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Involvement of the α/β Isoform of p38 MAP Kinase in Chemotactic Responses of Human Eosinophils to Eotaxin (CCL11) and RANTES (CCL5)

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Abstract: Eosinophils are the principal effector cells for allergic inflammation in a variety of diseases, in which they contribute to tissue damage and remodelling processes via the secretion of cytotoxic granular proteins and cytokines. The intracellular mechanisms that control the activation, recruitment and survival of eosinophils are fundamental in understanding these disease processes. Phosphoinositide 3-kinase (PI3K) has been shown previously to be essential for eosinophil chemotactic responses to some stimuli but not others. Human blood neutrophils have been shown to utilize two antagonistic signalling pathways for chemotaxis: PI3K and p38 mitogen-activated protein kinase (p38 MAPK). In the present study, the role of p38 MAPK in chemotactic responses of an eosinophil-differentiated myeloid leukaemia cell line (EOL-1) and human peripheral blood eosinophils to a range of stimuli - platelet-activating factor (PAF), eotaxin 1 (CCL11), RANTES (CCL5), interleukin 8 (IL8, CXCL8) and IL16 - was explored through the use of the p38 MAPK α/β isoform inhibitor, SB 203580. SB 203580 caused significant inhibition of chemotactic responses of both EOL-1 cells and blood eosinophils to eotaxin 1 and RANTES (≥75% inhibition at 1 μM SB 203580, $p<0.01$) but had no effect on the migration induced by PAF and IL16 (<25%) and little or no effect on responses to IL8. Responses to PAF - but not eotaxin - have been shown previously to be suppressed by PI3K inhibition. The complementary pattern of inhibition observed in the present study provides evidence that distinct PI3K-dependent and p38 MAPK-dependent chemoattractants may also exist for eosinophils.

Keywords: Eosinophils, Chemotaxis, Cell Signalling, p38 MAP Kinase

Introduction

Eosinophils are initiator and effector cells of Th2-mediated immunity (Ravin and Loy, 2016). They are associated with key components of inflammation in a variety of chronic diseases affecting the airways (Eng and DeFelice, 2016), heart (Séguéla *et al*., 2015) and gastrointestinal system (Hogan *et al*., 2013). Eosinophils are characterized by secretory granules that contain multiple cytotoxic proteins (Ravin and Loy, 2016). Additionally, eosinophils produce reactive oxygen metabolites, lipid mediators and cytokines, all of which are capable of causing severe host tissue damage in eosinophilic inflammation (Barnes, 2011).

Eosinophil migration appears to be modulated by two fundamental processes: Cell adhesion systems located at the site of inflammation in local endothelium and epithelium and chemotactic signals elicited through cytokine, chemokine and other chemoattractant receptors (Lampinen *et al*., 2004). A range of inflammatory mediators and cytokines have been identified as eosinophil attractants and activators, including platelet-activating factor, CC chemokines including eotaxin (CCL11) (Mishra *et al*., 2005) and regulated on activation normal T-cell expressed and secreted (RANTES, CCL5) (Svensson *et al*., 2009), CXC chemokines including interleukin 8 (IL8, CXCL8) (Bates *et al*., 2010) and the lymphocyte chemoattractant factor interleukin 16 (IL16)

(Rand *et al*., 1991). The cellular signalling pathways responsible for evoking and regulating eosinophil chemotaxis toward these chemoattractants are incompletely understood, although a role for receptor-mediated activation of phosphoinositide 3-kinase (PI3K) in responses to certain stimuli has been identified (Mishra *et al*., 2005).

The p38 MAP kinase (MAPK) signalling pathway plays an important role in inflammation and other physiological processes. Specific inhibitors of p38 MAPK α and β block production of the major inflammatory cytokines (e.g. tumour necrosis factor α and interleukin 1) and other proteins (e.g. cyclooxygenase 2) and are anti-inflammatory in animal models of disease (Ono and Han, 2000). A major function of the pathway is post-transcriptional control of inflammatory gene expression. Inhibitors of p38 MAPK have been shown to reduce inflammatory cytokine production and eosinophil infiltration into the lungs in animal models of asthma (Underwood *et al*., 2000) and to inhibit angiogenesis in a murine model of rheumatoid arthritis (Jackson *et al*., 1998).

SB203580 is a selective inhibitor of the α and β isoforms of p38 MAPK that inhibits the cytokine-induced adhesion, shape change and transmigration of eosinophils (Ip *et al*., 2003), as well as inhibiting eotaxin 1 production in human primary lung fibroblasts (Rokudai *et al*., 2006) and capsaicin-induced production of PAF in oesophageal epithelial cells (Ma *et al*., 2010). Pharmacological inhibition of receptors and signal transduction molecules in human eosinophils has shown that the release of oxygen free radicals in response to eotaxin 1 is relatively more dependent on the p38 MAPK pathway than the response to bacterial formyl peptide chemoattractants (Svensson *et al*., 2009).

Studies in human neutrophils revealed a signalling hierarchy for chemoattractants in which responses to specific attractants exhibited a dependence upon either PI3K or p38 MAPK (Heit *et al*., 2007). Since we have shown previously that eosinophil responses to certain attractants are dependent upon PI3K while others are not (Mishra *et al*., 2005; Hasan *et al*., 2010), we undertook a study to determine whether eosinophil chemotactic responses to a number of factors-including those known to be PI3K-independent-involved p38 MAPK.

Materials and Methods

Cells

The human leukaemia cell line, EoL-1 was cultured and differentiated to an eosinophilic phenotype as described previously (Al-Rabia *et al*., 2004). Cell viability was determined every 2 days by trypan blue dye exclusion. Viability was maintained at >90% throughout cultures.

Human peripheral blood eosinophils were isolated from non-asthmatic volunteers as described previously (Dent *et al*., 1998). Blood donors gave informed consent to the use of their cells and the project was approved by the North Staffordshire Local Research Ethics Committee (reference 06/Q2604/15).

Chemotaxis Assay

Chemotactic responses of differentiated EoL-1 cells and human peripheral blood eosinophils were measured in a 96-well blind chamber assay using 5 μm pore-size filters, as described previously (Mishra *et al*., 2005).

Statistical Analysis

Data are expressed as arithmetic mean ± standard error of the mean (SEM) from the indicated numbers of experiments. Statistical analyses were performed using SPSS version 21 for PC (IBM Corp., Armonk NY, USA). For comparisons of multiple groups (i.e. varying concentrations of SB 203580), repeated-measures analysis of variance (ANOVA) was followed by *post hoc* pairwise comparisons with untreated (control) cells using Dunnett's test for multiple comparisons. A probability (P)<0.05 was defined as significant throughout.

Results

Effect of α/β p38 MAPK Inhibitor (SB 203580) on Chemotactic Responses of EOL-1 Cells and Human Peripheral Blood Eosinophils to Eotaxin 1 and PAF

Eosinophil-differentiated EoL-1 cells exhibited chemotactic responses to both PAF and eotaxin 1, with optimal concentrations of 100 nM and 30 nM, respectively (Dent *et al*., 1998). Chemotactic responses to eotaxin 1 were inhibited in a concentration-dependent manner by SB 203580. In contrast, inhibition of PAF-induced chemotactic responses by SB 203580 did not achieve statistical significance at concentrations up to 10 μM (Fig. 1A).

Both PAF and eotaxin 1 stimulated migration of human peripheral blood eosinophils, with optimal concentrations of 30 nM for each stimulus (Dent *et al*., 1998). The response to eotaxin was inhibited concentration-dependently by SB 203580, while the drug had no significant effect on the response to PAF at concentrations of 100 μM (Fig. 1B).

Effect of SB 203580 on Chemotactic Responses of EOL-1 Cells to IL8, IL16 and RANTES

In preliminary experiments, the optimal concentration of IL8 for induction of both

differentiated EOL-1 cell and blood eosinophil chemotaxis was found to be 10 nM, while that for RANTES and IL16 was 0.1 nM (Hasan, 2008). Chemotactic responses to RANTES were inhibited by SB 203580 in a concentration-dependent manner (Fig. 2A). SB 203580 had no significant effect on chemotactic responses to IL16 or IL8 at concentrations up to 10 μM (Fig. 2B and 2C).

Effect of SB 203580 on Chemotactic Responses of Human Peripheral Blood Eosinophils to IL8, IL16 and RANTES

SB203580 inhibited the chemotactic response of human peripheral blood eosinophils to RANTES in a concentration-dependent manner (Fig 3A). SB 203580 had no significant effect on chemotactic responses to IL16 or IL8 at concentrations up to 10 μM (Fig 3B & C).

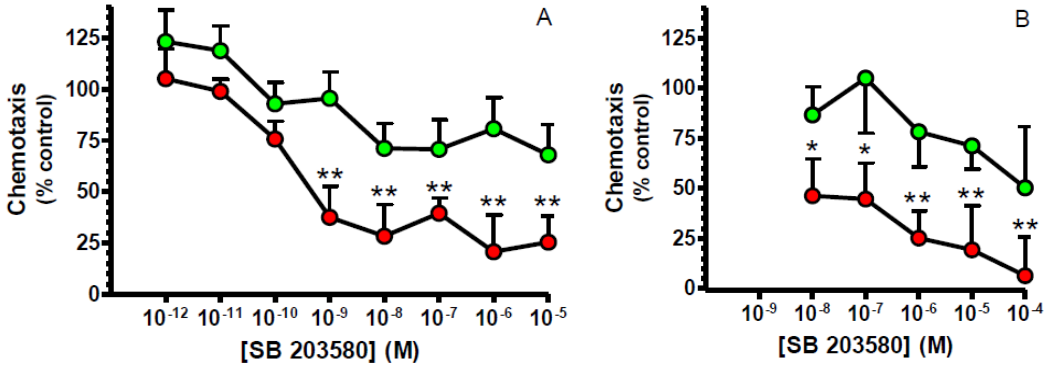

Fig. 1. Effects of SB 203580 on (A) eosinophil cell line EoL-1 and (B) human peripheral blood eosinophil chemotactic responses to 100 nM PAF (●) and 30 nM eotaxin 1 (●). Data are shown as mean ± SEM from six experiments conducted in triplicate for each inhibitor combination. *p<0.05, **p<0.01 compared with control response in the absence of inhibitor by repeated-measures ANOVA and *post hoc* Dunnett's test.

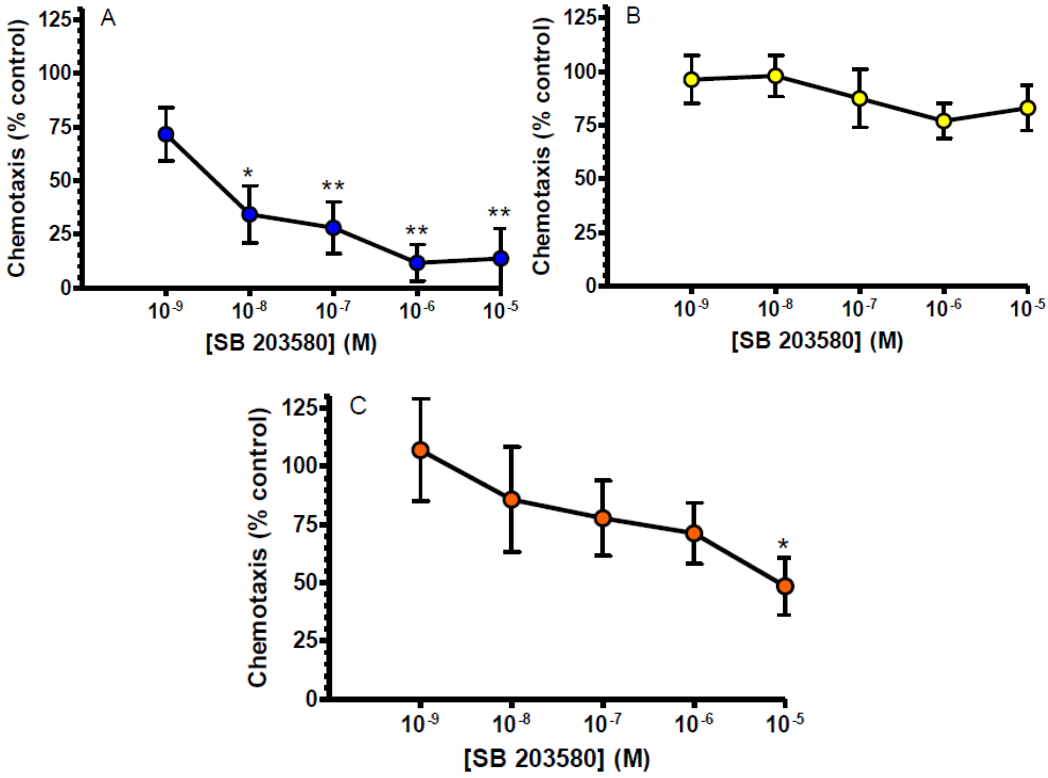

Fig. 2. Effects of SB 203580 on EoL-1 cell chemotactic responses to (A) 0.1 nM RANTES, (B) 0.1 nM IL16 and (C) 10 nM IL8. Data are shown as mean ± SEM from four experiments conducted in triplicate for each inhibitor combination. *p<0.05, **p<0.01 compared with control response in the absence of inhibitor

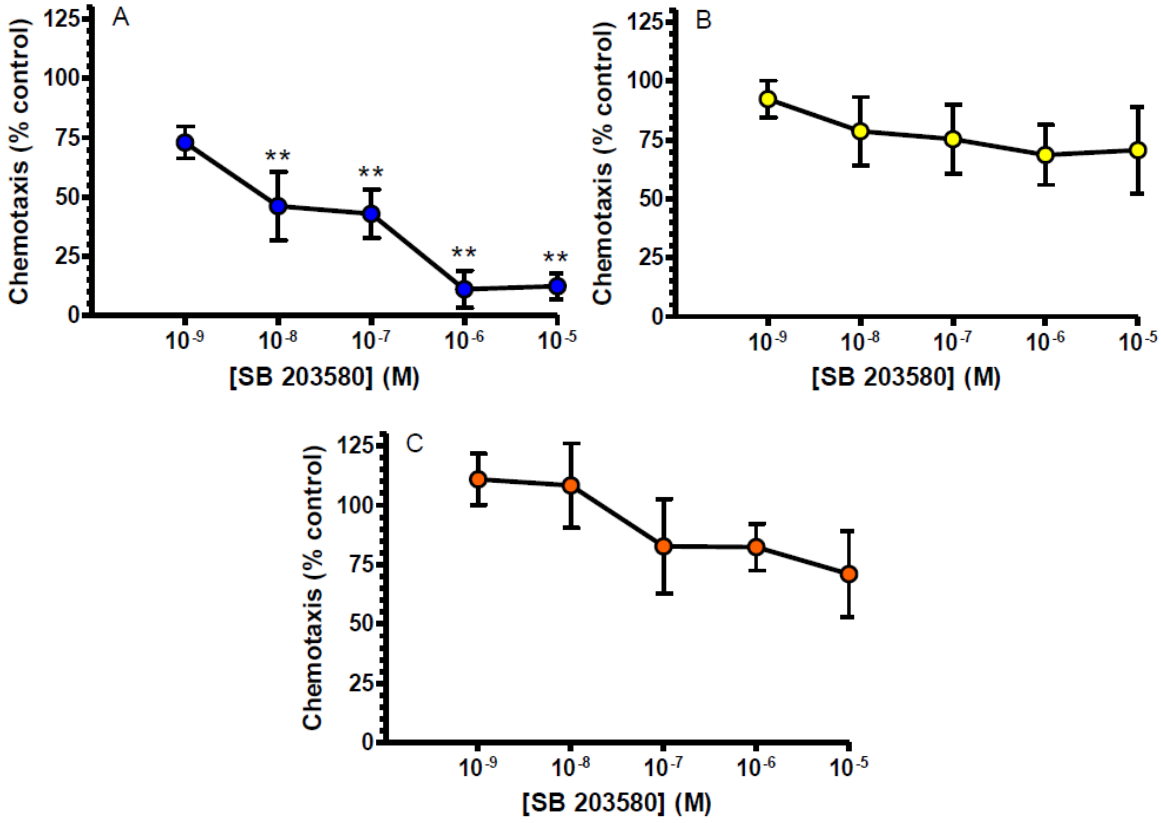

Fig. 3. Effects of SB 203580 on blood eosinophil chemotactic responses to (A) 0.1 nM RANTES, (B) 0.1 nM IL16 and (C) 10 nM IL8. Data are shown as mean ± SEM from four experiments conducted in triplicate for each inhibitor combination. ***p*<0.01 compared with control response in the absence of inhibitor

Discussion

Previous studies have shown a differential dependence of chemotactic responses in human eosinophils and eosinophil-differentiated EOL-1 cells upon PI3K. Responses to the phospholipid inflammatory mediator platelet-activating factor (PAF) are highly sensitive to suppression by PI3K inhibitors (Mishra *et al*., 2005), with the PI3Kγ isoform specifically essential to these responses (Hasan *et al*., 2010). In contrast, responses to the CC chemokine eotaxin 1 - a key eosinophil chemoattractant in airway secretions in severe asthma (Dent *et al*., 2004) - are unaffected by PI3K inhibitors (Mishra *et al*., 2005; Hasan *et al*., 2010). It is, therefore, apparent that chemotactic responses of eosinophils are not mediated by a single intracellular signalling pathway.

In a landmark study Heit *et al*. (2002) demonstrated the existence of a signalling hierarchy that allows neutrophils to migrate towards higher concentrations of specific chemoattractants *against* concentration gradients of other attractants. Heit *et al*. (2002) classified these two types of substance as intermediary chemoattractants (e.g. IL8 and leukotriene B_4), with actions mediated by PI3K and end-stage chemoattractants (e.g. complement anaphylotoxin C5a and bacterial formyl peptide fMet-Leu-Phe) whose actions are mediated by p38 MAPK.

In the present study, chemotactic responses of eosinophils to a range of stimuli were assessed to determine their sensitivity to an inhibitor of the α/β isoforms of p38 MAPK, SB 203580.

In a complementary finding to that of our previous reports (Mishra *et al*., 2005; Hasan *et al*., 2010), we found that responses to eotaxin 1 were highly sensitive to inhibition by SB 203580 while those to PAF were not. Among the other stimuli studied, another CC chemokine - RANTES - also exhibited chemotactic responses sensitive to p38 MAPK inhibition. While both of these chemokines are able to exert actions through multiple receptors, they have a single common target expressed in eosinophils: the CCR3 receptor (Elsner *et al*., 2004). This may account for the shared dependence of responses to these two stimuli upon the same signalling pathway.

Eotaxin may stimulate chemotaxis through activation of one or more protein kinases, which then activate effector molecules that recruit p38 MAPK. These in turn activate downstream molecules which induce

polarization and cytoskeletal reorganization. Thus, eotaxin and RANTES may induce the chemotaxis of eosinophils by a p38 MAPK-dependent mechanism. The results of the present study are similar to those reported by Stubbs *et al* in 2002, which showed evidence of involvement of p38 MAPK in multiple eosinophil responses to eotaxin (Stubbs *et al*., 2002).

While the CXC chemokine IL8 is recognized as having chemoattractant activity for eosinophils, the signalling pathways involved in this response are poorly understood. IL8 activates MAP kinases 3 and 1 (ERK1 and ERK2) in IL5-primed eosinophils and this action is necessary for the induction of leukotriene C_4 synthesis (Bates *et al*., 2000), however the signal transduction events involved in chemotaxis have not been identified. Although IL8 was classified as an intermediate (PI3K-dependent) attractant for neutrophils (Heit *et al*., 2002), the receptor presumed to mediate this response in neutrophils - CXCR1 - has not been shown to be expressed by eosinophils (Liu *et al*., 2003). We demonstrate here that IL8-induced eosinophil chemotaxis does not exhibit the same degree of dependence upon p38 MAPK as the CCR3 agonists eotaxin 1 and RANTES, with only marginal levels of inhibition (up to approximately 25%) observed in the presence of SB 203580 at concentrations that produce approximately 75% inhibition of responses to the CC chemokines. It remains to be determined which other signalling pathways-possibly including PI3K-dependent mechanisms-contribute to CXC chemokine chemotaxis responses in eosinophils.

The lymphocyte chemoattractant factor, IL16, induces activation and suppressor events in T lymphocytes through a variety of mechanisms involving multiple receptors (Lynch *et al*., 2003). While CD4 is the key receptor for IL16 actions on T lymphocytes, non-lymphoid immune effector cells may utilize other receptors for this cytokine and a role for PI3K in chemotactic responses to IL16 in mast cells has been demonstrated (Qi *et al*., 2006). In the present study there was no apparent dependence upon p38 MAPK of eosinophil chemotactic responses to IL16, with no inhibition of responses observed at any concentration of SB 203580. While this cannot be taken as evidence of involvement of another specific signalling pathway, it does contribute to an overall picture of stimuli shown elsewhere to evoke PI3K-dependent responses exhibiting no dependence upon p38 MAPK in our studies.

Future research studies should focus on stimulus, response and cell-type heterogeneity of p38 MAPK dependence. The present study has utilized a single inhibitor of the α/β isoforms of the enzyme. Further studies are required to investigate the functions of the individual isoforms α, β, δ and γ of p38 MAPK and the mechanisms by which they contribute to activation of human eosinophils. The interference or cross-talk of this pathway (p38 MAPK pathway) with the other pathways that influence the migration and accumulation of human eosinophils will be an interesting field for future studies.

Conclusion

Chemotactic responses of human eosinophils to CC chemokines (eotaxin 1 and RANTES) are dependent upon α/β p38 MAPK. Responses to PAF and IL16 are not dependent on α/β p38 MAPK, while responses to a CXC chemokine (IL8) show partial dependence. The dependence of responses of PAF and eotaxin 1 on α/β p38 MAPK are a mirror image of the dependence of these responses on PI3K, suggesting that different classes of chemoattractants utilizing different intracellular signaling pathways may exist for eosinophils, as has been described for human neutrophils.

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Author's Contributions

Anwar M Hasan: Conducted the laboratory work and the background literature review, summarized the data and drafted the manuscript.

Gordon Dent: Formulated the research question, designed the experimental protocol, conducted statistical analyses, produced the final graphs and edited the manuscript for submission.

Ethics

This work does not present any ethical issues.

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