



Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm



Akt cross-links IL-4 priming, stem cell factor signaling, and IgE-dependent activation in mature human mast cells

Katrin Feuser^a, Katharina Feilhauer^b, Ludger Staib^c,
Stephan C. Bischoff^a, Axel Lorentz^{a,*}

^a Department of Nutritional Medicine, University of Hohenheim, Fruwirthstraße 12, 70593 Stuttgart, Germany

^b Clinic for Visceral Surgery, Katharinen Hospital, Kriegsbergstraße 60, 70174 Stuttgart, Germany

^c Clinic for Visceral Surgery, Klinikum Esslingen, Hirschlandstraße 97, 73730 Esslingen, Germany

ARTICLE INFO

Article history:

Received 10 August 2010

Received in revised form

10 September 2010

Accepted 22 October 2010

Available online xxx

Keywords:

Cytokines

Human

Mast cell

Mucosa

Signal transduction

ABSTRACT

Challenge of human mast cells with both stem cell factor (SCF) and IL-4 enhances antigen-dependent mediator release raising the assumption of intracellular crosstalk between the IL-4, SCF, and FcεRI signaling pathways. Here, we analyzed the intracellular crosstalk of IL-4-, SCF-, and IgE-dependent activation pathways in mucosal mast cells isolated from human intestine. The release of β-hexosaminidase, leukotriene C₄, and IL-8, but not IL-6, was strongly enhanced in response to sequential challenge of mast cells with IL-4, SCF and FcεRI cross-linking compared to stimulation by FcεRI cross-linking alone. Previous studies revealed that MAPK and other serine/threonine kinases are involved in mast cell activation processes. Here we found that activation of mast cells by FcεRI cross-linking alone results in phosphorylation of ERK and p38, but not of Akt. Stimulation with SCF alone also induced phosphorylation of ERK and p38, and additionally of Akt. IL-4 priming enhanced activation of ERK, but blocked activation of p38. Activation of p38 was required for IL-6 production explaining the inhibitory effect of IL-4 on IL-6 expression in human mast cells. Moreover, IL-4 priming that anteceded FcεRI cross-linking induced activation of Akt. The combined challenge of mast cells with IL-4, SCF and FcεRI cross-linking substantially up-regulated activation of Akt, whereas blocking of Akt inhibited the pronounced production and release of IL-8 in response to the three mast cell agonists. In summary, our data demonstrate that ERK, p38, and especially Akt play an important role in cross-linking IL-4 priming, SCF signaling, and IgE-dependent activation of mature human mast cells.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

The role of mast cells in immediate type allergic reactions and other inflammatory processes are well established. They exert their biological functions by releasing inflammatory mediators as a consequence of IgE-dependent and IgE-independent activation. Such mediators derive pre-synthesized from granules, like histamine and proteases, or are *de novo* synthesized upon mast cell activation like leukotriene (LT) C₄, prostaglandins, chemokines and cytokines (Bischoff, 2007; Galli et al., 2008; Kalesnikoff and Galli, 2008).

A receptor mediating most effective human mast cell activation is FcεRI, the high-affinity IgE receptor (Blank and Rivera, 2004; Rivera and Gilfillan, 2006). In addition, particular cytokines

have been identified as important regulators of human mast cell function, such as the mast cell growth factor stem cell factor (SCF) and IL-4 (Babina et al., 2004; Bischoff et al., 1997, 1999; Hu et al., 2007; Lorentz et al., 2000). *In vitro*, SCF induces mast cell mediator release only at high concentration above 5–10 ng/ml and low efficacy compared to IgE-dependent activation; however, it is the most important mast cell survival factor both *in vitro* and *in vivo* (Bischoff et al., 1997). Moreover, SCF substantially enhances antigen-dependent mediator release from human mast cells (Bischoff et al., 1997; Bischoff and Dahinden, 1992). In contrast to SCF, IL-4 fails to induce mediator release *per se* in human mast cells, but enhances IgE-dependent mediator release, IgE-dependent Th2-type cytokine generation, and cell proliferation in the presence of SCF (Bischoff et al., 1999; Lorentz et al., 2000). Interestingly, IL-4 affects mast cells *via* a mechanism that is independent of STAT6 (Lorentz et al., 2005). Here, we hypothesized that there must be some overlapping as well as distinct interactions between the signaling pathways of FcεRI, c-Kit and IL-4 receptor independent of STAT6 that are required for the priming effects of SCF and IL-4

Abbreviations: LT, leukotriene; SCF, stem cell factor; β-hex, β-hexosaminidase.

* Corresponding author. Tel.: +49 0711 459 24391; fax: +49 0711 459 24343.

E-mail address: lorentz@uni-hohenheim.de (A. Lorentz).

on antigen-dependent mast cell mediator release (Hundley et al., 2004; Lorentz et al., 2005). These interactions could include activation of PLC, D, and A2, PI3K and its downstream target Akt/protein kinase B (referred as Akt hereafter), MAPK, and JAK/STAT signaling pathways (Gilfillan and Tkaczyk, 2006; Gilfillan and Rivera, 2009; Nelms et al., 1999; Rivera and Gilfillan, 2006). Intracellular signaling transduction is mediated, by activation or deactivation of regulatory proteins through phosphorylation or dephosphorylation by protein kinases and phosphatases. One major mechanism that is responsible for transduction of extracellular signals into a cellular response is the highly conserved MAPK superfamily. The MAPK superfamily includes three important members, namely ERK, JNK, and p38 (Raman et al., 2007).

Signaling interfaces connecting anti-IgE-, SCF-, and IL-4-dependent activation might be of importance for understanding the molecular basis of allergic reactions and for the definition of new therapeutic targets. We therefore analyzed mediator release and cytokine/chemokine expression in human mucosal mast cells in response to IL-4-, SCF-, and IgE-dependent stimulation, either separately or in combination, and examined the underlying intracellular signals. We found differential activation of ERK and p38 – two important MAPK family members – and identified Akt as important kinase of the intracellular crosstalk of IL-4, SCF, and FcεRI cross-linking.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: RPMI1640 + GlutaMax[®]I from Invitrogen/Gibco (Paisley, UK); serum from Biochrom AG (Berlin, Germany); antibiotics from PAN Biotech GmbH (Aidenbach, Germany); CD117 MicroBead Kit and Dead Cell Removal Kit from Miltenyi Biotec (Bergisch-Gladbach, Germany); cytokines from PeproTech (London, UK); Sybr[®]Green PCR Master Mix from Applied Biosystems (Foster City, USA); LTC₄ ELISA Kit from Assay Designs (Ann Arbor, USA); Procarta Cytokine-assay from Affymetrix (Santa Clara, USA); Human Phosph-MAPK Array Kit from R&D Systems (Abingdon, USA); Akti-1/2 from Merck KGaA (Darmstadt, Germany); all other inhibitors from Alexis (Lausen, Switzerland). The inhibitors were dissolved in dimethyl sulfoxide (DMSO) as concentrated stocks and stored according to the manufactures' instructions and diluted by at least 1000-fold with PBS before use to yield a final concentration of <0.1% DMSO.

2.2. Isolation and culture of mature human mast cells

Mature human mast cells were isolated from surgical tissue specimens (macroscopically normal tissue) derived from patients who underwent bowel resection because of cancer by mechanical and enzymatic tissue dispersion (Sellge and Bischoff, 2006). Permission to conduct the study was obtained from the local ethical committee (Stuttgart, Germany). After overnight incubation in culture medium (RPMI1640 + GlutaMax[®]I supplemented with 10% heat-inactivated foetal calf serum, 100 µg/ml streptomycin, 100 µg/ml gentamycin, 100 µg/ml penicillin and 0.5 µg/ml amphotericin B) mast cells were enriched by positive selection of *c-Kit* expressing cells using magnetic cell separation system, CD117 microbead kit after dead cell removal kit (MACS[™] system). The fraction containing the *c-Kit*-positive cells was cultured at a density of 2×10^5 mast cells per ml in medium supplemented with 25 ng/ml of rhSCF. Generally, after 2–3 weeks mast cell purity increases to 95–100%. Only cell preparations with purity >95% were used for experiments. Pure mast cells were cultured in the presence of SCF

alone or in combination with 2 ng/ml rhIL-4 for 10 d before used in the described experiments.

2.3. Stimulation of mast cells and inhibitors

SCF was withdrawn overnight and cells were treated with myeloma IgE for 60 min and stimulated with 1 µg/ml anti-human IgE, 100 ng/ml SCF or a combination of both for 10 min to determine phosphorylation status of MAPK and other serine/threonine kinases, for 90 min to analyze the mRNA expression of cytokines/chemokines, or for 6 h to examine the release of cytokines/chemokines. The pathway inhibitors SP600125 (20 µM), SB203580 (5 µM), Akti-1/2 (10 µM), and PD98059 (50 µM) were added in the indicated concentrations 60 min prior to FcεRI cross-linking. The inhibitory components were solubilized in 100% DMSO. Controls were carried out with DMSO at a concentration equivalent to the dilution of the inhibitor (=0.1%).

2.4. RNA preparation and real-time RT-PCR

Total RNA was prepared from 1×10^5 mast cells and real-time RT-PCR was performed as described (Lorentz et al., 2005). The following specific sense and anti-sense primer were used for the PCR: GAPDH: 5'-TGGTCTCTCTGACTTCAAC-3', 5'-CCTGTTGCTGTAGCCAAATT-3', product size: 128 bp; IL-6: 5'-GATGGATGCTTCCAATCTGG-3', 5'-TGGCATTGTGTTGGGTCA-3', product size: 226 bp; IL-8: 5'-CTGAGAGTGATTGAGAGTGG-3', 5'-ACAACCCTCTGCACCCAGTT-3', product size: 122 bp. To quantify mRNA expression real-time RT-PCR reactions containing 1.5 µl of cDNA template (sample or standard), 8 µl of H₂O, 10 µl of SYBR[®]Green PCR Master Mix, and 0.25 µl 20 µM sense and anti-sense primer were performed in optical tubes (Applied Biosystems). Reaction mixture without cDNA was used as negative control in each run. All reactions were performed using the ICycler (Bio-Rad Laboratories, Munich, Germany). Specificity of the reaction was controlled by melt curve and separation of amplified fragments on 1% agarose gel.

2.5. LTC₄ and β-hexosaminidase release

Concentration of LTC₄ in the supernatant collected after stimulation for 90 min was determined by ELISA kit in accordance to the manufactures' instructions. Degranulation response of mast cells was quantified by determining the amount of released β-hexosaminidase (β-hex) in the supernatant by color enzyme assay (Schwartz et al., 1979). The percent degranulation of mast cells was estimated by the following formula: (β-hex activity in the supernatant fraction/total β-hex activity in the cellular and supernatant fraction) × 100.

2.6. Cytokine release

Cytokine release was measured by multiplex bead immunoassay (Luminex 100, Luminex Corporation, Austin, TX, USA) according to the manufactures' instructions and using Procarta Cytokine-assay. The cytokines were quantified (pg/ml) by using standards supplied with the kits.

2.7. Proteome Profiler[™] Array

Relative phosphorylation levels of all three major families of MAPK (ERK1/2, p38α/β/γ/δ, JNK1/2/3/pan) and Akt 1/2/3/pan, RSK1/2, GSK-3α/β, MSK2, HSP27 as well as p70 S6 kinase were determined by Human Phospho-MAPK Array Kit according to the manufactures' instructions. The antigen-antibody complexes were visualized using electrochemiluminescence detection system

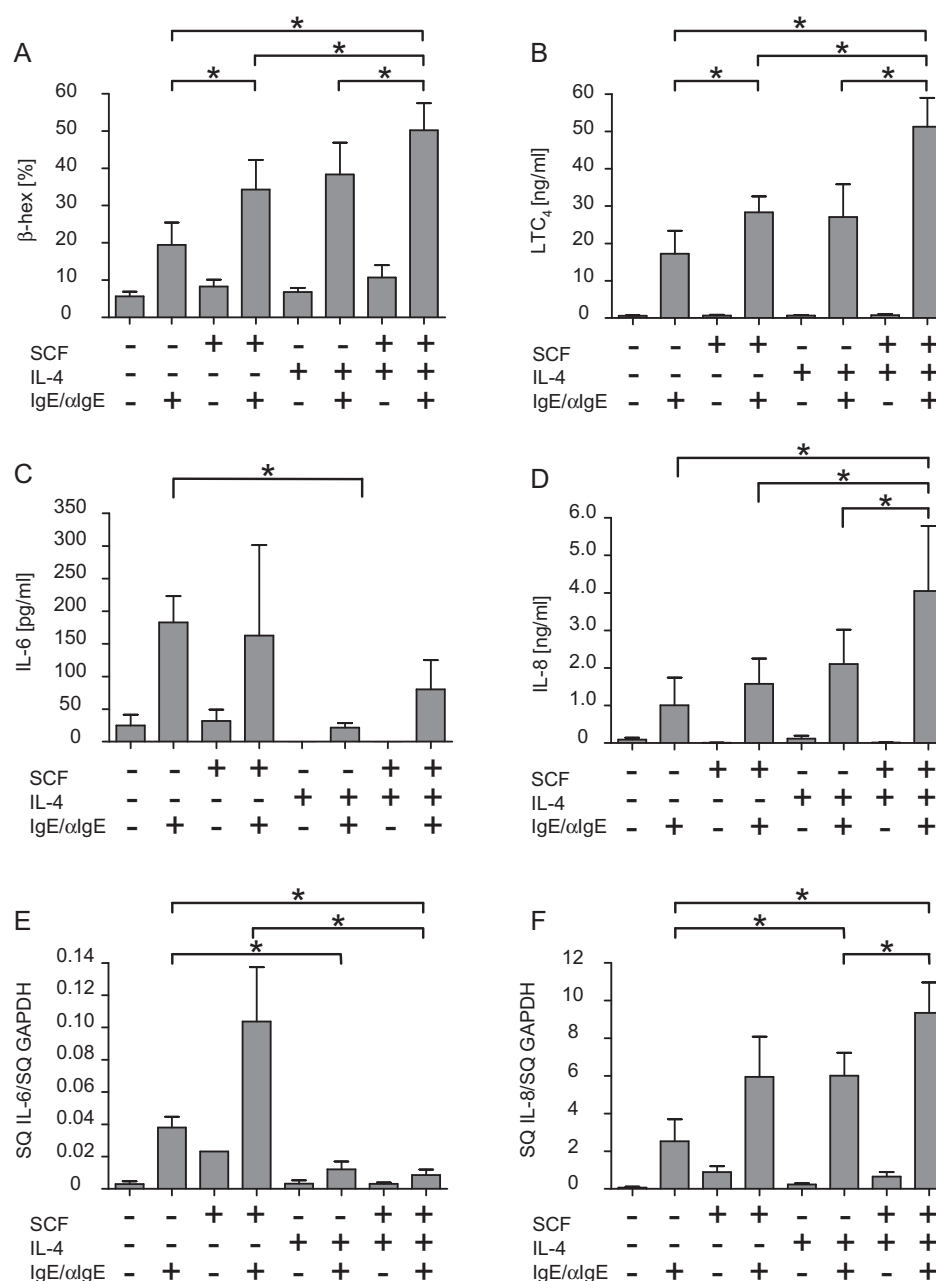


Fig. 1. Stimulation by Fc ϵ RI cross-linking, SCF, and IL-4 induce potentiated release and expression of inflammatory mediators in mast cells. Mast cells were cultured with SCF alone or with IL-4 (+) in addition to SCF for 10 d. SCF was withdrawn overnight and cells were treated with myeloma IgE for 60 min and stimulated with anti-human IgE (IgE/ α IgE) (+), SCF (+) or a combination of both for 6 h to determine the release of cytokines/chemokines, or for 90 min to analyze the release of β -hex as well as LTC₄, and to determine the mRNA expression of IL-6 and IL-8. (A) β -hex release measured by enzymatic color reaction expressed in percent of total content ($n=5$). (B) LTC₄ release analyzed by conventional ELISA expressed as pg/ 10^6 cells ($n=6$). (C and D) Cytokine and chemokine release measured by multiplex bead immunoassay and ELISA for IL-6 ($n=3$) (C), and IL-8 ($n=8$) (D) expressed as pg/ 10^6 cells. (E and F) Mast cell cytokine mRNA expression analyzed by real-time RT-PCR ($n=4$) for IL-6 (E), and IL-8 (F). To calculate the relative transcription level of the genes of interest, the copy number for the mRNA of the gene of interest was calculated and divided by that of GAPDH mRNA. Means $\pm$ SEM; * indicates $p < 0.05$ for significant difference, n means independent experiments using mast cells derived from independent surgical tissue specimens.

as described by the manufacturer (Fluor Chem, Biozym Scientific GmbH, Germany). The obtained signals were measured by bioimaging analyzer (Alpha Innotech Corporation, San Leandro, CA, USA).

2.8. Statistics

Data from multiple experiments were expressed as mean $\pm$ SEM. *T*-test was used to analyze differences between two groups. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. IL-4, SCF, and Fc ϵ RI cross-linking act synergistically in triggering inflammatory mediator release

Sequential challenge of mast cells with IL-4, SCF or IL-4 and SCF, in combination with Fc ϵ RI cross-linking led to a more pronounced mediator release than Fc ϵ RI cross-linking alone. This holds true for degranulation, assessed by β -hex release (Fig. 1A), and for *de novo* synthesis and release of LTC₄ (Fig. 1B) and IL-8 (Fig. 1D and F), but not for IL-6 (Fig. 1C and E). The release of IL-6 was attenuated in the

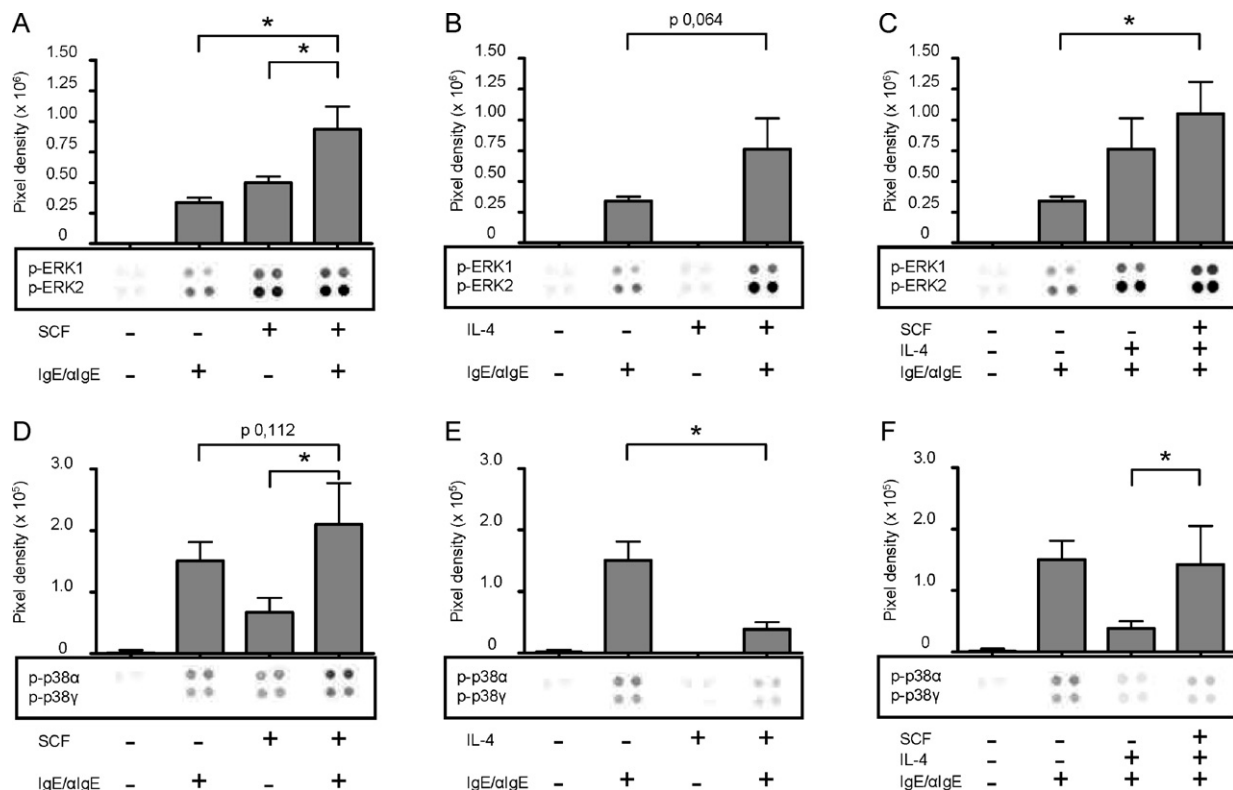


Fig. 2. Phosphorylation of ERK and p38 in response to FcεRI cross-linking, SCF, and/or IL-4. Mast cells were cultured with SCF or with IL-4 (+) in addition to SCF for 10 d. SCF was withdrawn overnight and cells were treated with myeloma IgE for 60 min and stimulated with anti-human IgE (IgE/αIgE) (+), SCF (+) or a combination of both for 10 min. Phosphorylation levels of ERK and p38 was measured by Proteome Profiler Antibody Array. Activation levels of these MAPK as detected were quantified using densitometry. (A and D) Phospho-ERK1/2 (A) and phospho-p38α/γ (D) in response to IgE/αIgE and SCF. (B and E) Phospho-ERK1/2 (B) and phospho-p38α/γ (E) in response to IgE/αIgE and IL-4. (C and F) Phospho-ERK1/2 (C) and phospho-p38α/γ (F) in response to IgE/αIgE, SCF, and IL-4. Data of the activation of ERK1 and ERK2, respectively p38α and p38γ were combined evaluated since they show equal tendency. The phospho-antibody signals were normalized against positive controls. Representative autoradiographs are shown (means + SEM, $n = 4$; * indicates $p < 0.05$ for significant difference).

presence of IL-4. Challenge with SCF or IL-4 *per se* without further stimulation by FcεRI cross-linking did not induce or enhance the release of pro-inflammatory mediators in mature human mast cells. Except IL-6, the release reaction was more pronounced in mast cells pre-treated with SCF and IL-4 compared to treatment with SCF or IL-4 alone prior to FcεRI cross-linking.

3.2. SCF and IL-4 both account for enhanced IgE-dependent ERK activation but have distinct effects on p38

In the next set of experiments, signaling pathways corresponding to the different stimulation procedures were analyzed. The MAPK family members ERK, p38, and JNK are known to be involved in cell proliferation and cytokine expression (Johnson and Lapadat, 2002; Lorentz et al., 2005). Therefore, we wondered if these MAPK family members become activated through FcεRI cross-linking, SCF, or IL-4 *per se* or in combination. Both, SCF and FcεRI cross-linking induced phosphorylation of ERK as well as phosphorylation of p38 (Fig. 2A and D). The combined stimulation by SCF and FcεRI cross-linking strongly increased the activation of ERK. IL-4 alone had no visible effect on MAPK phosphorylation, but in combination with IgE/anti-IgE IL-4 accounted for an increased phosphorylation of ERK, but suppressed activation of p38 (Fig. 2B and E). Combined treatment with SCF, IL-4 and FcεRI cross-linking resulted in an additionally enhanced activation of ERK compared to those cells stimulated by FcεRI cross-linking or FcεRI cross-linking and IL-4 (Fig. 2C). Phosphorylation of p38 was also increased by combined treatment with IL-4, SCF, and FcεRI cross-linking in comparison to IL-4 primed and IgE/anti-IgE stimulated cells, but was less pronounced than in those cells treated with SCF and FcεRI cross-linking

(Fig. 2F). We determined only low activation of JNK and observed no significant differences between SCF, FcεRI cross-linking or IL-4 treatment (data not shown).

3.3. Inhibition of p38 blocks IL-6 expression

To further assess the role of MAPK on cytokine release we applied specific inhibitors for p38 and MEK. The addition of the specific p38 inhibitor SB203580 attenuated the IL-6 mRNA expression in mast cells (Fig. 3B). SB203580 inhibited the SCF response and especially the combined SCF and IgE response, but did not affect the IgE response alone. However, SB203580 obviously did not block the kinase activity of other MAPK like ERK and JNK (Davis et al., 2004). Neither the release of β-hex nor the expression of IL-8 mRNA were significantly affected by SB203580 (Fig. 3A and C). Pre-incubation with the specific MEK inhibitor PD98059 resulted in a down-regulation of IL-8 mRNA expression without affecting expression of IL-6 and release of β-hex (Fig. 3D–F).

3.4. Sequential stimulation with IL-4 and FcεRI cross-linking, but not FcεRI cross-linking *per se* induces phosphorylation of Akt

Besides MAPK family members, several other intracellular serine/threonine kinases are of relevance in the signaling cascade of c-Kit, FcεRI, and IL-4 receptor (Gilfillan and Rivera, 2009; Nelms et al., 1999; Rivera and Gilfillan, 2006). Therefore we analyzed activation of different serine/threonine kinases in response to treatment with anti-IgE, SCF, and/or IL-4. Noteworthy, the serine/threonine kinase Akt was phosphorylated in response to SCF stimulation, but nearly no Akt phosphorylation was measured in

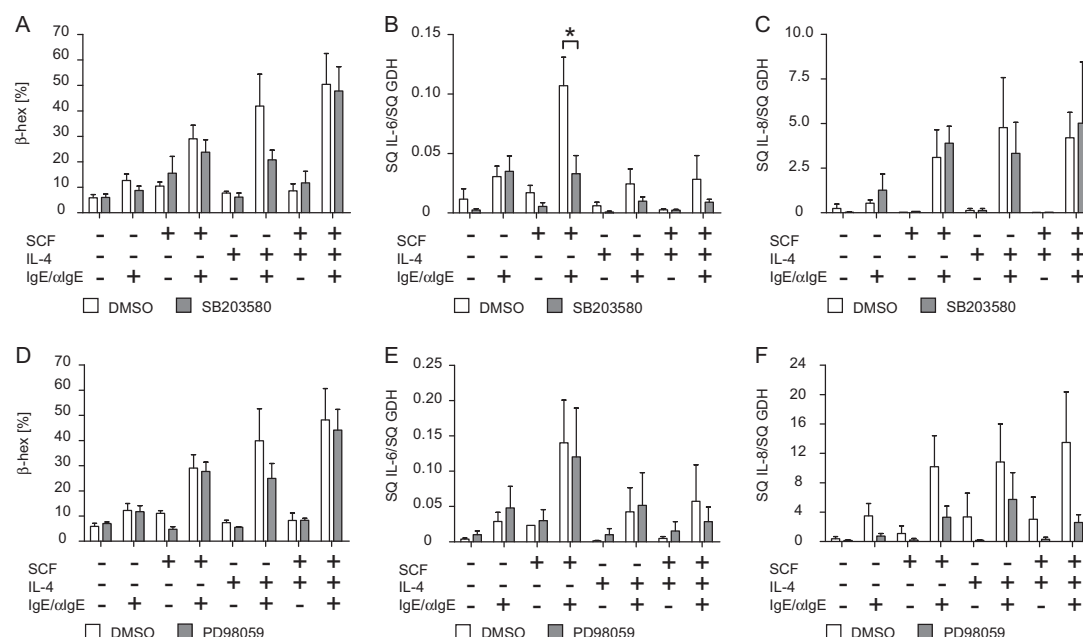


Fig. 3. Effect of specific inhibitors on the expression and release of inflammatory mediators. (A and D) β -hex release was determined in mast cells stimulated with IgE/ α IgE (+), SCF (+), and/or IL-4 (+) in the presence of the specific p38 inhibitor SB203580 (A), and the specific MEK inhibitor PD98059 (D) expressed in percent of total content. The mRNA expression was analyzed by real-time PCR in mast cells cultured with SCF alone or with IL-4 (+) in addition to SCF for 10 d and stimulated with IgE/ α IgE (+), SCF (+) or a combination of both in the presence of specific inhibitors. Expression of mRNA for IL-6 (B), and IL-8 (C) of mast cells stimulated in the presence of 5 μ M SB203580, and for IL-6 (E), and IL-8 (F) in response to stimulation in the presence of 50 μ M PD98059. Means $\pm$ SEM from four (β -hex release) or three (mRNA expression) experiments using mast cells derived from different surgical tissue specimens are shown. * Indicates $p < 0.05$ for significant difference.

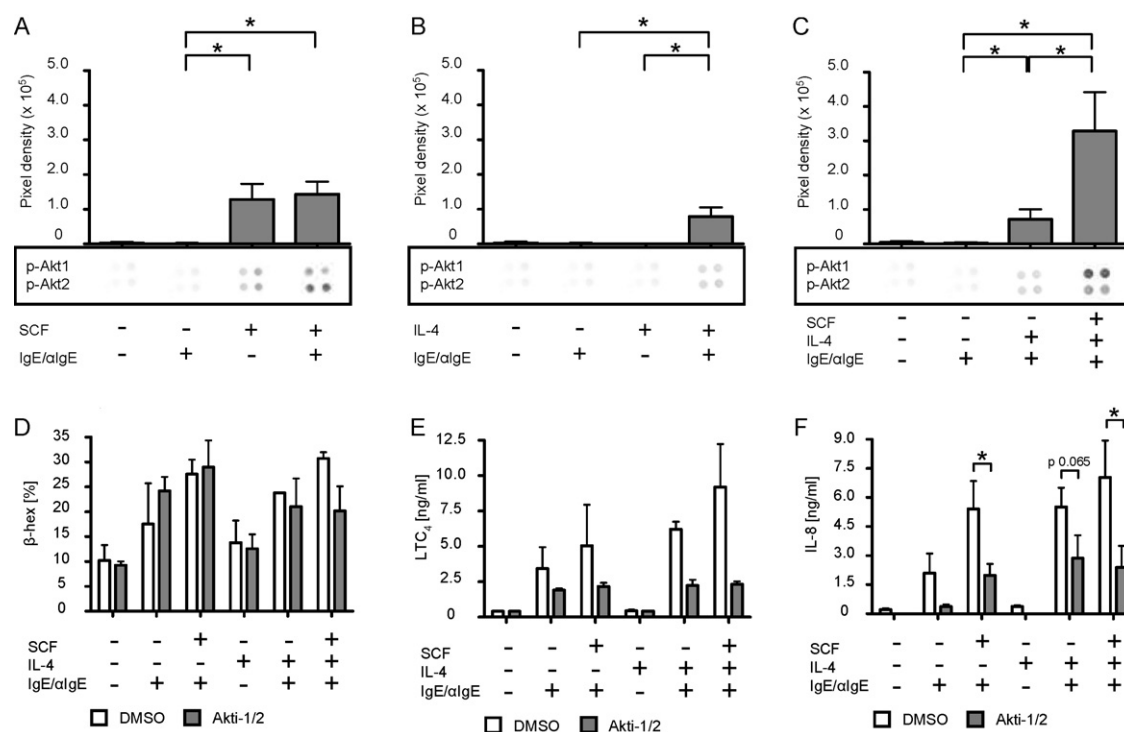


Fig. 4. Phosphorylation of Akt1/2 in response to Fc ϵ RI cross-linking, SCF, and IL-4 and effect of Akti-1/2 on IL-8 release. (A–C) Phosphorylation status of Akt in response to Fc ϵ RI cross-linking, SCF, and IL-4. Mast cells were cultured with SCF or SCF and IL-4 (+) for 10 d. SCF was withdrawn overnight and cells were treated with myeloma IgE for 60 min and stimulated with anti-human IgE (IgE/ α IgE) (+), SCF (+) or a combination of both for 10 min. Phosphorylation levels of Akt were measured by Proteome Profiler Antibody Array. Activation levels of Akt as detected with the array were quantified using densitometry. Phosphorylation levels of Akt1 and Akt2 were combined calculated since the data show equal tendency. (A) Phospho-Akt1/2 in response to antigen and SCF. (B) Phospho-Akt1/2 in response to antigen and IL-4. (C) Phospho-Akt1/2 in response to antigen, SCF, and IL-4. Representative autoradiographs are shown. The phospho-antibody signals were normalized against positive controls. Means $\pm$ SEM; $n = 4$. (D–E) Mast cells cultured with SCF alone or with IL-4 (+) in addition to SCF for 10 d were treated with myeloma IgE and 10 μ M Akti-1/2 for 60 min and stimulated with anti-human IgE (IgE/ α IgE) (+), SCF (+) or a combination of both in the presence of Akti-1/2 for 6 h, means $\pm$ SEM are shown. (D) β -hex release measured by enzymatic color reaction expressed in percent of total content ($n = 2$). (E) LTC₄ release analyzed by conventional ELISA expressed as pg/10⁶ cells ($n = 2$). (F) IL-8 release was determined by ELISA ($n = 5$). * indicates $p < 0.05$ for significant difference.

response to FcεRI cross-linking alone. Consistent with these findings, the combined treatment with IgE/anti-IgE and SCF did not cause an increased Akt phosphorylation, compared to stimulation with SCF alone (Fig. 4A). IL-4 by itself did not induce any detectable phosphorylation of Akt, but pre-treatment of human intestinal mast cells with IL-4 prior to FcεRI cross-linking resulted in Akt phosphorylation. We observed a substantially enhanced Akt activation in response to combined treatment with IL-4, SCF, and FcεRI cross-linking in comparison to those cells stimulated by SCF, SCF and IgE or IL-4 and IgE (Fig. 4C). The most pronounced chemokine/cytokine production in response to FcεRI cross-linking combined with SCF and/or IL-4 priming was found for IL-8. To analyze whether this effect is due to Akt activation, mast cells were treated with the specific Akt inhibitor Akti-1/2 prior to stimulation with SCF, IL-4 and/or FcεRI cross-linking. The enhanced production of IL-8 in response to SCF and IL-4 priming was mostly inhibited by pre-treatment with Akti-1/2 (Fig. 4D). Moreover, the enhanced LTC₄ generation induced by the combined stimulations was also down-regulated. Taken together, our findings support a central role of Akt in the crosstalk of downstream signaling events of FcεRI, c-Kit, and IL-4 receptor in mature human mast cells.

4. Discussion

During the last years it has been recognized that SCF or IL-4, respectively, prime human mast cells for enhanced IgE-dependent mediator release and cytokine generation (Bischoff et al., 1999; Gilfillan and Rivera, 2009; Hundley et al., 2004; Lorentz et al., 2005; Lorentz and Bischoff, 2001). Here, we report that the combined challenge with IL-4 and SCF causes a further enhancement of IgE-dependent mediator release in mature human mast cells and we provide insights in the underlying signaling cascades.

FcεRI, c-Kit, and the IL-4 receptor share common downstream signaling pathways. One of the major mechanisms responsible for the transduction of extracellular signals into a cellular response is the highly conserved MAPK superfamily comprising ERK, JNK, and p38 (Weston et al., 2002). The combined stimulation with SCF and FcεRI cross-linking strongly enhanced ERK and p38 activation as well as the production of cytokines and chemokines. These results indicate that the induced ERK and p38 activity correlate with the release and production of cytokines/chemokines following FcεRI cross-linking and SCF stimulation. However, SCF alone caused phosphorylation of ERK and p38, but did not induce release of β-hex or LTC₄ on its own in human mucosal mast cells. This implicates that ERK or p38 activation *per se* is not sufficient to initiate inflammatory mediator release in mast cells, but requires the activation of a second, distinct signal transduction pathway. Hundley et al. (2004) suggested that SCF fails to induce degranulation because of its inability to activate PKC, which is an important primary signal for the degranulation of mast cells (Gilfillan and Tkaczyk, 2006). Consistently, own observations using the inhibitor Gö 6975, a selective inhibitor of PKC, demonstrated that inhibition of this kinase did not affect the SCF induced expression of cytokines/chemokines, such as IL-5, IL-8, or MIP-1β, but inhibited IgE-mediated histamine release (Martiny-Baron et al., 1993, own unpublished data). These results indicate that the induced expression of cytokines/chemokines and the potentiated antigen-mediated expression by SCF appear independent from PKC.

Surprisingly, neither FcεRI cross-linking nor SCF stimulation caused a clearly increased phosphorylation of JNK in human intestinal mast cells. These observations are contrary to findings in rodent mast cells and human umbilical cord blood mast cells but match with findings in human basophils (Gibbs et al., 2005; Ishizuka et al., 1996; Kimata et al., 2000). On the other side, usage of JNK inhibitor SP600125 resulted in a down-regulated release of pre-stored β-hex

and mRNA expression of IL-8 and IL-6 in human intestinal mast cells (data not shown). Tanemura et al. (2009) revealed in their recent study that SP600125 abrogates FcεRI-induced degranulation and cytokine gene expression by inhibiting the kinase activity of PI3K in mouse mast cells, suggesting that SP600125 does not only specifically block JNK, but also PI3K. However, SP600125 had no effect on the phosphorylation of Akt, ERK, and p38 in human HMC-1 mast cells (Wang et al., 2006). Hence, we analyzed the release of β-hex as well as the expression of IL-8 of mast cells treated with wortmannin – a selective PI3K inhibitor – prior to FcεRI cross-linking. PI3K is a key effector of the downstream serine/threonine kinase Akt (Gilfillan and Tkaczyk, 2006). Wortmannin inhibited the release of β-hex and the expression of IL-8 (own unpublished data). Possibly, SP600125 inhibits mediator release and IL-8 mRNA expression by blocking PI3K.

In accordance with earlier findings in mice, we observed that IL-4 priming of mast cells failed to activate ERK, p38, and JNK by itself (Foltz et al., 1997). Noteworthy, treatment of human intestinal mast cells with IL-4 enhanced the IgE-dependent activation of ERK but blocked the IgE-dependent activation of p38. Observations in different cell lines and in primary mouse mast cells demonstrated that IL-4 did not induce tyrosine phosphorylation of p38 in mast cells but in other cell types, such as T- and pro-B-lymphocyte cell lines (Hunt et al., 2002). Thus, it appears that activation of MAPK pathways in response to IL-4 stimulation is highly cell type dependent. Similar to our findings in human mast cells, pre-treatment with IL-4 blocked the LPS-induced p38 activity in RAW 264.7 murine macrophages (Hunt et al., 2002). Interestingly, IL-4 in synergy with FcεRI cross-linking resulted in an increased mediator release and cytokine and chemokine production, but in an inhibited IL-6 expression, suggesting that IL-6 expression and release depends on p38 activation in human intestinal mast cells. Indeed, using p38 inhibitor SB203580 we found an inhibition of IL-6 expression, but not of IL-8. Moreover SB203580 did not affect the release of prestored mediators. It has been shown that p38 promote transactivation of the transcription factor NF-κB which is an important regulator of the IL-6 expression (Boudreau et al., 2004). The inability of IL-4 to induce phosphorylation, respectively the inhibition of p38 phosphorylation, despite causing human intestinal mast cell proliferation and enhanced mediator release and production besides IL-6, indicates that p38 MAPK activation is indeed not necessary for this enhancement.

Besides the MAPK family members, other serine/threonine kinases like Akt are of importance for the transduction of extracellular signals into a cellular response. The intracellular serine/threonine kinase Akt is implicated in a variety of cellular functions, such as survival, metabolism, and cell growth (Sale and Sale, 2008). Akt was shown to be activated upon cross-linking of FcεRI, or SCF activation in primary cultured mouse mast cells as well as in the rat mast cell line RBL-2H3, and to be involved in the FcεRI-induced production of IL-2 and TNF-α (Ishizuka et al., 1999; Kitaoura et al., 2000). In contrast to previous findings in rodent mast cells, we found that FcεRI cross-linking did not effect phosphorylation of Akt in human intestinal mast cells. Our findings indicate that Akt is not responsible for mediator expression and/or release in human intestinal mast cells in response to antigen-dependent activation alone.

In contrast to FcεRI cross-linking, treatment of human intestinal mast cells with SCF caused a strong activation of Akt. Most interestingly, we found that priming with IL-4 before IgE-stimulation resulted in a strong activation of Akt, indicating that SCF or IL-4 are necessary for the activation of Akt. Moreover, a combined addition of IL-4 and SCF further potentiated the activation of Akt following FcεRI cross-linking. These results strongly suggest Akt being an important element for the intracellular communication between the three different receptors, FcεRI, c-Kit, IL-4. Consistently, inhibi-

tion of Akt by the specific inhibitor Akti-1/2 resulted in a markedly down-regulated SCF and IL-4 induced IL-8 release, suggesting that Akt cross-links the signaling pathways of SCF, IL-4, and antigen in mature human mast cells. Although we observed no further Akt phosphorylation in response to FcεRI cross-linking in comparison to unstimulated cells, treatment with Akti-1/2 slightly attenuated IgE-mediated IL-8 production. This could be due to the Akti-1/2-caused complete inhibition of the basal Akt phosphorylation we measured in IgE-stimulated mast cells.

In the underlying study we analyzed signaling molecules in mast cells with regard to their activation levels in response to priming with IL-4 for several days. It could be assumed that cells incubated for 10 days in the presence of SCF alone will differ in the expression of numerous signaling molecules from cells incubated for 10 days in the presence of both SCF and IL-4; other changes than those found here could contribute to different responses to various ligands. Thus, a detailed analysis of the whole transcription could bring further insight in such changes induced by different priming factors and will be object of future studies.

In conclusion, SCF and IL-4 are required for the activation of Akt in IgE-stimulated human intestinal mast cells. Moreover SCF enhances phosphorylation of p38 whereas IL-4 inhibits it, showing common and distinct pathways of SCF and IL-4 in human mast cell activation. Our findings reveal a differential activation of MAPK and Akt following mast cell challenge with SCF and IL-4 and triggering by FcεRI cross-linking, providing new insights into the crosstalk of signaling pathways responsible for mast cell activation.

Acknowledgements

The work was supported by the Deutsche Forschungsgemeinschaft (BI 424-2-1 to Stephan C. Bischoff and Axel Lorentz).

The authors thank Yvonne Soltow and Arben Mrasori for excellent technical assistance.

References

- Babina, M., Guhl, S., Stärke, A., Kirchhof, L., Zuberbier, T., Henz, B.M., 2004. Comparative cytokine profile of human skin mast cells from two compartments—strong resemblance with monocytes at baseline but induction of IL-5 by IL-4 priming. *J. Leukoc. Biol.* 75, 244–252.
- Bischoff, S.C., 2007. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat. Rev. Immunol.* 7, 93–104.
- Bischoff, S.C., Dahinden, C.A., 1992. c-Kit ligand: a unique potentiator of mediator release by human lung mast cells. *J. Exp. Med.* 175, 237–244.
- Bischoff, S.C., Schwengberg, S., Raab, R., Manns, M.P., 1997. Functional properties of human intestinal mast cells cultured in a new culture system: enhancement of IgE receptor-dependent mediator release and response to stem cell factor. *J. Immunol.* 159, 5560–5567.
- Bischoff, S.C., Sellge, G., Lorentz, A., Sebald, W., Raab, R., Manns, M.P., 1999. IL-4 enhances proliferation and mediator release in mature human mast cells. *Proc. Natl. Acad. Sci. U.S.A.* 96, 8080–8085.
- Blank, U., Rivera, J., 2004. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* 25, 266–273.
- Boudreau, R.T., Hoskin, D.W., Lin, T.J., 2004. Phosphatase inhibition potentiates IL-6 production by mast cells in response to FcεRI-mediated activation: involvement of p38 MAPK. *J. Leukoc. Biol.* 76, 1075–1081.
- Davis, B.J., Flanagan, B.F., Gilfillan, A.M., Metcalfe, D.D., Coleman, J.W., 2004. Nitric oxide inhibits IgE-dependent cytokine production and Fos and Jun activation in mast cells. *J. Immunol.* 173, 6914–6920.
- Foltz, I.N., Lee, J.C., Young, P.R., Schrader, J.W., 1997. Hemopoietic growth factors with the exception of interleukin-4 activate the p38 mitogen-activated protein kinase pathway. *J. Biol. Chem.* 272, 3296–3301.
- Galli, S.J., Grimaldeston, M., Tsai, M., 2008. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat. Rev. Immunol.* 8, 478–486.
- Gibbs, B.F., Wolff, H.H., Zillikens, D., Grabbe, J., 2005. Differential role for mitogen-activated protein kinases in IgE-dependent signaling in human peripheral blood basophils: in contrast to p38 MAPK, c-Jun N-terminal kinase is poorly expressed and does not appear to control mediator release. *Int. Arch. Allergy Immunol.* 136, 329–339.
- Gilfillan, A.M., Rivera, J., 2009. The tyrosine kinase network regulating mast cell activation. *Immunol. Rev.* 228, 149–169.
- Gilfillan, A.M., Tkaczyk, C., 2006. Integrated signalling pathways for mast cell activation. *Nat. Rev. Immunol.* 6, 218–230.
- Hu, Z.Q., Zhao, W.H., Shimamura, T., 2007. Regulation of mast cell development by inflammatory factors. *Curr. Med. Chem.* 14, 3044–3050.
- Hundley, T.R., Gilfillan, A.M., Tkaczyk, C., Andrade, M.V., Metcalfe, D.D., Beaven, M.A., 2004. Kit and FcεRI mediate unique and convergent signals for release of inflammatory mediators from human mast cells. *Blood* 104, 2410–2417.
- Hunt, A.E., Williams, L.M., Lali, F.V., Foxwell, B.M., 2002. IL-4 regulation of p38 MAPK signalling is dependent on cell type. *Cytokine* 18, 295–303.
- Ishizuka, T., Chayama, K., Takeda, K., Hamelmann, E., Terada, N., Keller, G.M., Johnson, G.L., Gelfand, E.W., 1999. Mitogen-activated protein kinase activation through Fcε receptor 1 and stem cell factor receptor is differentially regulated by phosphatidylinositol 3-kinase and calcineurin in mouse bone marrow-derived mast cells. *J. Immunol.* 162, 2087–2094.
- Ishizuka, T., Oshiba, A., Sakata, N., Terada, N., Johnson, G.L., Gelfand, E.W., 1996. Aggregation of the FcεRI on mast cells stimulates c-Jun amino-terminal kinase activity. A response inhibited by Wortmannin. *J. Biol. Chem.* 271, 12762–12766.
- Johnson, G.L., Lapadat, R., 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 298, 1911–1912.
- Kalesnikoff, J., Galli, S.J., 2008. New developments in mast cell biology. *Nat. Immunol.* 9, 1215–1223.
- Kimata, M., Inagaki, N., Kato, T., Miura, T., Serizawa, I., Nagai, H., 2000. Roles of mitogen-activated protein kinase pathways for mediator release from human cultured mast cells. *Biochem. Pharmacol.* 60, 589–594.
- Kitaura, J., Asai, K., Maeda-Yamamoto, M., Kawakami, Y., Kikkawa, U., Kawakami, T., 2000. Akt-dependent cytokine production in mast cells. *J. Exp. Med.* 192, 729–740.
- Lorentz, A., Bischoff, S.C., 2001. Regulation of human intestinal mast cells by stem cell factor and IL-4. *Immunol. Rev.* 179, 57–60.
- Lorentz, A., Schwengberg, S., Sellge, G., Manns, M.P., Bischoff, S.C., 2000. Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4. *J. Immunol.* 164, 43–48.
- Lorentz, A., Wilke, M., Sellge, G., Worthmann, H., Klempnauer, J., Manns, M.P., Bischoff, S.C., 2005. IL-4-induced priming of human intestinal mast cells for enhanced survival and Th2 cytokine generation is reversible and associated with increased activity of ERK1/2 and c-Fos. *J. Immunol.* 174, 6751–6756.
- Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marmè, D., Schächtele, C., 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. *J. Biol. Chem.* 268, 9194–9197.
- Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., Paul, W.E., 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* 17, 701–738.
- Raman, M., Chen, W., Cobb, M.H., 2007. Differential regulation and properties of MAPKs. *Oncogene* 26, 3100–3112.
- Rivera, J., Gilfillan, A.M., 2006. Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.* 117, 1214–1225.
- Sale, E.M., Sale, G.J., 2008. Protein kinase B: signalling roles and therapeutic targeting. *Cell Mol. Life Sci.* 65, 113–127.
- Schwartz, L.B., Austen, K.F., Wasserman, S.I., 1979. Immunologic release of β-hexosaminidase and β-glucuronidase from purified rat serosal mast cells. *J. Immunol.* 123, 1445–1450.
- Sellge, G., Bischoff, S.C., 2006. Isolation, culture, and characterization of intestinal mast cells. *Methods Mol. Biol.* 315, 123–138.
- Tanemura, S., Momose, H., Shimizu, N., Kitagawa, D., Seo, J., Yamasaki, T., Nakagawa, K., Kajihio, H., Penninger, J.M., Katada, T., Nishina, H., 2009. Blockage by SP600125 of Fcε receptor-induced degranulation and cytokine gene expression in mast cells is mediated through inhibition of phosphatidylinositol 3-kinase signaling pathway. *J. Biochem.* 145, 345–354.
- Wang, B., Tsukada, J., Higashi, T., Mizobe, T., Matsuura, A., Mouri, F., Sawamukai, N., Ra, C., Tanaka, Y., 2006. Growth suppression of human mast cells expressing constitutively active c-Kit receptors by JNK inhibitor SP600125. *Genes Cells* 11, 983–992.
- Weston, C.R., Lambright, D.G., Davis, R.J., 2002. Signal transduction. MAP Kinase signaling specificity. *Science* 296, 2345–2347.