UK).

Data analysis

Data are expressed as mean ± s.e.m of n observations. Statistical significance was determined using single or multiple comparisons using GraphPad Prism 5 software (specific details are in the Figure legends). A P value < 0.05 was taken as significant and all treatments were compared with the appropriate control group.

RESULTS

Bubbling smoke through culture medium did not appear to alter pH or osmolarity, neither did it seem to impact on cell viability (See Figure S1).

LPS challenged caused a significant increase in BALF IL-1β levels and neutrophilia in the wild type mice, these were not altered in mice missing functional TRPV1, TRPV4, TRPA1 or pannexin-1 channels (See Figures S2-5). CS challenge caused significant neutrophilia in the wild type which was not altered in the mice missing functional TRPA1 channels (See Figure S5).

FIGURE LEGENDS

Figure S1: The effect of CS on culture medium and cell viability

Osmolarity and pH was measured in vehicle treated culture medium and that exposed to smoke (A and B). The culture medium was added to human cells and cell viability assessed twenty-four hours later (C).

Figure S2: The role of Trpv4 in LPS-induced murine airway inflammation

Trpv4$^{-/-}$ mice were exposed to aerosolised LPS (1 mg/ml) or vehicle (saline) for 30 minutes alongside wild-type controls. BALF was collected 6 hours after the exposure for measurement of neutrophil and IL-1β levels. Data are represented as mean ± S.E.M. for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = $P < 0.05$, denoting a significant difference between the smoke exposed and air exposed wild-type groups; * = $P < 0.05$, denoting a significant difference between the smoke exposed knock-outs and wild-types.

Figure S3: The role of Trpv1 in LPS-induced murine airway inflammation

$Trpv1^{-/-}$ mice were exposed to aerosolised LPS (1 mg/ml) or vehicle (saline) for 30 minutes alongside wild-type controls. BALF was collected 6 hours after the exposure for measurement of neutrophil and IL-1β levels. Data are represented as mean ± S.E.M. for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = $P < 0.05$, denoting a significant difference between the smoke exposed and air exposed wild-type groups; * = $P < 0.05$, denoting a significant difference between the smoke exposed knock-outs and wild-types.

Figure S4: The role of pannexin-1 in LPS-induced murine airway inflammation

$Pannexin\text{-}1^{-/-}$ mice were exposed to aerosolised LPS (1 mg/ml) or vehicle (saline) for 30 minutes alongside wild-type controls. BALF was collected 6 hours after the exposure for measurement of neutrophil and IL-1β levels. Data are represented as mean ± S.E.M. for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = $P < 0.05$, denoting a significant difference between the smoke exposed and air exposed wild-type groups; * = $P < 0.05$, denoting a significant difference between the smoke exposed knock-outs and wild-types.

Figure S5: The role of Trpa1 in CS- and LPS-induced murine airway inflammation

A: $Trpa1^{-/-}$ mice were exposed to CS or room air (control) twice daily for 3 consecutive days alongside wild-type controls. BALF was collected 24 hours after the last exposure for measurement of neutrophil levels. B: $Trpa1^{-/-}$ mice were exposed to aerosolised LPS (1 mg/ml) or vehicle (saline) for 30 minutes alongside wild-type controls. BALF was collected 6 hours after the exposure for measurement of neutrophil levels. Data are represented as mean ± S.E.M. for n=8 animals in each group. Statistical significance was determined using Mann-Whitney U test. # = $P < 0.05$, denoting a significant difference between the smoke/LPS

exposed and air/saline exposed wild-type groups; * = $P < 0.05$, denoting a significant difference between the smoke/LPS exposed knock-outs and wild-types.

REFERENCES

1 Solle M, Labasi J, Perregaux DG, *et al.* Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 2001;**276**:125–32.

2 Eltom S, Stevenson CS, Rastrick J, *et al.* P2X7 receptor and caspase 1 activation are central to airway inflammation observed after exposure to tobacco smoke. *PLoS One* 2011;**6**:e24097–e24097.

Figure S1

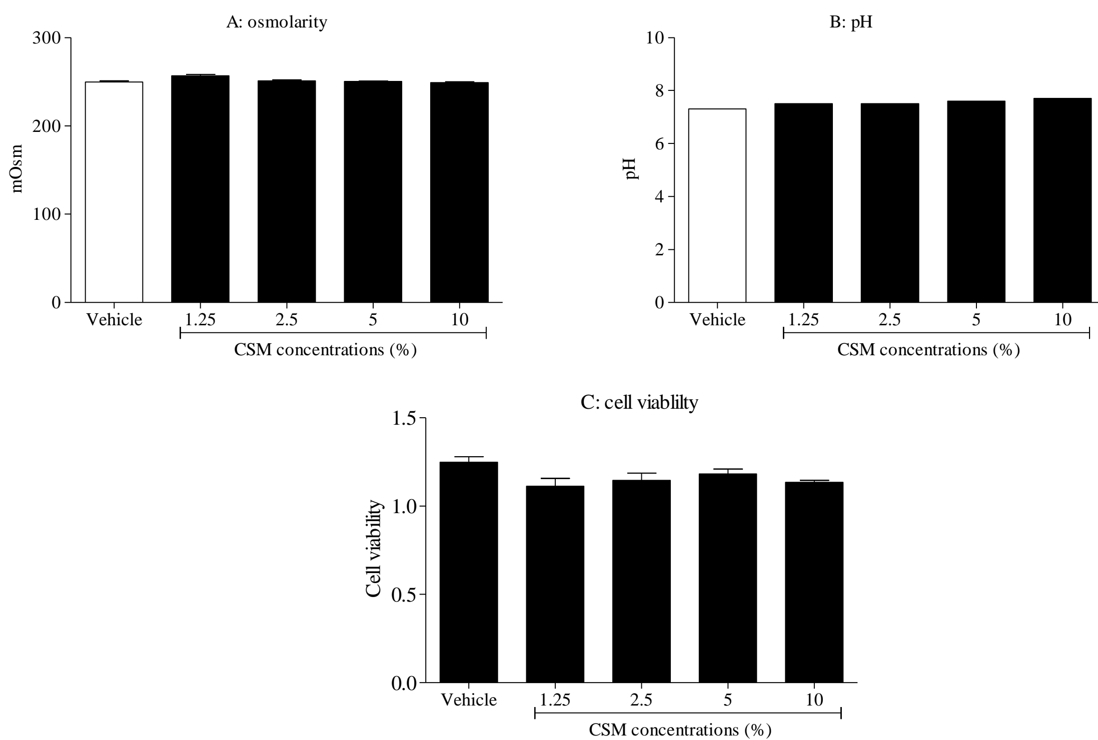
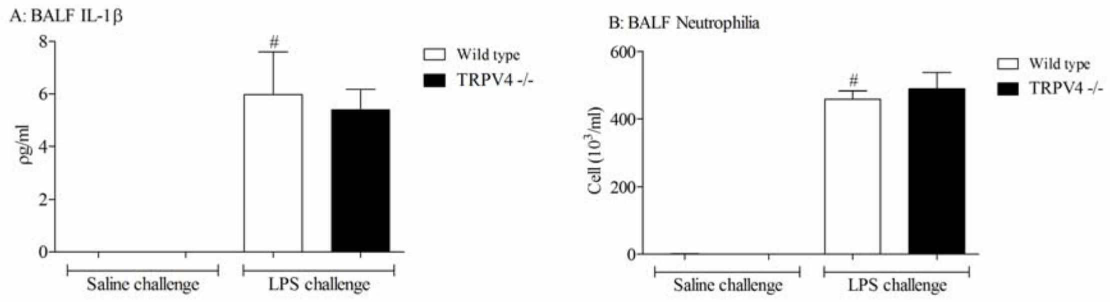


Figure S2



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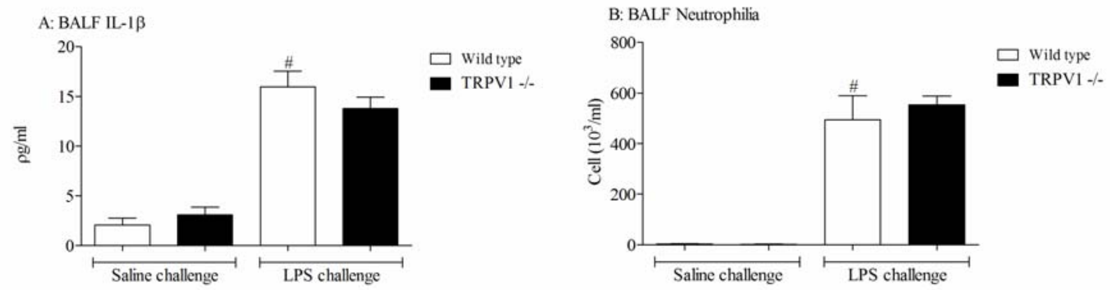

Figure S4

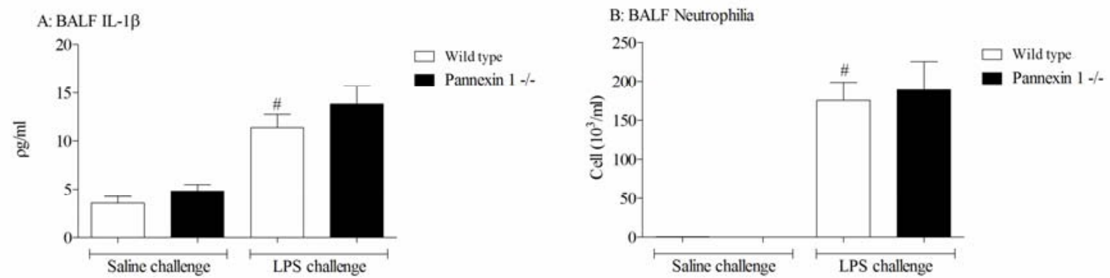

Figure S5