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Short communication

Differential regulation of TNFα and GM-CSF induced activation of P38 MAPK in neutrophils and eosinophils

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Abstract

P38 MAPK is a central mediator in cytokine signalling in human leukocytes. P38 MAPK is activated by phosphorylation of a conserved Thr180-X-Tyr182 motif by dual phosphorylation via the upstream kinases MKK3 and MKK6. Alternatively, P38 MAPK can be activated via autophosphorylation when associated with TAB1. In this study P38 MAPK phosphorylation and activation (measured via phosphorylation of P38 MAPK downstream target MK2) were investigated upon engagement of the GM-CSF- and TNFα-receptors expressed on both eosinophils and neutrophils. The MKK3/MKK6 pathway mediated neutrophil P38 MAPK activation after stimulation with TNFα (100 U/ml) or GM-CSF (10^{-10} M). Under these conditions the activation but not phosphorylation of P38 MAPK could be inhibited by SB203580 (10^{-5} M or 10^{-6} M). In eosinophils SB203580 (10^{-6} M) inhibited both the phosphorylation and activation of P38 MAPK after stimulation with several doses of TNFα (10–10000 U/ml) or GM-CSF (10^{-11} to 10^{-9} M), indicating that P38 MAPK activation is mediated via autophosphorylation in eosinophils. This hypothesis was supported by the finding that in marked contrast to neutrophils, MKK3/MKK6 did not show enhanced phosphorylation in eosinophils after cytokine stimulation, but were constitutively phosphorylated. Therefore, the involvement of TAB1 was investigated with regard to this cytokine-induced autophosphorylation. Co-immunoprecipitation experiments showed that TAB1 was constitutively associated with P38 MAPK in eosinophils and neutrophils and that cytokine-induced autophosphorylated P38 MAPK was co-precipitated with TAB1. These findings are consistent with the hypothesis that cytokine-induced autophosphorylation of P38 MAPK in primary granulocytes depends on the interaction with TAB1.

Keywords: Cytokine; Eosinophil; Neutrophil; TNFα; GM-CSF; Signal transduction; p38 MAP kinase; Regulation; Autophosphorylation; TAB1; MKK

1. Introduction

Aberrant activation of P38 mitogen-activated protein kinase (MAPK) is involved in a variety of pathophysiological responses and has been associated with inflammatory responses and tissue remodelling (Kumar et al., 2003). Recently, inhibitor studies using small molecule inhibitors against P38 have indicated that P38 is a central mediator in pulmonary inflammatory diseases (Barnes, 2006; Underwood et al., 2000a,b; Wong, 2005; Lee et al., 2000; Kankaanranta et al., 1999), such as chronic obstructive pulmonary disease (COPD) and asthma. In these diseases, innate immune cells, such as eosinophils and neutrophils, play an important role in the pathogenesis of chronic persistent inflammatory processes seen in the bronchial tissue (Oudijk et al., 2003; Douwes et al., 2002; Jatakanon et al., 1999; Gibson et al., 2001; Luijk et al., 2005). Therefore, proper regulation of activation of the P38 pathway in eosinophils and neutrophils during the inflammatory processes is required to prevent exaggerated damage to host tissue. Regulation of P38 activation occurs via dual phosphorylation on a conserved Thr180-X-Tyr182 motif via the upstream kinases mitogen-activated protein kinase kinase (MKK)3 and MKK6. However, alternative pathways for P38 activation were recently described that result in phosphorylation of P38 on its conserved Thr-X-Tyr motif. P38 can be activated independently of MKK3/MKK6 by

Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; TNFα, tumor necrosis factor-α; Ab, antibody; TAB1, TAK1-binding protein 1; MAPK, mitogen-activated protein kinase; MKK, mitogen-activated protein kinase kinase

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the association with the scaffold protein TAK1-binding protein 1 (TAB1) (Ge et al., 2002). TAB1 binding to P38 enhances the intramolecular P38 kinase activity. A second alternative pathway was identified in T-cells and involves the phosphorylation of a previously unappreciated residue on P38, Tyr323 (Salvador et al., 2005). In these cells, T-cell receptor (TCR) ligation results in activation of the Zap70 pathway, which subsequently results in the phosphorylation of Tyr323 on P38 and induction of the intramolecular P38 activity (Salvador et al., 2005).

The dual phosphorylation of P38 mediated via MKKs is insensitive to inhibition of P38 by SB203580, which action lies in occupation of the ATP-binding pocket within the P38 kinase domain (Lee et al., 2000; Kumar et al., 1999; Gum et al., 1998; Frantz et al., 1998). However, P38 phosphorylation under conditions of TAB1 association or Tyr323 phosphorylation is sensitive to SB203580 (Ge et al., 2002; Salvador et al., 2005; Kim et al., 2005) indicating that this phosphorylation of P38 occurs via P38 mediated autophosphorylation. It has been shown in neutrophils that the inflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNFα) can induce activation of P38 through activation of the upstream kinases MKK3/MKK6, confirming a MKK regulated mechanism towards P38 in these cells (Suzuki et al., 1999). For eosinophils it is uncertain which upstream kinases are involved in this process. A role for phophatidylinoisitol 3′-kinase (PI3K) activation upstream from P38 has been implicated in interleukin (IL)-4 and IL-5 induced immunoglobulin-A binding in eosinophils (Bracke et al., 1998). Nothing is known regarding the upstream signals in TNFα activated P38 in eosinophils.

In this study the GM-CSF- and TNFα-induced regulation and activation of P38 in neutrophils and eosinophils were investigated. The cytokines TNFα and GM-CSF were investigated in these processes because they use distinct receptors that are expressed on both cell types. In addition, both cytokines have been associated with chronic inflammation in inflammatory diseases, such as asthma and COPD (Oudijk et al., 2003, 2005; Thomas, 2001; Ritz et al., 2002).

2. Materials and methods

2.1. Reagents

Recombinant human GM-CSF was a gift from Prof. A. Lopez (IMVS, Adelaide Australia). Recombinant human TNFα was purchased from Roche (Indianapolis, IN). Antibodies used were: antiphospho-P38 (Thr180/Tyr182), antiphospho-MK2 (Thr334) and antiphospho-MKK4 (Thr261) from Cell Signaling (Beverly, MA), anti-P38 (C-20) and anti-TAB1 (N-19) from Santa Cruz (Santa Cruz, CA) and HRP-coupled swine anti-rabbit from Dako (Denmark). Pharmacological inhibitor SB203580 was purchased from Kordia Life Sciences (Leiden, The Netherlands).

2.2. Granulocyte isolation and stimulation

Granulocytes were isolated from 100 ml whole blood of healthy donors as described before (Oudijk et al., 2005). Negative immunomagnetic selection was used to purify eosinophils from granulocytes using anti-CD16-conjugated microbeads (Miltenyi Biotec; Auburn, CA) (Hansel et al., 1991). In addition, anti-CD3- and CD14-conjugated microbeads (Miltenyi Biotec) were added to the granulocyte suspension to avoid mononuclear cell contamination. Purity of eosinophils was >97%.

Neutrophils (less then 3% eosinophils) and eosinophils were resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6.0 mM KCl, 1.0 mM $MgSO_4$, 1.2 mM KH_2PO_4, supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) Human serum albumin from Sanquin (Amsterdam, the Netherlands)) and allowed to recover for 15 min at 37 °C with or without pharmacological inhibitor SB203580. Subsequently, cells were mock-stimulated or stimulated with TNFα or GM-CSF for 15 min at 37 °C. After stimulation, cells were washed two times in PBS at 4 °C. Cells were subsequently lysed in sample buffer (60 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol and 2% 2-mercaptoethanol) and boiled for 5 min or lysed in lysis buffer and used in immunoprecipitations.

2.3. Immunoprecipitation

Immediately after stimulation of 20×10^6 neutrophils or 2×10^6 eosinophils, cells were lysed in 1 ml of ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM PMSF; to neutrophil samples also 2 mM diisopropylphosphorofluoride was added) and samples were vortexed for 10 s and subsequently agitated for 30 min at 4 °C. Thereafter the lysates were centrifuged (14,000 × *g*, 20 min, 4 °C). Supernatants were incubated with 25 μl Protein G Sepharose 4 Fast Flow beads (Sigma; St. Louis, MO) for 1 h at 4 °C to preclear the samples. After centrifugation (14,000 × *g*, 30 s, 4 °C), supernatants were incubated with 5 μg of anti-TAB1 antibody (Ab) and agitated for 12 h at 4 °C. Subsequently, 25 μl Protein-G sepharose beads were added to the samples and samples were agitated for 8 h at 4 °C. Then the pellets of the immunoprecipitates were washed three times with lysis buffer and resuspended in sample buffer and boiled for 5 min.

2.4. Western blotting

Protein samples were analyzed on 10% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P (Millipore; Bedford, MA) for total cell lysates and to Hybond-LPF (Amersham, UK) for immunoprecipitates. The blots were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl, and 0.3% Tween-20) containing 5% bovine serum albumin (BSA) for 1 h at RT followed by incubation with antiphospho-P38 (1/1000), antiphospho-MK2 (1/1000), antiphospho-MKK4 (1/1000) or anti-P38 (1/1000) in hybridization buffer with 5% BSA overnight at 4 °C. After incubation with the first antibody, the blots were washed six times 4 min in hybridization buffer. Second antibody (HRP-coupled swine anti-rabbit; 1/3000) was incubated in hybridization buffer with 5% BSA for 1 h followed by washing five times 4 min in incubation buffer and a last wash

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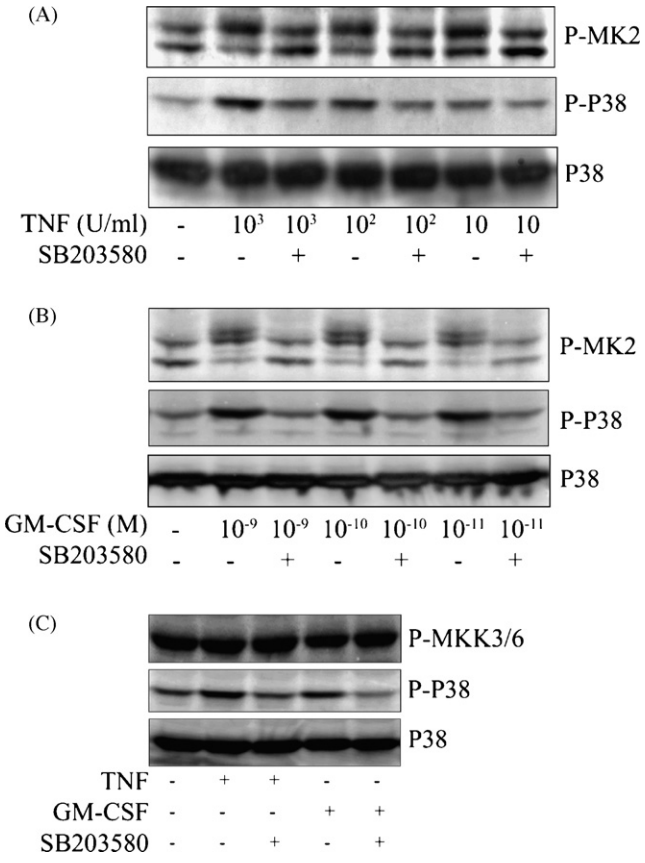

Fig. 1. P38 MAPK phosphorylation and activation are mediated via autophosphorylation and not via MKK3/MKK6 in eosinophils. Eosinophils (0.5×10^6) were preincubated with or without P38 catalytic site inhibitor SB203580 (10^{-6} M) for 15 min and stimulated for 15 min with several concentrations of (A) TNFα (1000–10 U/ml) or (B) GM-CSF (10^{-9} to 10^{-11} M) or (C) TNFα (100 U/ml) and GM-CSF (10^{-10} M). The experiment shown is representative for at least three experiments.

step in PBS. Detection of all western blots was performed by enhanced chemiluminescence-plus (Amersham) and detected using a Typhoon-9410 (Amersham).

3. Results and discussion

Currently little is known regarding the different routes towards P38 in primary cells upon engagement of proinflammatory cytokine receptors, such as the receptors for IL-3, IL-5 and GM-CSF sharing the common beta-c (CD131) chain, or the TNFα receptor. By comparing the mechanisms of P38 activation in neutrophils and eosinophils stimulated with inflammatory cytokines GM-CSF and TNFα, distinct upstream pathways towards P38 were found (Figs. 1 and 2). In Fig. 1A and B it is shown that activation of eosinophils with TNFα (10–1000 U/ml) or GM-CSF (10^{-11} to 10^{-9} M) for 15 min induced a dose-dependent phosphorylation of P38. This TNFα- (Fig. 1A) and GM-CSF- (Fig. 1B) induced phosphorylation of P38 was sensitive to SB203580 (10^{-6} M) indicating that P38 regulation in eosinophils occurs via autophosphorylation. Activity of P38 *in situ* was investigated by measuring the phosphorylated status of the specific P38 physiological substrate MAPK-activated protein kinase 2 (MK2) (Kumar et al., 1999). Phosphorylation of MK2 in eosinophils was also dose dependently increased after stimulation with both stimuli and this increase was also inhibited by SB203580 (Fig. 1).

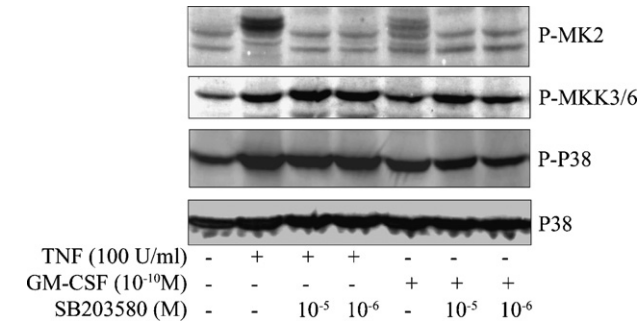

Fig. 2. P38 MAPK activation and phosphorylation correlates with phosphorylation of MKK3/MKK6 in human neutrophils. Neutrophils (2×10^6) were preincubated with or without P38 catalytic site inhibitor SB203580 (10^{-5} or 10^{-6} M) for 15 min and stimulated for 15 min with TNFα (100 U/ml) or GM-CSF (10^{-10} M). The experiment shown is representative for at least three experiments.

TNFα (100 U/ml) and GM-CSF (10^{-10} M) did not induce activation of the P38 upstream kinases MKK3/MKK6 in eosinophils, as the kinetics of this autophosphorylation of P38 in eosinophils were not mirrored by similar kinetics in phosphorylation of MKK3/MKK6 (Fig. 1C). The findings suggest that MKK3 and MKK6 are constitutively phosphorylated in eosinophils (Fig. 1C), while this activation does not lead to P38 phosphorylation. Eosinophil P38 is, therefore, activated by an alternative pathway(s) leading to autophosphorylation of the kinase.

In marked contrast to eosinophils, SB203580 (10^{-5} or 10^{-6} M) did not inhibit the TNFα- (100 U/ml) and GM-CSF- (10^{-10} M) induced P38 phosphorylation in human neutrophils (Fig. 2), but both concentrations of SB203580 inhibited P38 activation measured via the phosphorylated status of MK2 (Fig. 2). For neutrophils the upstream kinases are most likely MKK3 and MKK6 as the kinetics of the phosphorylation of these MKKs after stimulation were similar to the kinetics of P38 phosphorylation and activation (Fig. 2). Furthermore, neutrophil P38 phosphorylation was insensitive to SB203580 (Fig. 2) discarding the importance of autophosphorylation.

Several recent publications have suggested that TAB1 association is important in controlling the P38 autophosphorylation (Ge et al., 2002; Kim et al., 2005). To evaluate the possibility that autophosphorylation of P38 in eosinophils was associated with an interaction with TAB1, co-immunoprecipitations were performed. Fig. 3 demonstrates that phospho-P38 coprecipitates with TAB1 in lysates from TNFα- (Fig. 3A; 100 U/ml) and GM-CSF- (Fig. 3B; 10^{-10} M) stimulated eosinophils, which phosphorylation was inhibited by SB203580 (10^{-6} M) for both stimuli (Fig. 3A and B). Inhibition of autophosphorylation by SB203580 (10^{-6} M) did not lead to dissociation of the complex as equal amounts of co-precipitated total P38 were present in unstimulated and stimulated samples (Fig. 3A and B). We next addressed the question whether TAB1 was also constitutively associated with P38 in neutrophils. As in eosinophils, P38 in

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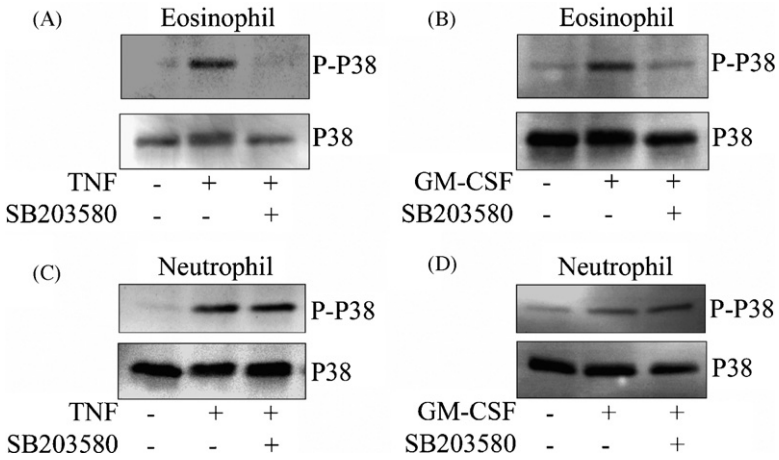

Fig. 3. TAB1 is constitutively associated with P38 MAPK in eosinophils and neutrophils and both autophosphorylation and phosphorylation of P38 occur in the P38/TAB1 complex. (A and B) Eosinophils and (C and D) neutrophils were preincubated with or without P38 catalytic site inhibitor SB203580 (eosinophils: 10^{-6} M; neutrophils 10^{-5} M) for 15 min and stimulated for 15 min with (A and C) TNFα (100 U/ml) or (B and D) GM-CSF (10^{-10} M). After stimulation, cells were subjected to co-immunoprecipitation with an Ab (N-19; Santa Cruz) to TAB1. Precipitates were prepared for immunoblotting and incubated with an Ab to phospho-P38 and reprobed with an Ab recognizing total P38. The experiment shown is representative for three experiments.

neutrophils was found in co-immunoprecipitations with TAB1 antibodies (Fig. 3C and D). P38 associated with TAB1 was phosphorylated upon activation with TNFα (Fig. 3C; 100 U/ml) and GM-CSF (Fig. 3D; 10^{-10} M), but SB203580 (10^{-5} M) did not influence the phosphorylation status of P38 under these conditions (Fig. 3C and D).

In marked contrast to *in vitro* data showing that TAB1 interaction with P38 is responsible for P38 autophosphorylation and activation (Ge et al., 2002), we show that interaction *per se* does not lead to autophosphorylation of P38 in human neutrophils and eosinophils. These data demonstrate that, in contrast to the *in vitro* data, the interaction between P38 and TAB1 is not sufficient to activate the kinase *in situ* in primary granulocytes.

Interestingly, differential activation of P38 leads to differences in activation of downstream targets. Lu et al. (2006) showed that TAB1 mediates MKK-independent P38 activation while negatively modulating the MKK-route. This latter mechanism might lead to modulation of phosphorylation of MKK3/MMK6 in cytokine activated neutrophils in the presence of SB203580 (see Fig. 2). Further research is needed to identify these potential different signalling modules downstream of P38 among eosinophils and neutrophils.

In this study we have shown that identical cytokine receptors in different granulocytes can induce two different pathways towards the phosphorylation and activation of P38. In neutrophils a MKK-driven activation of P38 dominates whereas in eosinophils a MKK3/MKK6-independent autophosphorylation regulates P38 activation. TAB1 was constitutively associated with P38 in both neutrophils and eosinophils and is likely an essential but not a sufficient condition for the autophosphorylation of P38. The cytokine-induced signals that lead to autophosphorylation of the P38/TAB1 complex in eosinophils remain to be identified. However, our data clearly show that the cellular context of cytokine receptors determines which signalling paradigms control the P38 pathway in primary cells.

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