Chapter 4

Regulation of interleukin-4 expression in mast cells under hypoxic conditions

Maciej B. Olszewski[1,2], Edward F. Knol[2]

[1]Department of Molecular Biology, International Institute of Molecular and Cell Biology, 4 Ks. Trojdena St., 02-109 Warsaw, Poland
[2] Department of Dermatology/Allergology, University Medical Center Utrecht, Heidelberglaan 100, 3584CX Utrecht, The Netherlands

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Introduction

Hypoxia is a state occurring in organisms under many, mostly pathological, conditions e.g. chronic hypoxia related to coronary heart disease. A consequence of this state is sometimes a heart attack resulting in an acute hypoxic state. Another common occurrence of hypoxic state are sites of rapid tumour growth where angiogenesis is lagging behind.

In hypoxic areas or their immediate proximity often the presence of mast cells is noted. In heart post-ischaemic reperfusion-induced inflammation there's a prominent role for mast cells (1), (2). It has also been observed that stabilisation of mast cells with pharmacologic agents can contribute to limiting this injury (3). These treatments, however, influence rapid, degranulation-related response of mast cells. More prolonged response of mast cells to hypoxic condition may contribute to sustained inflammatory state (2), but may also augment angiogenesis which is profitable in post-ischaemic state in injured heart (4). The same process however is detrimental to the patient an correlates with worse prognosis when tumour progression-related angiogenesis is considered (5, 6). In the course of reperfusion-related processes subsets of lymphocytes have been shown to release IL-10 which exerts anti-inflammatory activity and may help in limiting inflammatory injury (7).

Depending on timescale considered and stimuli present in microenvironment mast cells can exert both pro- and anti-inflammatory activities. It has been shown that mast cells store and rapidly release TNF (8) initiating inflammatory response (9). To date, presence of preformed anti-inflammatory cytokines available for immediate release from human mast cells has not been unequivocally demonstrated but these cells, when appropriately stimulated release a range of anti-inflammatory cytokines, including IL-4, -10 and -13 which makes them a potential target of a therapy aimed at reducing excessive inflammation in the tissue. Importantly, a critical immunoprotective role of mast cells in skin graft rejection model has recently been reported (10). Thus it has to be noted that mast cells presence and activity can be both beneficial as well as detrimental to the tissue in which the immune response is developing.

HIF-1α is a transcription factor that has been implicated as a major hypoxic signal transducer (11), its degradation rate being dependent on oxygen availability. Hypoxia-induced effect of stabilization of HIF-1α is often mimicked by desferrioxamine, an iron chelator inhibiting HIF-prolyl hydroxylase, an enzyme initiating HIF-1α degradation pathway. This model, while reflecting HIF-1α-related effects of hypoxia, does however not account for HIF-1α-independent responses to hypoxia such as Akt-related signal transduction pathways.

In this work we approached the question of mast cells response to hypoxia. It has been reported that DFX-stimulated HMC-1 mast cells exhibit inflammatory response (12). We aimed at establishing whether the long-term cellular response in actual hypoxia is unequivocally proinflammatory. We report that mast cells incubated in

1% oxygen release increased amounts of IL-4 and IL-13 as opposed to TNF-alpha and IFN-γ. This response does not correlate with HIF-1α induction, but seems to be related to Akt and GSK-3 activity and NFAT-mediated IL-4 promoter induction.

Results

Hypoxic stress induces cytokine release from mast cells

In order to determine the influence of hypoxia on cytokine release profile of HMC-1 mast cells, the cells were incubated in normoxic (21% oxygen) or hypoxic (1% oxygen) conditions in the presence or absence of 1 μM ionomycin and 100 ng/ml PMA. Cell-free supernatant was collected after 6 and 24 hours of culture, and IL-4, IL-6, IL-10, IL-13, IFN-γ and TNF were assayed. The results obtained are presented in Fig. 1. The cells incubated for 24 hours in hypoxic conditions released significantly more IL-4 and IL-13 than cells incubated in 21% oxygen. No increase in release of IL-6, IL-10, IFN-γ and TNF was demonstrated following 24 hours (Fig. 1C) or 6 hours (not shown) of incubation in hypoxic conditions.

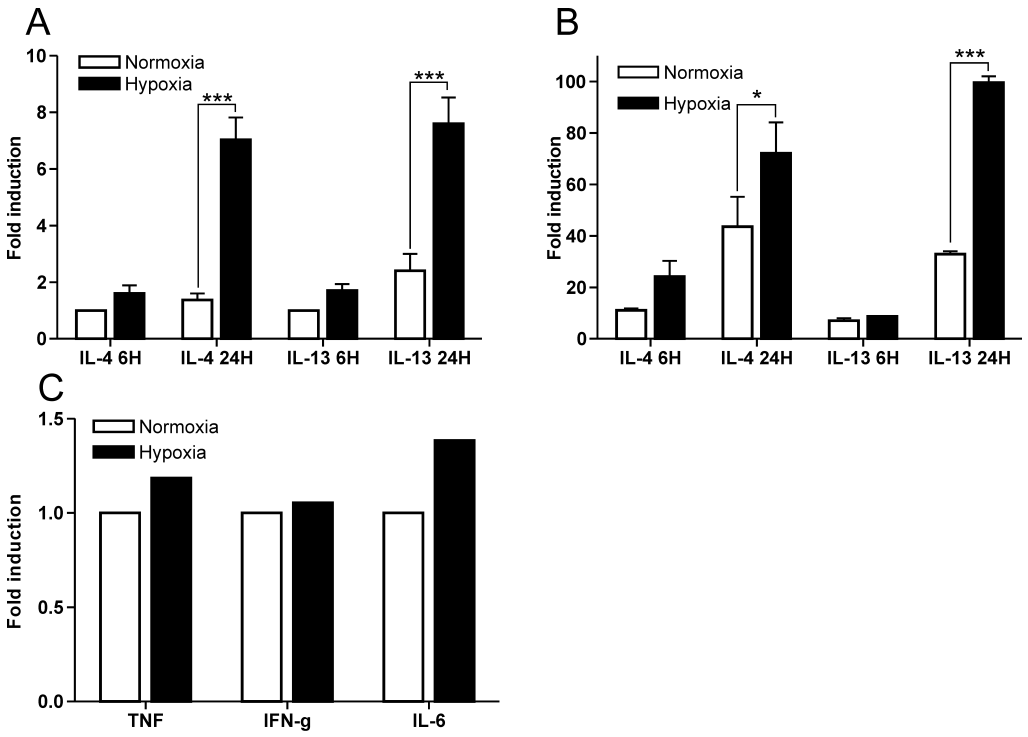

Figure 1 Mast cells release IL-4 and IL-13 in hypoxic conditions. HMC-1 mast cells were incubated in normoxic (21% O2) or hypoxic (1% O2) conditions for 6 or 24 hours in the absence (A) or presence (B) of 100 ng/ml PMA and 1 μM ionomycin. Concentration of IL-4 and IL-13 in the culture medium was assayed by ELISA. (*) denotes P<0.05 and (***) denotes P<0.001. (C) HMC-1 mast cells were incubated in normoxic (21% O2) or hypoxic (1% O2) conditions for 24 hours and concentrations of TNF, IFN-γ, IL-6 and IL-10 were assayed by ELISA. (n.d.) denotes none detected. Reference concentrations used for normalization (relative 1) were: (panels A and B) 6 hours/normoxia 24 ng/ml (IL-4) and 32 ng/ml (IL-13); (panel C) 24 hours/normoxia 30 ng/ml (TNF), 75 ng/ml (IFN-γ) and 136 ng/ml (IL-6)

IL-4 induction in hypoxia is dependent on calcineurin and GSK-3 activity

In order to identify the pathway responsible for hypoxia-dependent IL-4 induction HMC-1 cells were incubated for 1 hour with the following inhibitors: 5 FK506 (calcineurin), SB216763 (GSK3), PD98059 (MEK1) or with medium alone. Subsequently, cells were transferred to fresh medium containing respective inhibitors supplemented with ionomycin, PMA or no stimulus. Cells were incubated in normoxic or hypoxic conditions and culture medium samples for IL-4 assay were taken after 6 and 24 hours. In addition, cellular lysates were analyzed by western blot using anti-HIF-1α antibodies. As expected, both ionomycin and PMA induced IL-4 expression. This effect was additive to the induction caused by hypoxia, although relatively least pronounced in cells stimulated with ionomycin for 24 hours in both hypoxic and normoxic conditions. In contrast, the effect of PMA was most pronounced after 24 hours in hypoxia (Fig. 2A). Inhibition of GSK3 with SB216763 caused general elevation of IL-4 levels, particularly at 24 hours of hypoxic treatment combined with ionomycin or PMA (Fig. 2B). Addition of FK506 significantly decreased IL-4 release regardless of stimulus used, however an effect of hypoxia was still observable (Fig. 2C). Blocking of MEK1 with PD98059 caused general decrease in IL-4 release as compared to non-inhibited cells except for cells incubated for 24 hours in hypoxic conditions, regardless of stimulus used. In that case IL-4 levels were higher than in non-inhibited cells, but lower than in SB216763-inhibited cells (Fig. 2D).

Stimulation of IL-4 expression in hypoxia is HIF-1α-independent.

Comparing IL-4 levels in culture supernatants and HIF-1α levels we observed that changes in these two values were not correlated. Hypoxia alone did not cause significant elevation of HIF-1α level. The combination of hypoxia and ionomycin was very effective at maintaining high level of this transcription factor for 24 hours (Fig. 2A). When cells were treated with SB216763, high level of HIF-1α could be observed following 6 hours of stimulation with ionomycin in both normoxia and hypoxia and also following 6 hours of stimulation with PMA, but only in hypoxic conditions. Contrary to non-inhibited cells, ionomycin-dependent HIF-1α induction was relatively transient (6 hours) with long-term (24 hours) level similar to non-treated normoxic cells (Fig. 2B). In cells treated with FK506 HIF-1α levels were generally lowered; small induction was only observed in cells both stimulated with ionomycin or PMA and incubated in hypoxia (Fig. 2C). Treatment with PD98059 for 6 hours resulted in elevated HIF-1α levels in cells stimulated with ionomycin or PMA the former being more effective (Fig. 2D). This effect was strongly enhanced in cells incubated in hypoxic conditions and the pattern of activation resembled the one observed in cells treated with SB216763 (Fig. 2B). After 24 hours of PD98059 treatment, however, levels of HIF-1α remained high only in cells treated with ionomycin or PMA and incubated in hypoxic conditions, in that respect resembling the pattern observed in non-inhibited cells (Fig. 2A).

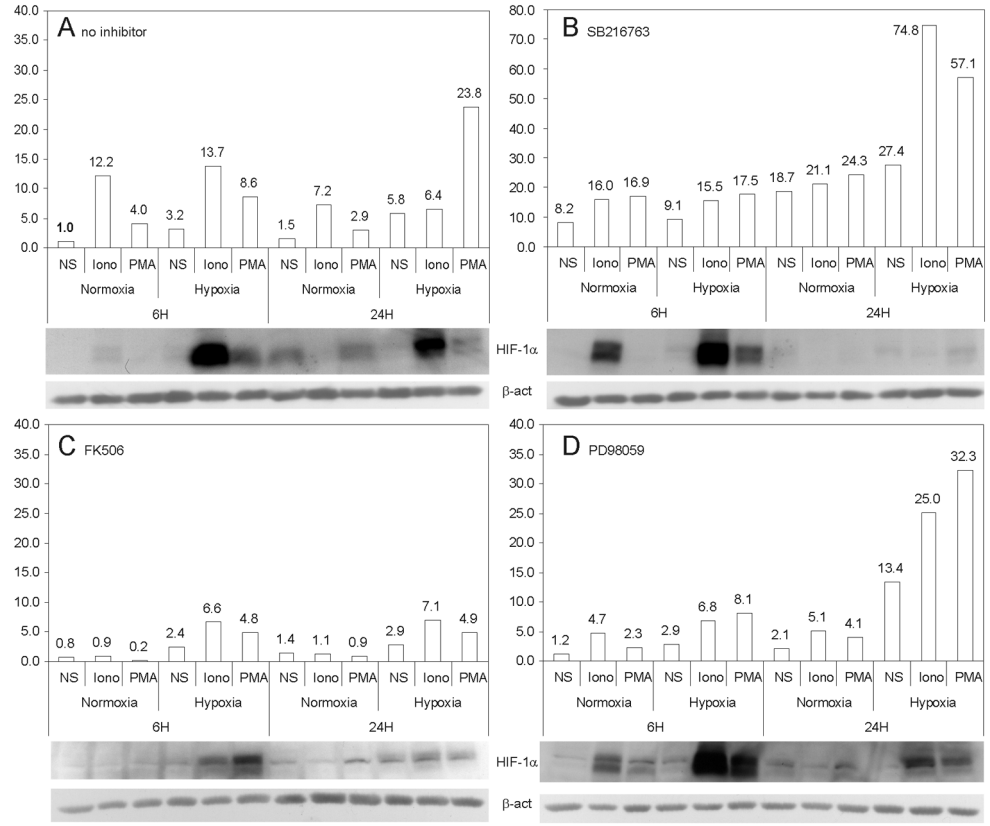

Figure 2 IL-4 release in hypoxia is dependent on calcineurin and GSK-3 but not on HIF-1α. HMC-1 mast cells preincubated for 1 hour in culture medium (A), 10 μM SB216763 (B), 5 μM FK506 (C) or 50 μM PD98059 (D) were incubated for 6 or 24 hours in normoxic or hypoxic conditions with or without addition of 1 μM ionomycine or 100 ng/ml PMA. IL-4 concentration in supernatants was assayed by ELISA. Cell lysates were analyzed by Western Blot with antibodies detecting HIF-1α (upper panel) or β-actin (lower panel).

Hypoxia-induced IL-4 stimulation is partly dependent on NFAT

The relatively high induction of IL-4 by SB216763 treatment pointed to possible involvement of Akt pathway in regulation of NFAT transcriptional activity and thus IL4 expression level. In order to analyze GSK-3 and NFAT involvement in IL4 regulation HMC-1 cells were transfected with pNFAT-SEAP plasmid encoding for SEAP reporter gene under the control of NFAT responsive promoter. 24 hours post-transfection cells were transferred to fresh medium containing 5 μM FK506, 10 μM SB216763 or to culture medium alone for 1 hour and following that transferred to fresh medium containing respective inhibitor and 1μM ionomycin or 100 ng/ml PMA where applicable. Cells were incubated in normoxic or hypoxic conditions for 6 hours and supernatant samples were taken for IL-4 and SEAP assays. The results are summarized in Fig. 3A for IL-4 and Fig. 3B for NFAT.

An induction of both IL-4 and SEAP production was observed in hypoxic conditions although the extent of this induction differed significantly depending on stimulation or inhibition applied in parallel. In general, IL-4 promoter exhibited higher dynamics

of response than NFAT responsive element. This effect was particularly pronounced in response to ionomycin and SB216763 in both hypoxia and normoxia. Also IL-4 expression level was more susceptible to inhibition with FK506. It is of note, however, that the pattern of transcriptional activity of both genes was similar in response to both GSK-3 inhibition and hypoxia. This prompted us to define the influence of the regulatory proteins operating upstream of NFAT, namely calcineurin and Akt.

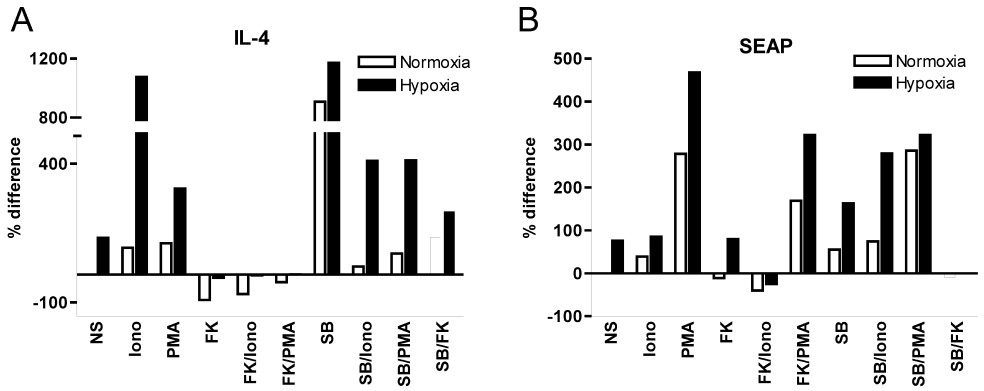

Figure 3 Hypoxia-induced IL-4 stimulation is partly dependent on NFAT. HMC-1 mast cells transfected with a plasmid encoding for SEAP under transcriptional control of NFAT responsive element and 24 hours post-transfection preincubated for 1 hour with 5 μM FK506 or 10 μM SB216763. Following preincubation cells were incubated for 6 hours with indicated combinations of the inhibitors and 1 μM ionomycin or 100 ng/ml PMA in normoxic or hypoxic conditions. IL-4 concentration (A) and SEAP activity (B) were measured in supernatants by ELISA and chemiluminescent assay, respectively. NS are non-treated cells.

IL-4 expression is regulated by Akt

HMC-1 cells were transfected with plasmids encoding for constitutively active Akt (myrAkt) and NFAT reporter (NFAT-SEAP). 24 hours post-transfection cells were transferred to fresh medium containing 5 μM FK506, 10 μM SB216763 or to culture medium alone for 1 hour and following that transferred to fresh medium containing respective inhibitor or 1μM ionomycin where applicable. Cells were incubated in normoxic or hypoxic conditions for 6 hours and supernatant samples were taken for IL-4 and SEAP assays. The results are presented in Fig. 4A for IL-4 and Fig. 4B for NFAT reporter.

The response to hypoxia could be observed in both systems and the effect of stronger response to FK506 and ionomycin by IL-4 promoter was reproduced. In contrast the influence of cotransfected myrAkt was more pronounced in case of NFAT reporter gene. In all experimental situations treatment with ionomycin or SB216763 caused the induction of both IL-4 and NFAT-driven SEAP. This effect was additionally enhanced by transfection with myrAkt. In case of IL-4 expression the most potent combination of stimuli was stimulation by ionomycin accompanied by myrAkt transfection and incubation in hypoxia. In case of NFAT reporter such combination was constituted by inhibition of GSK-3, myrAkt transfection and incubation in hypoxia. These results showed that, in addition to well-known calcineurin-mediated IL-4 expression regulation, Akt pathway is also involved in regulation of this cytokine expression.

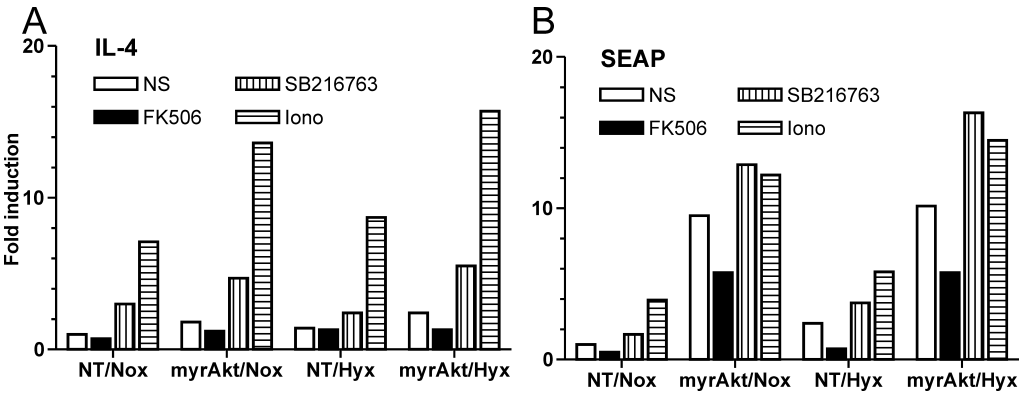

Figure 4 IL-4 expression is regulated by Akt. HMC-1 mast cells were transfected with plasmids encoding for constitutively active Akt (myrAkt) and SEAP under transcriptional control of NFAT responsive element and 24 hours post-transfection preincubated for 1 hour with 5 μM FK506 or 10 μM SB216763. Following preincubation, cells were incubated for 6 hours with indicated inhibitors or 1 μM ionomycin in normoxic or hypoxic conditions. IL-4 concentration (A) and SEAP activity (B) were measured in supernatants by ELISA and chemiluminescent assay, respectively.

Discussion

In this work we demonstrate a novel observation that HMC-1 mast cells, when exposed to hypoxic environment, release increased amounts of IL-4 and IL-13. Amounts of the cytokines released do not correlate with HIF-1α induction. We also present the data demonstrating involvement of Akt pathway and NFAT in the regulation of IL-4 expression under hypoxic conditions.

Mast cells can be a source of both pro- and anti-inflammatory cytokines and the release profile depends on direct intercellular interactions, soluble factors (such as cytokines) present in the local environment and biophysical factors, including oxygen availability. In various ischemia-reperfusion models it has been shown that mast cells become activated and degranulate, releasing both prestored and inducible factors, generally representing a proinflammatory profile (2, 3, 14, 15). This initial pulse contributes to the induction of the inflammatory state which is necessary for cleaning up the necrotic area. It is, however, important that at subsequent stages the expression of proinflammatory cytokines and chemokines is repressed and the transition to fibrous tissue deposition and angiogesis takes place. This may be achieved by expression of anti-inflammatory, fibro- and angiogenic agents such as interleukins 4, 10 and 13, VEGF and TGF-β by various subsets of cells, including T-cells, macrophages and mast cells. The latter might contribute to this phase by expressing some of the aforementioned mediators either following degranulation or in a process of differential release that involves mast cells activation and mediator release but not degranulation (reviewed in (16)). Some of the mediators released in degranulation-independent manner include corticotrophin-releasing hormone-stimulated VEGF (17) and TLR2-mediated release of IL-4, 6 and 13 in the absence of IL-1 (18). Overall, this implicates a novel role for mast cells as regulatory cells capable of not only inducing but also limiting the extent of immune response, including inflammation. It has been demonstrated that mast cells, through effects mediated at least in part by histamine, can contribute to the immunosuppressive actions of

ultraviolet-B irradiation on the expression of contact hypersensitivity responses in mice (19). Maurer et al. have shown that endothelin-A-receptor-dependent mast-cell activation can diminish both endothelin-1 levels and endothelin-induced pathology in vivo, and also can contribute to optimal survival during acute bacterial peritonitis (20). These findings seem to define a new role for mast cells: promotion of homeostasis by limiting the toxicity associated with an endogenous mediator. Another recent report describes a critical protective role of mast cells in skin graft rejection where tolerant allografts, which are sustained owing to the immunosuppressive effects of regulatory T cells, acquire a unique genetic signature dominated by the expression of mast-cell-gene products (10). The ability of mast cells to act as both pro- and anti-inflammatory regulators and their involvement in many inflammation-related pathological states makes them a potentially attractive target for pharmacological intervention shifting the balance of agents released towards the protective side.

Our data show that mast cells, when exposed to both short (6 hours) and prolonged (24 hours) hypoxia, release IL-4 and IL-13 while the expression of TNF, IFN-γ and IL-6 is not significantly induced (Fig. 1). Previous reports coming from both in vivo (2) and in vitro research (12) suggested that mast cells release predominantly proinflammatory agents. This research, however, was focused either on post-ischemic reperfusion or on desferroxamine-mimicked hypoxia, respectively. The latter method, while commonly used to simulate hypoxia has its limitations, primarily concerning selectiveness of pathways activated by chelating iron and possibly not reflecting all the branches of cellular response to hypoxic stress. One of the key sensors and signal transducers activated by hypoxia is HIF-1α transcription factor (11). In desferroxamine-stimulated mast cells the activation of HIF-1α and involvement of NF-kappaB in induction of proinflammatory cytokines has been reported (12). While the role of HIF-1α is beyond doubt, other signal transduction pathways might also be involved in initiating cellular responses to hypoxic environment.

It has been shown that both IL-4 and IL-13 gene expression is largely controlled by NFAT transcription factor family members (NFAT2 in particular) and that this regulation can be both stimulus- and cell type-specific (21-23). The members of this family of transcription factors are substrates of glycogen-synthase-kinase-3 (GSK-3) and, upon phosphorylation are exported from the nucleus and rendered transcriptionally inactive. GSK-3 in turn is a substrate for Akt kinase, which is activated in hypoxic conditions (24). NFAT is dephosphorylated and thus activated by calcineurin (reviewed in (25)) and this activation has been reported in cells subjected to hypoxic conditions (26). The above facts has prompted us to put forward a hypothesis whereby in hypoxic conditions calcineurin and/or Akt become activated and contribute to NFAT activation-the former by direct dephosphorylation of the transcription factor and the latter by phosphorylation and inhibition of GSK-3. The issue of signal transduction pathways leading to IL-4 expression induction was addressed by experiments presented in Fig. 2. The general stimulatory and inhibitory effects observed for SB216763 and FK506 respectively (Fig. 2B-C) fit well within the presented hypothesis. Additive stimulatory effect of hypoxia, GSK-3 inhibitor

and ionomycin or PMA (Fig. 2B) suggests that even in hypoxic conditions GSK-3 may not be completely inhibited and Akt inhibitory effect is enhanced by either pharmacological block of GSK-3 (SB216763) or increased phosphorylation of GSK-3 by PMA-stimulated PKC. The effect of ionomycin might be explained by concerted GSK-3 inhibition (hypoxia and SB216763) and calcineurin stimulation. The fact that in the presence of FK506 cells stimulated with hypoxia and/or ionomycin release relatively low amounts of IL-4 (Fig. 2C) may indicate that while calcineurin appears to be the major phosphatase responsible for IL-4 expression induction, there may be kinases other than GSK-3 phosphorylating and inactivating NFAT. Inhibition of MEK-1 kinase by PD98059 only had a marked inhibitory effect on IL-4 expression when cells were additionally stimulated with ionomycin (Fig. 2D) which is in agreement with reports stating that calcineurin and MEK-1 pathways are codependent in eliciting NFAT-mediated response (27). A noteworthy exception from this rule is a 24 hours hypoxia timepoint where treatment with PD98059 caused clear induction of IL-4 release. This might suggest that after prolonged hypoxic treatment MEK-1 exhibits inhibitory activity towards IL-4 expression. As far as HIF-1α is concerned, it appears that while treatments applied to the cells clearly influence this transcription factor levels, these levels do not correlate with IL-4 release, which, in the absence of HIF-1 binding site in IL-4 promoter, strongly suggests that while this cytokine expression is regulated by oxygen, this regulation is not mediated by HIF-1. Direct comparision of IL-4 and NFAT reporter construct expression levels revealed that the pattern of regulation of these two genes is very similar (Fig. 3). Of note is the fact that IL-4 promoter exhibited higher dynamics of stimulation with hypoxia, ionomycin and SB216763, but also higher sensitivity to inhibition with FK506. This might reflect the fact that IL-4 promoter, apart from NFAT-binding sites, contains other regulatory sequences e.g. AP-1-binding sites. Contrarily, in the reporter gene promoter there only are 3 copies of NFAT-binding sequence. Thus, possible hypoxia-induced activation of NFAT-independent signal transduction pathways might be reflected by IL-4 promoter activity and not by an element responsive to NFAT only. Interference with signal transduction pathways elements located upstream of NFAT by means of transfection of constitutively active Akt (myrAkt) confirmed that this kinase is involved in both IL-4 and NFAT reporter expression regulation (Fig. 4). The general pattern of stimulation and inhibition by ionomycin, SB216763 and FK506 was retained and the expression level of both analyzed genes was increased by transfection with myrAkt-expressing vector. In case of SEAP/myrAkt cotransfection the stimulatory effect seems to be more pronounced than in case of IL-4 expression which may reflect the fact that in SEAP/myrAkt-cotransfected cells every cell expressing the reporter also expresses the kinase which is not the case with IL-4 and thus IL-4 expression enhancement due to myrAkt activity is lower. Observation that SB216763 treatment and myrAkt transfection produce additive stimulatory effect may indicate that the myrAkt-induced inhibition of GSK-3 is not complete and pharmacological treatment provides further inhibition of this enzyme. Interestingly, in myrAkt-transfected and FK506-treated cells there is virtually no additional stimulation of IL-4 or SEAP due to hypoxia. This suggests that in the presence of saturating amount of Akt activity

(myrAkt transfection) any further increase of IL-4 or SEAP expression is mediated by the hypoxia-enhanced activity of calcineurin. The fact that in myrAkt-transfected and hypoxia-stimulated cells ionomycin treatment additionally induces expression of both genes remains in agreement with this model.

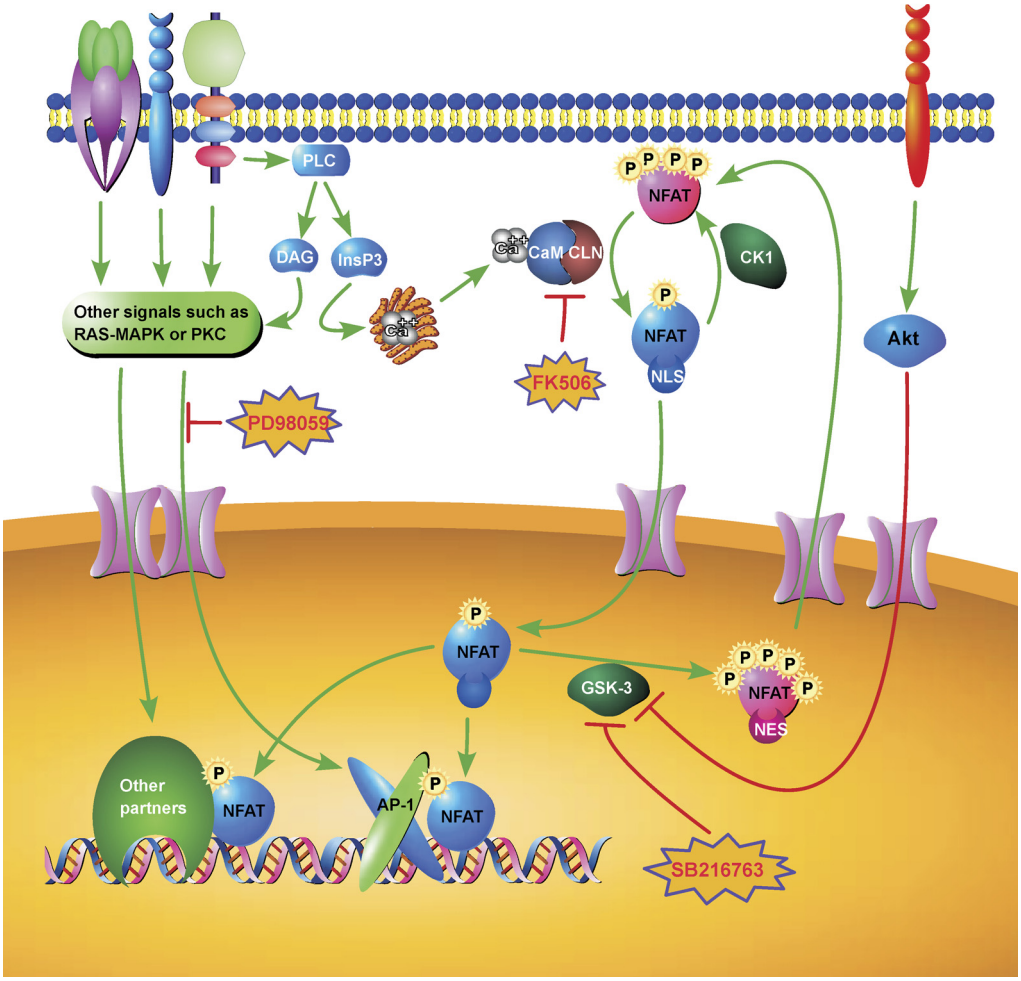

Figure 5 Scheme depicting the proposed model of IL-4 expression regulation in mast cells under hypoxic conditions

In this manuscript we present a novel observation that HMC-1 mast cells incubated in hypoxic conditions release anti-inflammatory cytokines IL-4 and IL-13 in the absence of pronounced induction of TNF, IFN-γ or IL-6. Analysis of signal transduction pathways leading to IL-4 expression induction revealed that this induction is mediated by both calcineurin and Akt/GSK-3 pathways and involves NFAT. Further research concerning signal transduction pathways regulating the profile of cytokines released by mast cells in hypoxic conditions is required in order to manage excessive inflammatory reactions occurring in many patophysiological conditions e.g. post-ischaemic tissue, keloids, arthritic joints or grafts undergoing rejection.

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Materials and Methods

Cell culture and transfection

The human mast cell line HMC-1 (13) was a generous gift from Dr. J. Butterfield, Mayo Clinic, Rochester, MN, USA. Cells were maintained in Iscove's medium supplemented with 10% heat-inactivated FBS and 2 mM glutamine. At the density of approximately $1.5x10^6$ cells/ml cells were diluted to $0.4x10^6$ cells/ml and used for the experiments. Cells were grown in 5% CO_2 at 37°C in fully humidified atmosphere. All media components were purchased from Sigma. For hypoxic treatment cells were incubated in 1% O2/5% CO2 fully humidified atmosphere in Ruskin Invivo2 Hypoxia Workstation 1000 (Biotrace International, UK)

For transfection 25-50 µg DNA was added to $2\text{-}10x10^6$ (HMC-1) 400µl culture medium and electroporated in Gene Pulser II (Biorad) at 250V (HMC-1), 950µF. Immediately following transfection cells were diluted to $5x10^5$ cells/ml in fresh medium.

Cytokine and SEAP assays

For cytokine release/inhibitor studies cells were incubated, where applicable, with 5µM FK506, 10 µM SB216763 or 50 µM PD98059, applied 60 minutes before stimulation. Subsequently, the cells were resuspended in fresh medium containing respective inhibitor and, where applicable, 1 µM ionomycin and/or 100 ng/ml phorbol 12-myristate 13-acetate (PMA). Cell suspension density was adjusted to $1x10^6$ cells/ml. Cytokine ELISA assays were performed using commercial kits (Sanquin, Amsterdam, The Netherlands) according to manufacturer's instructions. SEAP (SEcretable Alkaline Phosphatase) activity was assayed with Great EscAPe kit (Clontech) according to manufacturer's instruction.

Western Blot

For Western Blot analysis cell pellets were boiled in Laemmli buffer, resolved by SDS-PAGE and membranes were analyzed according to standard procedures. HIF-1α protein and β-actin were detected with monoclonal mouse antibodies (Becton Dickinson Transduction Laboratories and Santa Cruz Biotechnology, respectively).

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