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EPAC activation reverses neutrophil dysfunction induced by β_2 -agonists, corticosteroids and critical illness

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ABSTRACT

Background. Neutrophils play a role in the pathogenesis of asthma, chronic obstructive pulmonary disease and pulmonary infection. Impaired neutrophil phagocytosis predicts hospital-acquired infection. Despite this, remarkably few neutrophil-specific treatments exist.

Objectives. To identify novel pathways for the restoration of effective neutrophil phagocytosis, and to activate such pathways effectively in neutrophils from patients with impaired neutrophil phagocytosis.

Methods. Blood neutrophils were isolated from healthy volunteers and from patients with impaired neutrophil function. In healthy neutrophils, phagocytic impairment was induced experimentally using beta-2 agonists. Inhibitors and activators of cyclic AMP (cAMP)-dependent pathways were used to assess the influence on neutrophil phagocytosis *in vitro*.

Results. Beta-2 agonists and corticosteroids inhibited neutrophil phagocytosis. Impairment of neutrophil phagocytosis by beta-2 agonists was associated with significantly reduced RhoA activity. Inhibition of protein kinase A (PKA) restored phagocytosis and RhoA activity, suggesting that cAMP signals through PKA to drive phagocytic impairment. Cyclic AMP can however signal through effectors other than PKA, such as exchange protein directly activated by cAMP (EPAC). An EPAC-activating analog of cAMP (8CPT-2Me-cAMP) reversed neutrophil dysfunction induced by beta-2 agonists or corticosteroids, but did not increase RhoA activity. 8CPT-2Me-cAMP reversed phagocytic impairment induced by Rho kinase inhibition, but was ineffective in the presence of Rap1 GTPase inhibitors. 8CPT-2Me-cAMP restored function to neutrophils from patients with known, acquired impairment of neutrophil phagocytosis.

Conclusions. EPAC activation consistently reverses clinical and experimental impairment of neutrophil phagocytosis. EPAC signals through Rap1 and bypasses RhoA. EPAC activation represents a novel potential means by which to reverse impaired neutrophil phagocytosis.

CLINICAL IMPLICATIONS

EPAC activation and/or Rap-1 specific signaling represent potential ‘drugable’ targets for the restoration of functioning neutrophil phagocytosis, at a time when neutrophil-specific therapies are distinctly lacking in clinical practice.

CAPSULE SUMMARY

EPAC reverses impaired neutrophil phagocytosis via Rap-1. As neutrophil dysfunction in critical illness predicts infection, and as this restorative mechanism in neutrophils is previously unreported, EPAC homologs could theoretically prevent infection without recourse to antibiotics.

KEY WORDS

Neutrophil; beta-2 agonist; cyclic AMP; exchange protein directly activated by cAMP (EPAC); hospital-acquired infection.

ABBREVIATIONS

AKAP	A kinase anchoring protein
APACHE	Acute physiology and chronic health evaluation
6-Bnz-cAMP	N ⁶ -benzoyladenosine-cAMP
cAMP	Cyclic adenosine monophosphate
COPD	Chronic obstructive pulmonary disease
ELISA	Enzyme-linked immunosorbent assay
EPAC	Exchange protein directly activated by cAMP

GM-CSF	Granulocyte macrophage colony-stimulating factor
HAI	Hospital-acquired infection
IMDM	Iscove's modified Dulbecco's medium
MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBS	Phosphate-buffered saline
PGE1	Prostaglandin E1
PKA	Protein kinase A
SAPS	Simplified acute physiology score
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SOFA	Sequential organ failure assessment

INTRODUCTION

Neutrophils are central to the pathogenesis of a wide variety of common and important clinical conditions including chronic obstructive pulmonary disease (COPD) and asthma (particularly corticosteroid-resistant asthma).¹⁻³ Patients with COPD and asthma are commonly prescribed long-term inhaled beta-2 agonists and corticosteroids. The detailed effects of these treatments on neutrophil phagocytic function are relatively poorly understood, though it has been suggested that beta-2 agonists can impair neutrophil function.⁴

It is also increasingly recognized that acquired neutrophil dysfunction is common in critically ill patients,⁵ and is independently associated with a significantly increased risk of subsequent hospital-acquired infection (HAI).⁶ Impaired neutrophil function can be restored to normal, *ex vivo*, through administration of granulocyte-macrophage colony-stimulating factor (GM-CSF).⁶ This suggests the potential to develop pharmacological strategies to restore specific defects in neutrophil function for clinical benefit. The potential importance of developing non-antibiotic-based pharmacological prevention strategies is enormous. For example, at a conservative estimate there are 1.7 million HAIs in the US annually,⁷ at a time when antibiotic resistance is a global concern and few new antibiotics are in development. The aims of this study were therefore to characterize the effects of beta-2 agonists, corticosteroids and critical illness on neutrophil function, and to identify novel ways of modulating pathways involved in the impairment of neutrophil phagocytosis, in the hope of identifying candidates with the potential to restore efficient phagocytosis.

METHODS

Reagents

Salbutamol, salmeterol, isoprenaline, fluticasone, beclomethasone, budesonide, SQ 22536, ICI 118,551, atenolol, prostaglandin E1 (PGE1), Y27632, zymosan derived from *Saccharomyces cerevisiae* and Giemsa staining solution were from Sigma Aldrich (Gillingham, UK). KT5720, mouse anti-human GM-CSF receptor antibody (IgG2a), and murine IgG2a negative control antibody were from Merck (Darmstadt, Germany). St-Ht31 and St-Ht31 control peptide were from Promega (Madison, USA). Antibodies against RhoA and protein kinase A (PKA) were from Cell Signaling (Hitchin, UK). Dextran was from Pharmacosmos (Holbaek, Denmark). Percoll Plus was from GE Healthcare (Little Chalfont, UK). Iscove's Modified Dulbecco's Medium (IMDM) was from Life Technologies (Paisley, UK). 8CPT-2Me-cAMP, N⁶-benzoyladenine-cAMP (6-Bnz-cAMP) and GGTi 298 were from Tocris Bioscience (Bristol, UK). PKA inhibitor IV was from Santa Cruz Biotechnology (Dallas, TX, USA), ESI-09 was from Biolog (Bremen, Germany). Rho G-lisa was from Cytoskeleton (Denver, USA). PKA activity assay was from Abcam (Cambridge, UK).

Ethical approvals

Ethical approval to obtain neutrophils from healthy volunteers was granted by the County Durham and Tees Valley Research Ethics Committee. Approval to obtain neutrophils from patients was granted by the Yorkshire and the Humber - Leeds West Research Ethics Committee. Ethical approval relating to the BALTI-2 trial was granted by the Oxford A Research Ethics Committee.

Isolation of peripheral blood neutrophils

Neutrophils were isolated from whole blood by dextran sedimentation and fractionation over discontinuous Percoll gradients as previously described.⁸ Only samples yielding $\geq 95\%$ neutrophil purity (assessed by morphological analysis) and $\geq 95\%$ viability (assessed by trypan blue exclusion) were used.

Phagocytosis assay

Phagocytosis of serum-opsonized zymosan by neutrophils was assessed as previously described.⁵ A variety of reagents were incorporated into the phagocytosis assay, individually or in combination, to determine the effect on phagocytosis. Doses for each condition are described in figure legends or in the Results section. All incubations described were performed at 37°C in 5% CO₂. Unless otherwise stated, neutrophils were prepared in IMDM containing 1% autologous serum, and were adherent to tissue culture plastic. In all experiments in which salbutamol, salmeterol, corticosteroids or PGE1 were used to impair neutrophil phagocytosis, the application was for 30 minutes, prior to the addition of serum-opsonized zymosan for a further 30 minutes. Phagocytosis was quantified in terms of the proportion of neutrophils containing 2 or more zymosan particles at light microscopy.

Bacterial Killing Assay

The ability of neutrophils to kill live bacteria was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay as previously described.⁹ Briefly, bacteria (laboratory strains of *Staphylococcus aureus* NCTC 8325 or *Pseudomonas aeruginosa* PA01, and clinical strains of *S. aureus* (methicillin sensitive – strain 97 STA) and *P. aeruginosa* (42 PSA) isolated from patients with ventilator-associated

pneumonia were added to 5×10^5 neutrophils at a multiplicity of infection (MOI) of 10 and incubated at $37^\circ\text{C}/5\% \text{ CO}_2$ for 30 minutes. Cells were then lysed with Triton X-100 before the addition of $20\mu\text{l}$ of MTT (5mg/ml). MTT metabolized to yield a purple color by live bacteria was solubilized in isopropanol and the OD_{560} measured. The percentage bacteria killed was determined by comparing values to a standard curve prepared by diluting the initial bacterial dose to represent 0, 30, 60 and 90% of bacteria killed versus the OD_{560} value.

RhoA and PKA activity assays

RhoA and PKA activity were measured using an enzyme-linked immunosorbent assay (ELISA; Rho G-lisa) as per the manufacturer's instructions. Neutrophils were adhered to tissue culture plates before being exposed to serum-opsonized zymosan or vehicle control for 5 minutes. Cells were washed in ice-cold phosphate-buffered saline (PBS) and then lysed. Aliquots were taken for estimation of total protein with the remainder snap frozen in liquid nitrogen and stored at -80°C . Samples were equalized for total protein concentration and analyzed using a BMG FluoStar Optima plate reader. Samples were assessed for total RhoA and total PKA by boiling in Laemmli sample buffer before Western blot analysis using anti-RhoA or anti-PKA antibodies.

Statistical Analysis

The Statistical Package for the Social Sciences 17.0 (IBM, USA) was used. In the figures shown, unless otherwise stated, results are derived from 6 independent experiments (ie conditions performed using neutrophils from 6 different donors). Statistical comparisons were by one-way ANOVA with Bonferroni's post-hoc correction, and data expressed as mean (bar) and standard error of the mean (error bars). P values <0.05 were considered statistically significant, and in the figures * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

RESULTS

With the exception of results shown in Figure 7 C and D, all data relate to neutrophils derived from healthy volunteers.

Incubation of neutrophils from healthy volunteers with the short-acting beta-2 agonist salbutamol resulted in a dose-dependent impairment of phagocytosis (Figure 1A). The long-acting beta-2 agonist salmeterol induced a similar effect (Figure 1A). Neither salbutamol nor salmeterol influenced neutrophil viability at the concentrations used (data not shown). The selective beta-2 antagonist ICI 118,551 prevented the anti-phagocytic effects of salbutamol and salmeterol, without inhibiting the impairment of phagocytosis induced by the beta-1 agonist isoprenaline. In contrast, atenolol (a predominantly beta-1 antagonist with partial beta-2 antagonist activity) prevented the anti-phagocytic effect of isoprenaline, but not the effects of salbutamol or salmeterol (Figure 1B). The inhibitory effects of salbutamol on neutrophil phagocytosis were also reduced, in a dose-dependent manner, by the adenylate cyclase inhibitor SQ22536, (Figure 1C). Salbutamol at 10 μ M was used to induce reproducible phagocytic impairment in later experiments, unless otherwise stated.

Incubation of neutrophils with the glucocorticoids beclomethasone, fluticasone and budesonide resulted in a dose-dependent impairment of neutrophil phagocytosis (Figure 2A). The combination of beclomethasone and salbutamol produced an effect similar to salbutamol alone (Figure 2B).

The results in Figure 1 suggested that beta-2 agonist-induced impairment of neutrophil phagocytosis is mediated via cyclic adenosine monophosphate (cAMP), and that mechanisms

downstream of cAMP signaling may provide novel opportunities for pharmacologic restoration of neutrophil phagocytosis. PGE1 was selected as a separate stimulus known to increase intracellular cAMP. PGE1 was incubated with neutrophils, and produced a similar impairment of phagocytosis (Figure 3A). PKA is a key target for intracellular cAMP, and is located at strategic compartmentalized locations within cells through interaction with A kinase anchoring proteins (AKAPs).¹⁰ Pre-incubation of neutrophils with St-Ht31 (a cell-permeable inhibitor of AKAPs) prevented impairment of neutrophil phagocytosis mediated by salbutamol (Figure 3B). This suggested that cAMP impairs neutrophil phagocytosis predominantly via activation of PKA. To test this further, salbutamol-treated neutrophils were pre-incubated with two separate PKA inhibitors, KT5720 and PKA inhibitor IV. Both inhibitors prevented salbutamol-induced impairment of phagocytosis. (Figure 3C). We further demonstrated that a PKA-specific cAMP analogue, 6-Bnz-cAMP, significantly reduced neutrophil phagocytosis (Figure 3D).

In parallel with the studies of phagocytosis described, we confirmed that salbutamol increases PKA activity in neutrophils and that this effect can be blocked by a PKA inhibitor (Figure 4A). RhoA is inherently involved in cytoskeletal organization in the neutrophil,¹¹ and adequate function of RhoA is pivotal to effective phagocytosis. To investigate the downstream mechanisms of salbutamol-induced impairment of phagocytosis further, we therefore studied the effect of salbutamol on RhoA activity. Salbutamol significantly reduced RhoA activity in neutrophils exposed to zymosan, without affecting the total amount of RhoA proteins (Figure 4B). This effect could be significantly abrogated by PKA inhibition (Figure 4B). These findings suggested that salbutamol stimulates PKA, which inhibits RhoA activity, which in turn impairs phagocytosis of zymosan.

The data presented in Figures 3 and 4, in addition to our previous data,⁶ suggested that RhoA plays a part in regulating efficient phagocytosis. The next question was whether phagocytosis could be restored through other intracellular pathways, when RhoA activity was blocked. The role of PKA both in mediating beta-2-induced impairment of phagocytosis and in reducing RhoA activity was interesting in this regard, as cAMP can exert divergent functions in cells, depending on whether it engages PKA or alternative effectors. One such alternative effector is EPAC (exchange protein directly activated by cAMP), which is typically associated with Rap-1 signaling.¹²⁻¹⁴ We therefore used an analog of cAMP that selectively activates EPAC (8CPT-2Me-cAMP) to determine the effect of routing cAMP away from the RhoA pathway. 8CPT-2Me-cAMP reversed salbutamol-induced impairment of phagocytosis (Figure 5A, “salbutamol” columns). The protective effect was removed by an EPAC-specific inhibitor, ESI-09 (Figure 5A, “salbutamol” columns). GM-CSF, which can reverse impaired neutrophil phagocytosis induced by C5a or by critical illness,⁶ also reversed salbutamol-mediated phagocytic impairment (Figure 5A, “salbutamol” columns). However ESI-09 could not prevent the protective effect of GM-CSF, implying that GM-CSF does not act through EPAC (Figure 5A, “salbutamol” columns). All of these results could be replicated when PGE1 was substituted for salbutamol (Figure 5A, “PGE1” columns).

To test the role of RhoA in restoration of phagocytosis by GM-CSF and 8CPT-2Me-cAMP, the Rho kinase inhibitor Y27632 was also used to drive impairment of neutrophil phagocytosis. Under these circumstances 8CPT-2Me-cAMP, but not GM-CSF, was capable of restoring neutrophil phagocytosis (Figure 5A, “Y27632” columns). This indicated that GM-CSF exerts its restorative effect through RhoA, while 8CPT-2Me-cAMP does not.

We therefore went on to study the effects of EPAC activation on RhoA activity. Interestingly, application of 8CPT-2Me-cAMP to healthy neutrophils reduced RhoA activity by 63% (Figure 5B, left panel). Furthermore, 8CPT-2Me-cAMP had no effect on salbutamol-induced impairment of Rho activity (Figure 5B, right panel). Both these results contrasted with the effects of GM-CSF (Figure 5B), which were more like those for PKA inhibition (Figure 4B). This suggested that 8CPT-2Me-cAMP does not exert its protective effects on phagocytosis through RhoA. As EPACs can act as guanine-exchange proteins for the Rap family,¹³ we tested whether a Rap inhibitor (GGTi 298) could interfere with the protective effects of 8CPT-2Me-cAMP. GGTi 298 significantly abrogated the protective effects of 8CPT-2Me-cAMP on phagocytosis (Figure 5C). To confirm that 8CPT-2Me-cAMP exerted none of its effects through the GM-CSF receptor, we incubated neutrophils with an antibody that blocks the GM-CSF receptor. The blocking antibody prevented the phagocytosis-protective effects of GM-CSF, but not those of 8CPT-2Me-cAMP (Figure 5D). The effect of glucocorticoids on phagocytosis could also be partly restored by 8CPT-2Me-cAMP (Figure 5E).

Figure 5 indicates an effect of 8CPT-2Me-cAMP on phagocytosis of inert particles. In order to test a more relevant physiological response, the effect on bacterial killing was assessed. 8CPT-2Me-cAMP significantly restored the capacity of neutrophils to kill laboratory and clinical strains of the Gram positive pathogen *S. aureus* (Figure 6A and B) and the Gram negative pathogen *P. aeruginosa* (Figure 6C and D).

Collectively, the results above suggested that neutrophil phagocytosis can be impaired through a PKA-dependent pathway. They further suggested that selective EPAC activation can bypass this pathway, thereby representing a potentially novel means to restore neutrophil phagocytosis. They also suggested that the protective effects exerted by 8CPT-2Me-cAMP

and GM-CSF are independent of one another. We therefore sought to test the potential clinical relevance of these findings in two scenarios.

Firstly, plasma from patients in the BALTI-2 trial (a randomized controlled trial of intravenous salbutamol versus placebo in critically ill patients),¹⁵ was applied to healthy neutrophils. On six separate occasions plasma from patients (n=13 who received placebo, n=5 who received salbutamol) was applied in a blinded fashion to neutrophils isolated from healthy volunteers (ie six separate healthy volunteers provided neutrophils, the plasma applied was the same each time). Plasma from salbutamol-treated and placebo-treated patients impaired neutrophil phagocytosis to a similar degree (Figure 7A). Given the striking degree of phagocytic impairment induced by plasma from critically ill patients, these experiments were repeated to determine whether 8CPT-Me-cAMP could reverse this effect. Both 8CPT-2Me-cAMP and GM-CSF reversed the anti-phagocytic effect of the plasma (Figure 7B).

Secondly, neutrophils were isolated from the blood of 16 critically ill patients at high risk of nosocomial infection (8 male, 8 female; median age 66 (range 25-82) years). All patients were: admitted to ICU within the last 48 hours; met criteria for the systemic inflammatory response syndrome (SIRS); and required the support of at least one organ system. Clinical parameters included mean Acute Physiology and Chronic Health Evaluation (APACHE) II score of 23.1 (standard deviation (SD) 8.4), Simplified Acute Physiology Score II (SAPS II) of 42.3 (SD 10.0) Sequential Organ Failure Assessment (SOFA) score of 9.0 (SD 4.1, n=15 due to missing data in 1 patient), and PaO₂:FiO₂ ratio 173 mmHg (SD 78, n=15 due to missing data in 1 patient). The principal diagnoses were pneumonia (n=7), urinary sepsis (n=3), infective endocarditis, biliary sepsis, pancreatitis, multiple trauma, acute colitis, and

post-operative peritonitis (all n=1). Both treatments resulted in significant improvement in phagocytic capacity (Figures 7C and 7D).

A schematic summary of our main findings is shown in Figure 8.

DISCUSSION

The findings presented here may have potential clinical implications for critically ill patients at the highest risk of developing hospital-acquired infection,⁶ where EPAC activation and/or selective signaling through Rap-1 emerge as interesting areas for possible therapeutic exploration. The capacity to restore efficient killing of Gram positive and Gram negative pathogens commonly implicated in HAI is encouraging in this regard. While EPAC activators have not been used clinically to date, they have shown promise in, for example, *in vivo* models of macular degeneration.¹⁶

Our overall findings extend data showing that the generation of cAMP impairs neutrophil phagocytosis. In our hands, the beta-2 agonist effect could be abolished by selective inhibitors of PKA, suggesting that cAMP selectively targets PKA in neutrophils. In several cell types, cAMP is directed to PKA through the activity of AKAPs.^{17,18} This process appears to be replicated in neutrophils, which are known to contain AKAPs,¹⁹ and in our experiments an AKAP inhibitor was also capable of partially restoring phagocytosis in beta-2 agonist-treated neutrophils.

The anti-phagocytic activity of beta-2 agonists appeared to be mediated, at least in part, by inhibition of RhoA. Similar findings have been described for impaired phagocytosis induced by excessive C5a.⁵ This suggests a similar downstream mechanism for impairment of neutrophil phagocytosis mediated through the beta-2 receptor, PGE1 receptor, and CD88 (the receptor for C5a). The implication is that RhoA inhibition is a central downstream process in mediating the phagocytic impairment induced by engaging these receptors. A similarly

important role for RhoA in regulating complement-mediated phagocytosis has been described in murine macrophages.²⁰

A key finding of our study was that, in striking contrast to GM-CSF, EPAC activation not only failed to restore salbutamol-driven impairment of RhoA activation, but knocked down constitutive RhoA. Despite this, both GM-CSF and the EPAC activator could completely reverse beta-2 agonist- and PGE1-induced impairment of neutrophil phagocytosis. This formed the basis for the observation that EPAC and GM-CSF have alternative modes of action. In keeping with this, Rho kinase inhibition prevented phagocytic restoration caused by GM-CSF, but had no effect on the restorative effects of EPAC activation (Figure 5A). Taken together these findings suggested that GM-CSF and EPAC activation, while both capable of restoring phagocytosis, had opposing effects on RhoA. EPAC is known to signal through Rap-1 in other cell types,^{21,22} raising the possibility that EPAC may selectively signal through Rap-1, bypassing RhoA. The confirmation of two independent pathways for restoration of neutrophil phagocytosis is novel, to our knowledge, though interestingly a similar situation has been described in a murine macrophage cell line.²³

Certain cautions are required when considering our data. For example, our *ex vivo* conditions may be insufficiently representative of those *in situ*. Our experiments were performed in blood neutrophils adhered to tissue culture plastic, and it is unlikely that phagocytic capacity (and response to molecules such as EPAC analogues or GM-CSF) would be replicated identically in neutrophils within body tissues. Any implications around the significance of our findings in tissue neutrophils therefore remain necessarily speculative. In addition, our routinely used concentration of salbutamol, 10 μ M, is higher than that often described in clinical scenarios. The original BALTI trial generated estimated salbutamol concentrations in

the low micromolar range.²⁴ Our rationale for studying plasma from patients in the BALTI-2 trial related to the use of intravenous beta-2 agonists. In hindsight, while salbutamol concentrations were not specifically measured in the BALTI-2 study, we were always unlikely to observe any effect of salbutamol over and above the profound anti-phagocytic effect of plasma from critically ill patients.

In addition, we focused on neutrophil phagocytosis. The concentrations of salbutamol used have been shown to reduce neutrophil chemotaxis in other studies.²⁵ We concentrated on phagocytosis because impaired *ex vivo* phagocytosis of serum-opsonized zymosan predicts nosocomial infection in a clinical setting.⁶ We should emphasize that in our bacterial killing experiments (Figure 6) in particular we cannot infer that the effectiveness of GM-CSF or 8-CPT-2Me-cAMP is attributable to effects on phagocytosis alone, as effects on many other innate immune functions of neutrophils could be contributing.

Furthermore, interpretation of studies of this nature may be constrained by lack of absolute specificity of inhibitors. For example molecules such as KT5720 can influence targets other than PKA, and Y27632 certainly does not inhibit all RhoA functions. Finally, in advocating the further study of EPAC analogs or Rap-1 activators as potential therapeutics, we must obviously remain alert to the fact that EPAC and Rap-1 are involved in multiple homeostatic processes, and future work will be required to take due consideration of potential toxicities.

In summary, EPAC activation represents a novel potential therapeutic avenue for the restoration of neutrophil phagocytosis, and deserves further investigation.

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Competing interests. AJS has received funds to attend educational conferences (travel, accommodation and registration) from GlaxoSmithKline, Boehringer Ingelheim and Astra Zeneca. He has given non-promotional talks for GSK. No other author has any competing interest to declare.

Author contributions. JS, MHRS and AJS designed the study; JS, GH, EMB, JGM, JDW, SW and MHRS performed experiments; JS, ACM, DFM, MHRS and AJS undertook data analysis and interpretation; EMB, JGM, DRT, GDP, SVB, AIR, VCL and SEW obtained and provided clinical samples; JS, MHRS and AJS drafted the manuscript; all authors approved the manuscript prior to submission.

REFERENCES

- 1 Gadek JE, Fells GA, Zimmerman RL, Rennard SI, Crystal RG. Antielastases of the human alveolar structures. Implications for the protease-antiprotease theory of emphysema. *J Clin Invest* 1981;68:889-98.
- 2 Hoenderdos K, Condliffe A. The neutrophil in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2013;48:531-9.
- 3 Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of a subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002;57:875-9.
- 4 Ignarro LJ, Lint TF, George WJ. Hormonal control of lysosomal enzyme release from human neutrophils. Effects of autonomic agents on enzyme release, phagocytosis, and cyclic nucleotide levels. *J Exp Med* 1974;139:1395-1414.
- 5 Conway Morris A, Kefala K, Wilkinson TS, Dhaliwal K, Farrell L, Walsh T, et al. C5a mediates peripheral blood neutrophil dysfunction in critically ill patients. *Am J Respir Crit Care Med* 2009;180:19-28.
- 6 Conway Morris A, Brittan M, Wilkinson TS, McAuley DF, Antonelli J, McCulloch C, et al. C5a-mediated neutrophil dysfunction is RhoA-dependent and predicts infection in critically ill patients. *Blood* 2011;117:5178-88.
- 7 Klevens RM, Edwards JR, Richards CL Jr, Horan TC, Gaynes RP, Pollock DA, et al. Estimating health care-associated infections and deaths in US hospitals, 2002. *Public Health Rep* 2007;122:160-66.
- 8 Dransfield I, Buckle AM, Savill JS, McDowall A, Haslett C, Hogg N. Neutrophil apoptosis is associated with a reduction in CD16 (Fc gamma RIII) expression. *J Immunol* 1994;153:1254-63.

- 9 Stevens MG, Kehrl Jr ME, Canning PC. A colorimetric assay for quantitating bovine neutrophil bactericidal activity. *Vet Immunol Immunopathol* 1991;28:45-56.
- 10 Huang LJ, Durick K, Weiner JA, Chun J, Taylor SS. Identification of a novel protein kinase A anchoring protein that binds both type I and type II regulatory subunits. *J Biol Chem* 1997;272:8057-64.
- 11 Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* 2005;21:247-69.
- 12 Breckler M, Berthouze M, Laurent AC, Crozatier B, Morel E, Lezoualc'h F. Rap-linked cAMP signaling Epac proteins: compartmentation, functioning and disease implications. *Cell Signal* 2011;23:1257-66.
- 13 Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, et al. A family of cAMP-binding proteins that directly activate Rap1. *Science* 1998;282:2275-79.
- 14 de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 1998;396:474-77.
- 15 Perkins GD, Gates S, Lamb SE, McCabe C, Young D, Gao F, et al. Beta Agonist Lung Injury Trial-2 (BALTI-2) trial protocol: a randomised, double-blind, placebo-controlled trial of intravenous infusion of salbutamol in the acute respiratory distress syndrome. *Trials* 2011;12:113.
- 16 Wittchen ES, Nishimura E, McCloskey M, Wang H, Quilliam LA, Chrzanowska-Wodnicka M, et al. Rap1 GTPase activation and barrier enhancement in rpe inhibits choroidal neovascularization in vivo. *PLoS One* 2013;8:e73070.
- 17 Horvat SJ, Deshpande DA, Yan H, Panettieri RA, Codina J, DuBose TD Jr, et al. A-kinase anchoring proteins regulate compartmentalized cAMP signaling in airway smooth muscle. *FASEB J* 2012;26:3670-79.

- 18 Diviani D, Dodge-Kafka KL, Li J, Kapiloff MS. A-kinase anchoring proteins: scaffolding proteins in the heart. *Am J Physiol Heart Circ Physiol* 2011;301:H1742-H1753.
- 19 Jones SL. Protein kinase A regulates beta2 integrin avidity in neutrophils. *J Leukoc Biol* 2002; 71: 1042-1048.
- 20 Kamanova J, Kofronova O, Masin J, Genth H, Vojtova J, Linhartova I, et al. Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling. *J Immunol* 2008;181:5587-97.
- 21 Rangarajan S, Enserink JM, Kuiperij HB, de Rooij J, Price LS, Schwebe F, et al. Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. *J Cell Biol* 2003;160:487-93.
- 22 Wang Z, Dillon TJ, Pokala V, Mishra S, Labudda K, Hunter B, et al. Rap1-mediated activation of extracellular signal-regulated kinases by cyclic AMP is dependent on the mode of Rap1 activation. *Mol Cell Biol* 2006;26:2130-45.
- 23 Kim JG, Moon MY, Kim HJ, Li Y, Song DK, Kim JS, et al. Ras-related GTPases Rap1 and RhoA collectively induce the phagocytosis of serum-opsonized zymosan particles in macrophages. *J Biol Chem* 2012;287:5145-55.
- 24 Perkins GD, McAuley DF, Thickett DR, Gao F. The beta-agonist lung injury trial (BALTI): a randomized placebo-controlled clinical trial. *Am J Respir Crit Care Med* 2006;173:281-87.
- 25 Perkins GD, Nathani N, McAuley DF, Gao F, Thickett DR. In vitro and in vivo effects of salbutamol on neutrophil function in acute lung injury. *Thorax* 2007;62:36-42.

FIGURE LEGENDS

Figure 1: β adrenergic receptor agonists inhibit human neutrophil phagocytosis in an adenylate cyclase-dependent manner.

(A) Neutrophils were pre-incubated with the short-acting beta-2 agonist salbutamol or the long-acting beta-2 agonist salmeterol and phagocytosis of zymosan quantified. Statistical comparisons are with bar of the same color in the left-hand column. (B) In a variation, neutrophils were first treated with atenolol (100 μ M) or the selective beta-2 antagonist ICI 118,551 (10 μ M) for 30 minutes before exposure to either salbutamol (10 μ M), salmeterol (10 μ M) or the beta-1 agonist isoprenaline (10 μ M). Statistical comparisons are between the bars indicated. (C) In a separate variation, neutrophils were pre-incubated with the adenylate cyclase inhibitor SQ 22536 before incubation with salbutamol (10 μ M). Statistical comparisons are with the left-hand column..

Figure 2: Glucocorticoids inhibit neutrophil phagocytosis in a dose-dependent manner.

(A) Neutrophils were pre-incubated with fluticasone, beclomethasone and budesonide, and phagocytosis of zymosan quantified. Statistical comparisons are with the bar of the same color in the left hand panel. (B) Neutrophils were pre-incubated with salbutamol (10 μ M), beclomethasone (10 μ M) or a combination of the two, and phagocytosis quantified. Statistical comparison is with the left hand column.

Figure 3: Inhibition of neutrophil phagocytosis by β_2 agonists is PKA-dependent.

(A) Neutrophils were pre-incubated with salbutamol or prostaglandin E1 or a combination, and phagocytosis of zymosan quantified. Statistical comparisons are with

the left hand column. (B) Neutrophils were pre-incubated with the AKAP inhibitory peptide St-Ht31, control or peptide control prior to addition of vehicle control or salbutamol (10 μ M), and phagocytosis quantified. Statistical comparisons are as indicated by horizontal bars. (C) Neutrophils were pre-incubated with the PKA inhibitors KT5720 (final concentration 5 μ M) or PKA inhibitor IV (1 μ M) for 30 minutes prior to addition of salbutamol (10 μ M) or prostaglandin E1 (1 μ M), then phagocytosis was quantified. Statistical comparisons are as indicated by horizontal bars. (D) Neutrophils were pre-incubated with vehicle control, St-Ht31 (20 μ M) or the peptide control for St-Ht31 (20 μ M) prior to the addition of vehicle control or the PKA-selective cAMP analogue 6-Bnz-cAMP (10 μ M), and phagocytosis of zymosan quantified. Statistical comparisons are with the nearest black bar to the left.

Figure 4: Inhibition of neutrophil phagocytosis by β_2 agonists is associated with down-regulation of RhoA.

(A) Neutrophils were pre-incubated with the PKA inhibitor KT5720 (5 μ M) for 30 minutes, before addition of salbutamol or control. Zymosan (grey and white bars) or no zymosan (black bars) was then added for 5 minutes, and the reaction stopped. PKA activity was estimated by ELISA (upper panel) and total PKA protein and β -actin were assessed by Western blot (lower panel). (B) In a variation of (A), RhoA activity and protein were assessed instead of PKA. For both (A) and (B) statistical comparisons are with the nearest white column. Data are representative of 5 independent experiments.

Figure 5: β_2 agonist-induced dysfunction of neutrophil phagocytosis is rescued by an EPAC activator which acts independently of the GM-CSF receptor.

(A) Neutrophils were pre-incubated with salbutamol, prostaglandin E1 (1 μ M) or the Rho kinase inhibitor Y27632 (10 μ M) +/- the EPAC inhibitor ESI-09 (10 μ M) for 30 minutes, prior to the addition of the EPAC activator 8CPT-2Me-cAMP (final concentration 50 μ M) or GM-CSF (0.3nM) for 30 minutes. Phagocytosis was quantified. Statistical comparisons are with the nearest black bar to the left. (B) Neutrophils were pre-incubated with salbutamol before incubation with human recombinant GM-CSF (0.3 nM), or 8CPT-2Me-cAMP (50 μ M). Serum-opsonized zymosan was then added for 5 minutes. Cells were then placed on ice and lysed. RhoA activity was estimated by ELISA. Statistical comparisons are with the nearest black bar to the left. (C) Neutrophils were pre-incubated with the Rap-1 inhibitor GGTi 298 (10 μ M) or medium alone for 30 minutes before incubation with salbutamol with or without human recombinant GM-CSF (0.3nM) or 8CPT-2Me-cAMP (50 μ M) for 30 minutes. Phagocytosis was quantified. Statistical comparisons are indicated by the horizontal bars. (D) Neutrophils were pre-incubated with GM-CSF receptor blocking antibody (100 ng/ml) or negative control antibody (100ng/ml) for 30 minutes before exposure to salbutamol with or without GM-CSF (0.3nM) or 8CPT-2Me-cAMP (50 μ M) for 30 minutes. Phagocytosis was quantified.. Statistical comparisons are indicated by the horizontal bars. (E) Neutrophils were pre-incubated with salbutamol, beclomethasone (10 μ M) or a combination before exposure to vehicle control, GM-CSF (0.3nM) or 8CPT-2Me-cAMP (50 μ M) for 30 minutes. Phagocytosis was quantified. Statistical comparisons are with the nearest black bar to the left.

Figure 6: β_2 agonist-induced impairment of bacterial killing is prevented by EPAC activation.

Neutrophils were pre-incubated with salbutamol before incubation with human recombinant GM-CSF (0.3 nM), or 8CPT-2Me-cAMP (50 μ M). A *S. aureus* lab strain (Panel A), methicillin-sensitive *S. aureus* clinical strain (Panel B), *P. aeruginosa* lab strain (Panel C) or *P. aeruginosa* clinical strain (Panel D) were added (MOI 10) and incubated at 37°C/5% CO₂ for 30 minutes. Cells were lysed with Triton X-100 before the addition of 20 μ l of MTT (5mg/ml), the OD₅₆₀ was measured, and the percentage bacterial killing estimated from standard curves. Results are expressed as the percentage of starting bacteria killed. Values are mean +/- SEM from 7 and 8 independent experiments for *S. aureus* and *P. aeruginosa* respectively. Statistical comparisons are with the second (salb) column in each panel.

Figure 7: EPAC activation restores phagocytic function both to neutrophils exposed to anti-phagocytic plasma and to dysfunctional neutrophils from critically ill patients at high risk of nosocomial infection.

(A) Neutrophils were pre-incubated with plasma (1:10 dilution) from 18 patients involved in either limb of the BALTI-2 trial (5 had received intravenous salbutamol, 13 had received intravenous placebo), or with medium alone (control) for 30 minutes. Salbutamol added to neutrophils (second column) acted as a positive control. Zymosan (opsonized using serum from the healthy volunteer who provided the neutrophils on any given day) was added and phagocytosis quantified. Values are mean +/- SEM from six independent experiments [ie, on a given day, prior to addition of opsonized zymosan, one healthy donor's neutrophils were exposed to medium ("control" column), salbutamol ("salbutamol" column), plasma from each of 5 salbutamol-treated patients, with the mean rate of phagocytosis calculated ("salbutamol patients" column), or plasma from each of 13 placebo-treated patients, with the mean rate of phagocytosis calculated ("placebo patients" column); this experiment was repeated using neutrophils from 6 different healthy donors, using mean values from the 6 experiments]. (B)

Neutrophils were pre-incubated with plasma (1:10 dilution) from 6 patients involved in either limb of the BALTI-2 trial (3 had received intravenous salbutamol, 3 had received intravenous placebo), or with medium alone (control) for 30 minutes. Cells were then treated with human recombinant GM-CSF (0.3 nM) or 8CPT-2Me-cAMP (50 μ M) or medium alone for 30 minutes. Zymosan (opsonized using serum from the healthy volunteer who provided the neutrophils on any given day) was then added for 30 minutes, and phagocytosis quantified. (C and D) Neutrophils were isolated from the blood of 16 critically ill patients and adhered to tissue culture plastic before incubation with either (C) human recombinant GM-CSF (0.3 nM) or (D) 8CPT-2Me-cAMP (50 μ M) for 30 minutes prior to the addition of autologous serum-opsonized zymosan for a further 30 minutes. Phagocytosis was quantified. Data are shown as lines connecting individual data points from each individual patient, with or without the relevant treatment. The heavy horizontal line indicates the median value. Data were analyzed using the Wilcoxon rank sum test.

Figure 8: Schematic representation of main findings.

The picture represents a neutrophil, divided into 3 illustrative sections. Section 1 is a schematic of normal phagocytosis. Upon engagement of the appropriate phagocytic receptor, opsonized zymosan is thought to signal through RhoA, which plays a part in coordinating polymerization of filamentous actin (F-actin), allowing the efficient organization of the phagosome, with subsequent phagocytosis. Section 2 illustrates the proposed disruption of this system by beta-2 agonists or PGE1. These stimuli generate cAMP, which appears to preferentially activate PKA, through interaction with AKAP. Activation of PKA inhibits RhoA activation, preventing efficient phagocytosis. Other anti-phagocytic stimuli, such as an excess of C5a, also appear to exert their effects through inhibition of RhoA.⁶ Section 3 demonstrates the proposed restorative role for EPAC activation. We propose that cAMP

preferentially activates PKA in neutrophils in response to stimuli that impair phagocytosis. Directing cAMP through EPAC results in signaling through Rap-1, which allows efficient phagocytosis to proceed, even when RhoA is simultaneously blocked. On the left of the diagram, GM-CSF is seen engaging with its receptor on the neutrophil. GM-CSF can replenish RhoA activity, but if Rho kinase is blocked the effects of GM-CSF are abrogated. In contrast, even when Rho kinase is blocked, EPAC activation can still rescue phagocytosis, suggesting that EPAC activation and GM-CSF restore phagocytosis through independent mechanisms.