Chapter 6

General discussion

TNF in rodent and human mast cells

Involvement of TNF in pathogenesis of inflammatory diseases

TNF is a pleiotropic cytokine that plays multiple roles in the immune system and beyond it. At least part of this complexity may be attributed to differential activities of its soluble and transmembrane forms (1) and its two receptors (2). The *in vivo* significance of these pathways is not fully clear and the role of TNF in pathogenesis of disease, especially autoimmune, remains unpredictable. There is evidence that deregulation of TNF *in vivo* may have pathogenic as well as protective effects. For example, deregulation of TNF expression in transgenic mice leads to the development of local or multi-organ inflammation (3, 4). In humans there is an established link between TNF and pathogenesis of rheumatoid arthritis (RA) (5), multiple sclerosis (MS) (6, 7) and inflammatory bowel disease (IBD) (8) to name just a few.

The majority of joint inflammatory disorders, typified by the manifestations of rheumatoid arthritis, are characterized by hyperproliferation of synovial tissue and infiltration of immune cells resulting in progressing erosion of cartilage and bone. A broad range of pro-inflammatory cytokines has been detected in RA biopsies establishing their link to the pathogenesis of joint inflammation (9). The importance of TNF has been demonstrated by amelioration of arthritic lesions in anti-TNF-treated animals (10, 11) and humans (9). Interestingly, in all transgenic and mutant mice developing arthritis the pathogenic activity of TNF is mediated by TNFRI (p55) (5, 8, 12). The role of TNFRII (p75) is controversial and seems to depend on the molecular form of TNF present in the joint environment. In mice over expressing wild-type TNF in the absence of TNFRII a more aggressive form of the disease is observed (8). In contrast, in animals overexpressing the transmembrane (uncleavable) form of TNF in the absence of TNFRII, onset of the disease is significantly delayed (12). This effect could be explained by higher affinity of the transmembrane form of TNF to TNFRII and the modulation of overall TNF response by this receptor (2). In view of the fact that pathogenic effects of the disease are caused by synoviocytes that proliferate in response to TNF (13) and produce matrix-degrading enzymes and chemokines (14, 15), tight regulation of the amount and molecular form of TNF present in the joint environment is crucial for control of the disease. While specific control of TNF production *in situ* is not feasible as of now, biologicals neutralizing TNF have been developed and used in therapy with some success (16).

A critical role for TNF in pathogenesis of demyelinating inflammatory disease of CNS has been suggested in several studies of multiple sclerosis (MS) in humans and experimental autoimmune encephalomyelitis (EAE) that is an established murine model of MS. TNF is overproduced by cells infiltrating CNS (17) and detectable at elevated levels in plasma and cerebrospinal fluid of MS patients (18). Tissue-specific expression of TNF in CNS indicates the potential of this cytokine to induce demyelination (6, 7). Removal of the mature lymphocytic population (RAG-1 knockout) did not alter the pattern of primary demyelination indicating that adaptive arm of immune response is not necessary for TNF-induced pathology in this model (19). However, it has to be noted that in the EAE model, the disease could be induced

by adaptive transfer of myelin-specific T cells (20, 21). Studies in TNF-deficient mice indicated that in a myelin oligodendrocyte (MOG) peptide induced EAE that in the absence of TNF the onset of clinical symptoms is significantly delayed (22). This suggests that pro-inflammatory activities of TNF are required for normal induction of the disease. On the other hand, the fact that even in TNF-deficient mice severe EAE eventually develops (7, 22) demonstrates that other cytokines may compensate for TNF in demyelination.

Vasculitis is a clinicopathologic process characterized by inflammation and necrosis of blood vessels that leads to vessel occlusion and tissue ischemia. Vasculitis may occur as a primary process or as a component of other underlying diseases. In some human vasculitic syndromes elevated levels of circulating immune complexes (IC) and deposits of complement and immunoglobulins are observed. The mechanism of inflammation induction by IC and mast cells involvement in these syndromes was analyzed (23). TNF was previously established as crucial for the development of skin vasculitis using TNF/LTA-deficient mice (24). More in-depth analysis revealed, that the source of the pool of TNF necessary for vasculitic reaction induction are mast cells and their stimulation by IC is mediated predominantly by FcγRIII (23).

Examples discussed above document the involvement of TNF (also mast cell derived-TNF) in autoimmune inflammatory diseases. In addition, deregulated TNF expression has been implicated in pathogenesis of a number of other chronic and acute inflammatory diseases, such as septic shock, meningococcemia, adult-respiratory distress syndrome, otitis media, hepatitis B and C infection, Reyes' syndrome, and cerebral malaria, among others. Discussing all of these in detail is beyond the scope of this thesis. The important conclusion is that TNF is potentially a very attractive therapeutic target. The appreciation of this fact is reflected by the presence on the market of several anti-TNF medications. They are, however, not free of side effects and thus the invention of a way of delivering the bioactive compound at the exact place and time would be of great therapeutic potential in preventing the pathological roles of TNF.

Biological importance of mast cell-derived TNF

Considering the range of biological activities exhibited by this cytokine, the importance of tight regulation of its expression is compelling. When expression is defined as the presence of bioactive TNF in the environment (both local and systemic), all regulatory stages have to be considered, beginning with promoter activity, mRNA stability, translation rate, endoplasmatic reticulum (ER) translocation, posttranslational modifications (including proteolytic processing), intracellular trafficking and release. The scope of this thesis covers posttranslational modifications and intracellular trafficking of this cytokine. Mast cells (MC) were chosen as a model system since mast cell-derived TNF has been shown to form a functionally distinct pool, that is responsible for initiation of the inflammatory response (25-27). The importance of mast cell-derived TNF was further confirmed in animal model of inflammatory state, a reverse Arthus reaction (28). This applies in particular to preformed TNF stored in mast cell granules which can be released within minutes from cell activation,

influencing the subsequent course of immune response (27). Since mast cells are also capable of producing and releasing additional amounts of TNF with the use of constitutive secretory pathway (29, 30) there has to exist a mechanism that is regulating the amount of TNF directed for storage in the granules as opposed to direct release. We aimed at elucidating the nature of this molecular switch and defining features present in TNF molecule that take part in this regulation.

TNF processing and release were extensively studied in macrophages (31). These cells however do not store this cytokine and therefore, considering the functional importance of granular TNF, the question of choosing appropriate research model is of particular importance. Several rodent mast cell models including both *in vitro* differentiated mast cells (e.g. bone marrow derived mast cells - BMMC (32)) and cell lines such as C57 (33) and RBL-2H3 (34) are widely used in mast cell research. Human models, except from isolated mature MCs, comprise cord blood derived mast cells (CBMC), cell lines (e.g. HMC-1 (35) and LAD-2 (36)). Therefore, we investigated the process of TNF intracellular trafficking in rat RBL-2H3 cells that are adherent and thus particularly suitable for microscopic analysis (**chapter 2**). We also extended our research to human HMC-1 and LAD-2 cell lines (**chapter 3**). The approach based on expression of GFP-fused TNF allowed for imaging of the recombinant protein delivery to mast cell granules in living cells. The results obtained raise several interesting points for discussion.

TNF intracellular trafficking in mast cells

We have visualized intracellular trafficking of TNF to mast cell granules in both rodent and human mast cells. These granules are organelles related to lysosomes (37, 38), but unlike lysosomes, they are rapidly released upon appropriate stimulation of cells. Several mechanisms directing proteins to lysosomes have been described (39-41); they are based on either amino acid sequence or glycosylation of the transported protein. The motifs of type I transmembrane proteins within the cytoplasmic tails that are responsible for lysosomal trafficking (and a few known exceptions of type II) have been characterized (reviewed in (42)). These motifs have been demonstrated to direct ectopically expressed proteins to NK cell granules (43, 44). TNF however is a type II transmembrane protein (N-terminus on the cytoplasmic side) with no apparent sorting motifs in its cytoplasmic tail. Proteins lacking amino acid sorting motifs may still be delivered to lysosomes in a sorting process involving recognition of a mannose-6-phosphate moiety (present in a glycan chain of a protein being sorted) by a mannose-6-phosphate receptor (45). Previously, it has been reported that a MPR-dependent pathway is engaged in sorting of cathepsin D into cytoplasmic granules in mast cells (40). Interestingly, computer-assisted sequence analysis indicates a potential glycosylation site in both rodent and human TNF. However, the actual presence of this posttranslational modification in TNF molecule and its significance for the granular sorting has not been verified.

In case of rodent mast cell-associated TNF there is a combination of two mechanisms unusual for a cytokine: storage of preformed protein and a role of N-glycan in its intracellular trafficking (**chapter 2**). The implications of both may be

important for the regulation of the amount and the form of TNF available in the cell exterior. Posttranslational modifications such as glycosylation allow for functional modifications of protein fate and activities: its target compartment (46, 47) or receptor-ligand interactions (48). The dependence of a lysosomal enzyme tripeptidyl-peptidase folding, trafficking and stability on N-linked glycosylation has been reported (47). It has been postulated, as a mechanism alternative to MPR-based sorting, that N-glycans might provide structural support and prevent aggregation of the proteins bearing them, rather than interact directly with sorting machinery (46). Based on the data obtained we were not able to define unequivocally which mechanism is responsible for the delivery of TNF to rodent mast cell granules. Also, the question whether TNF in its transmembrane form is displayed on the outer membrane and later on endocytosed to the granules or retrieved from *trans*-Golgi network (TGN) for direct sorting to the granules remains unresolved.

A point of note is an unusual phenotype of the cells expressing nonglycosylable mutant TNF. As seen in **chapter 2, fig. 6**, in cells expressing 1-235N86S-mTNF-EYFP, the majority of fluorescence is found in the cytoplasm, while there is small but observable fraction still reaching granules. This might indicate, that ER-entry of this protein is impaired thus precluding any further sorting and attributing to N86 residue a role extending beyond glycosylation; alternatively, the mutated protein might not be stably retained in its target compartment and translocate to cytoplasm. The presence of the significantly decreased granular pool of N86S-mTNF may reflect the glycan-independent sorting of this cytokine. Such compensatory mechanisms have been described in MPR knockout mice (49).

Interestingly, human TNF does not possess a N-linked glycosylation motif. Instead computer-based prediction indicates that in the corresponding area (residue T83) there is a potential O-glycosylation site (http://www.cbs.dtu.dk/services/). Such modifications have previously been reported as important for trafficking of proteins (50, 51). Human TNF however does not seem to be glycosylated (**chapter 3**) which, in conjunction with the fact that O-linked glycosylation of TNF in B cell line has been reported (52), stresses the existence of cell type-specific modification patterns and trafficking mechanisms. Thus, it is not surprising that trafficking of a protein for storage in a unique compartment such as mast cell secretory granule may demand specialized solutions. We show that at least a fraction of human TNF is exposed on the outer membrane and reendocytosed on its way to granules. Similar mechanism of endocytosis of uncleaved TNF has been demonstrated in macrophages (53) although this obviously would not lead to granular accumulation of the cytokine.

Proposed model and implications

The discovery of such route allows for proposing a model, whereby TNF is constitutively transported to the outer membrane and reendocytosed to the granules with only a minor fraction undergoing proteolytic processing and release (Fig. 1). Upon stimulation granular stores are released and, in parallel, TNF sheddase (TACE) is induced in secretory pathway (54, 55). TNF is processed on its way to the outer membrane and released upon exposure to the exterior. Any remaining

transmembrane molecules are then processed by other metalloproteinases present in inflamed tissue, such as MMP-7 (56, 57). When homeostasis is restored, granular stores are refilled and constitutive release of TNF is stopped. Proposed TNF trafficking models are presented in Fig. 1A (rodent) and 1B (human).

Cross-species analysis of TNF protein sequences reveals an interesting feature (Fig.2). Overall the sequence is very well conserved with the exception of a region spanning approximately 35 amino acid residues located near the membrane on lumenal side. Both N- and O- linked glycosylation motifs found in rodent and primate TNFs, respectively, are located in this area. Interestingly, N-linked glycosylation site that is important for TNF trafficking in rodents is absent in other taxons. This finding is yet another indication that conclusions drawn from research conducted in rodent models should be applied to humans cautiously.

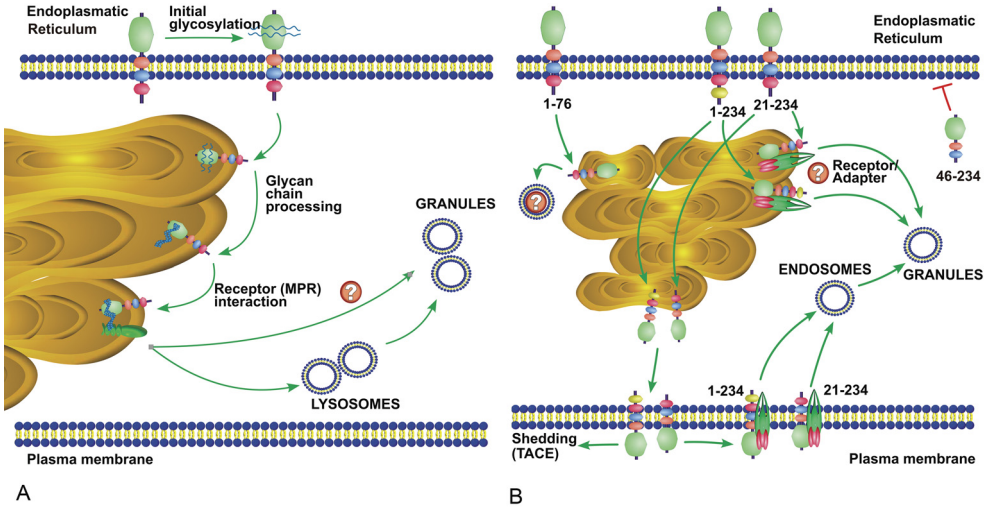


Figure 1 Proposed models of TNF trafficking to mast cell granules in rodents (A) and human (B)

Potential clinical implications of defining TNF trafficking route and the motifs responsible for this trafficking include constructing biologicals that might be delivered to and stored in mast cell granules. Such bioactive proteins would be released in exactly the same time and space the protein serving as a trafficking model, e.g. TNF. In this way response of mast cells could be modulated *in situ*, e.g their pathogenic anaphylactic or pro-inflammatory activity could be limited. Similar approach has been applied for delivery of soluble TNF receptor to NK cell granules (44). Such level of specificity is unlikely to be achieved by the currently available pharmacological agents, given the often disputable specificity of such agents and the redundancy of the functions to be influenced both on the level of single cell regulation and immune system. This makes it also difficult to design pharmacological inhibitors of TNF routing in mast cells to inhibit their pro-inflammatory role. The case of TNF is particularly encouraging since several anti-TNF biologicals are already available in clinics and the role of mast cell-derived TNF has been established in many disorders involving autoimmune and chronic inflammation components (23), reviewed in (58).

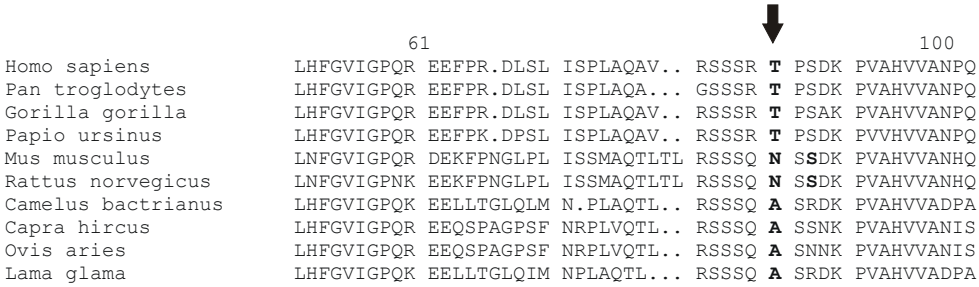


Figure 2 Multiple alignment of TNF sequences representing Primates, Rodentia and Artiodactyla, showing low conservation of potentially glycosylated residues (indicated by an arrow)

Regulation of cytokine expression in mast cells exposed to stress conditions

Immunomodulatory potential of IL-4

In many cases a response of the immune system may be elicited by non-immune triggers such as irradiation, hypoxia, oxidative stress or chemicals. Since the immunomodulatory role of mast cells in limitation of excessive inflammation is increasingly appreciated, we examined mast cells behaviour in various stress conditions and their potential contribution to the development of immune response. Mast cells are capable of releasing mediators that, at least potentially, can exhibit anti-inflammatory activity, such as TGF-β (59), IL-4 (60), IL-10 (61) and histamine (62). We have chosen IL-4 as a subject of more in-depth analysis for several reasons. Firstly, it is one of the central cytokines skewing the immune response towards the Th2 type. Additionally it causes antibody class switching towards IgE, which are a primary class recognized by mast cells. Moreover, there is ample literature concerning immunoregulatory functions of IL-4 (discussed below); in some cases also mast cell-derived IL-4. Finally, IL-4 promoter regulation has been researched extensively, leading to conclusions pointing out cell type specific mechanisms ruling its activity (63).

Anti-inflammatory effects of IL-4 were first demonstrated through its capacity to suppress Th1 responses and protective immunity against intracellular pathogens. In murine model of *Leishmania major* infection, complete neutralization of IL-4 shifts the immune response balance from Th2 to Th1 and thus provides the immunity against the pathogen (64). The understanding of IL-4 functions was complicated by the reports that Th2 cells may cause autoimmune diseases and directly mediate tissue destruction when transferred to immunodeficient animals (65, 66). These data indicated that IL-4 anti-inflammatory activity depends on several co-factors that define the final outcome of immune responses. In leishmaniasis model Th1, but not Th2 cells, elicited delayed-type hypersensitivity reactions (DTHRs) that provided protective immunity from the pathogen. The same Th1-mediated DTHRs may lead to autoimmune inflammatory diseases in which case antigen-specific shifting of immunity from Th1 to Th2 might be therapeutically beneficial and possibly devoid of side effects associated with current immunosuppressive treatments. This hypothesis was tested in a model of experimental autoimmune encephalomyelitis (EAE) which

is a rodent model of multiple sclerosis (MS). It was demonstrated that antigen-specific Th2 cells did not induce EAE and that a Th2-inducing treatment prevented the development of EAE (67, 68). Among many models of inflammatory diseases contact hypersensitivity responses of the skin (CHS) to exogenous haptens in sensitized animals is of special interest. CHS may be more easily and rapidly elicited and monitored than organ-specific autoinflammatory diseases. In these models it was demonstrated that even in an ongoing CHS skin inflammation the IFN-γ/IL-4 ratio may be reduced by IL-4 administration (69). Additionally, such result may also be achieved by adoptive transfer of antigen-specific Th2 cells together with their respective antigen although this effect is observable only after repeated CHS challenge; this delayed effect indicates that Th2 cells may interfere with Th1 DTHRs indirectly by deviating naïve Th cells or even Th1 cells towards Th2 phenotype.

The question of applicability of these findings to treatment of autoimmune inflammatory diseases in humans was addressed in a study evaluating therapeutic effects of IL-4 in psoriasis, a Th1-associated disease of skin and joints (70). Six weeks of continuous IL-4 treatment strongly reduced clinical score in patients and decreased IL-8, IFN-γ and IL-19 expression in skin biopsies. After the treatment Th cells were still detectable in psoriatic skin although they no longer expressed the Th1-marker chemokine receptor CCR5; additionally IL-4 became detectable in skin samples. Thus IL-4 decreased prototypic pro-inflammatory Th1 cytokines, induced IL-4 and improved human autoimmune inflammatory disease.

Experiments with IL-4-deficient mice and IL-4 neutralizing antibodies revealed that, under certain conditions IL-4 may paradoxically promote Th1 differentiation and DTHRs (71-73). For instance IL-4 deficient mice do not develop severe EAE or Th1 responses when infected with certain strains of *L. major* or *C. albicans* (72, 74). This phenomenon was only understood with better description of dendritic cells (DC) maturation process showing that there exist distinct DC phenotypes that promote either Th1 or Th2 differentiation. It was demonstrated that under the influence of IL-4 DCs acquire DC1 phenotype that produces IL-12 (75, 76). The role of IL-4 in DC1 development and at different stages of immune response was investigated in a model of *Leishmania* infection of BALB/c that are Th2-prone and thus *Leishmania*-susceptible (77). Mice given IL-4 during the first 6 hours of infection developed DC1 and Th1 populations and protective immunity. Importantly, when IL-4 administration was extended to the period of Th-cell activation, Th2 phenotype was induced and protective immunity abrogated.

Together these analyses demonstrate, that the same cytokine may exert opposing effects on immune response depending on primary target cells of this cytokine and time and amount of cytokine produced.

Mast cell-derived IL-4 and regulation of its expression

IL-4 is produced by a relatively small subset of immune cells. Apart from Th2 cells, some CD8+, NK1+ and γδT cells produce IL-4 upon antigen activation (78, 79). Cross-linking of FcεRI in mast cells, basophils and eosinophils also leads to a

significant release of IL-4 (80-82). In contrast to T cells, mast cells do not require differentiation in a specialized tissue environment to acquire the capability of releasing IL-4. They differentiate significantly in bone marrow under the influence of SCF and IL-3 (83, 84) and complete their maturation at their tissue of residence (85). Murine mast cells, unlike T cells, do not require prior antigen exposure for IL-4 production. Upon FcεRI cross-linking they initiate IL-4 mRNA transcription within 15 minutes (86). In case of primary human mast cells there is an ongoing discussion as to whether they are capable of releasing IL-4 (87, 88). Human cell lines, however, produce and release IL-4, indicating that learning more on IL-4 biology in human mast cells may lead to defining the regulatory mechanisms allowing for the regulation of such release. Although generally recognized as tissue fixed cells, mast cells migration to secondary lymphoid organs was reported (89, 90). Their ability to release IL-4 in this environment makes them perfect candidates for IL-4 source for freshly primed naïve T cells skewing their differentiation towards Th2 phenotype. Additionally IL-4 may enhance B cells proliferation in germinal centres and, in conjunction with CD40L present on mast cells, induce isotype switching to IgE and IgG (91).

Initial studies concerning IL-4 promoter regulation in murine mast cells were conducted on an 800 bp fragment directly preceding transcription initiation site (TIS) (92). Deletion analysis revealed that the most important region starts at -87 bp (relative to TIS) and the region -87 to -70 contains NFAT-binding site termed P1, previously defined as important for IL-4 expression in T cells (93). A 300 bp region upstream of TIS contains 5 NFAT-binding sequences termed P0-P4. Several features of the factor that binds to P1 in mast cells indicate that it is unlike the NFAT found in T cells (63). Schematic representation of murine and human IL-4 *cis*- and *trans*-acting regulatory elements is presented in Fig. 3; binding sites for several important transcription factors are marked. The most important cell type-specific features are:

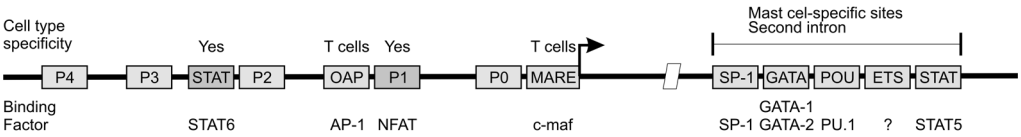
1. STAT binding site that is not required for IL-4 expression in mast cells (94), but is an obligatory element in Th2 cells development (95, 96)
2. c-maf binding site (MARE) that is specifically required by Th2 cells, but not for mast cells for IL-4 expression (97)
3. P1 NFAT binding site (see below).

The analysis of NFATs that bind to -87 - -70 promoter region indicated that the mast cell specific factor, as opposed to T cells, does not bind AP-1 factor and has a molecular weight of approximately 41kD (80-100 kD in T cells) (98).

Mast cell cDNA library screening revealed 2 predominant forms of NFAT2 (NFATc) designated α and β. NFAT2.α is expressed in spleen and its level is strictly dependent on activation in both T cells and mast cells. NFAT2.β is expressed at very low levels in all cells, but its mRNA level is raised only in mast cells activated via FcεRI crosslinking or calcium ionophore. An additional, mast-cell specific enhancer is located in intronic sequence (intron II) (86) and overall transcriptional activity of the Th2 gene cluster

(IL-4, 5 and 13) seems to be regulated by cell type-specific balance between STAT3 and Ikaros factors that recruit histone acetylotransferase or histone deacetylase, respectively, and open or close chromatin structure (99).

A



B

Figure 3 (A) Alignment of the sequences of 300 bp human and murine IL-4 promoter (B) Binding sites of the important transcription factors in IL-4 promoter. P0-P4 are NFAT-binding sites, OAP and MARE are T cell specific sites, P1 and STAT sites bind different transcription factor isoforms in mast cells and T cells; intron-located regulatory element is mast cell-specific, ETS element is mouse-specific. Adapted from (63)

Overall, although the biological importance of some of the cell type-specific mechanisms in IL-4 gene expression regulation are not clear it is possible they are responsible for the precise spatial and temporal pattern of IL-4 expression, whose importance was discussed in a previous section.

Mast cells in hypoxic stress

In many pathological situations mast cells are exposed to hypoxic condition. The model that provided most complete picture of mast cell behaviour probably is post-ischaemic reperfusion of myocardium (100, 101). Mast cells contribution to inflammation developing in such tissue has been established and attributed, at least in part, to prestored TNF that is released upon reperfusion (100). Mast cell stabilizing agents have been shown to limit post-ischaemic inflammation-related injury (102) although it has to be noted that they only influence the early, degranulation-related events. As discussed above, at these early stages of immune response mast cells play, mainly through TNF and lipid mediators, a key role in the induction of inflammation and so therapeutic value of such drugs should not be overlooked. However, there is a separate, degranulation independent, phase of cytokine production that involves *de novo* synthesis and release of these mediators. This process is predominantly regulated at the level of promoter activity. We decided to investigate the capability of mast cells to release IL-4 in the longer (6 to 24 hours) term following hypoxic stimulation. Such activity might augment limitation and resolution of the inflammatory process once it is no longer necessary. It has been reported that although mast cells take part in the initiation of inflammation by TNF release (100), they do not contribute to the late peak of TNF that follows (103). This is consistent with our observation that HMC-1 human mast cells when subjected

to hypoxia do not release significantly higher amounts of TNF when compared to cells incubated in normoxic conditions (**chapter 4**). The lack of induction is also observed in case of IL-6 and IFN-γ. We, however, detected increased amounts of IL-4 and IL-13 released after 6 and 24 hours of incubation in hypoxic conditions. This hypoxia-induced stimulation was further enhanced by inhibition of glycogen synthase 3 (GSK-3) or overexpression of constitutively active Akt kinase. On the other hand, hypoxia-related increase in IL-4 production was effectively inhibited by the calcineurin (CaN) inhibitor FK506. These two pathways meet at the level of NFAT that is a substrate of both GSK-3 and CaN. The results obtained thus suggested that NFAT might be the transcription factor responsible for hypoxia-induced increase in IL-4 production. We also showed, by using a reporter construct expressing secretable alkaline phosphatase (SEAP) under control of NFAT-responsive element, that NFAT was induced by hypoxia, GSK-3 inhibition and active Akt overexpression. Although general patterns of IL-4 promoter and NFAT reporter were similar, 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. It is also noteworthy that under some of the experimental conditions tested, HIF-1α transcription factor was significantly induced, but the level of this induction did not correlate with the levels of IL-4 release. A proposed model of IL-4 transcriptional activity in hypoxic conditions is presented in Fig. 4

The notion that mast cells subjected to hypoxia release a brief, degranulation-related pulse of TNF (100) followed by a switch to IL-4 production might suggest that they initiate an inflammatory response to alert the immune system, but ultimately contribute to its resolution by deviating Th cells towards Th2 phenotype and disarming macrophages.

A commonly used replacement for hypoxic conditions is an iron-chelating agent desferroxamine (DFX). It has been reported that in mast cells treated with DFX, NFkB pathway is activated and pro-inflammatory cytokines are released (104) and that this effect is mediated through HIF-1α transcription factor. The lack of NFAT-dependent genes in this system may be a result of iron-chelating activity of DFX that may render iron-containing calcineurin inactive. The decrease of IL-4 and IgE production following DFX administration to mice has been reported (105). In the setup employing actual hypoxia activation of HIF-1α did not correlate with IL-4 expression increase (**chapter 4**). This indicates that effect of hypoxia may extend beyond HIF-1α signaling and that chemicals such as DFX may not reproduce these effects accurately due to several reasons.

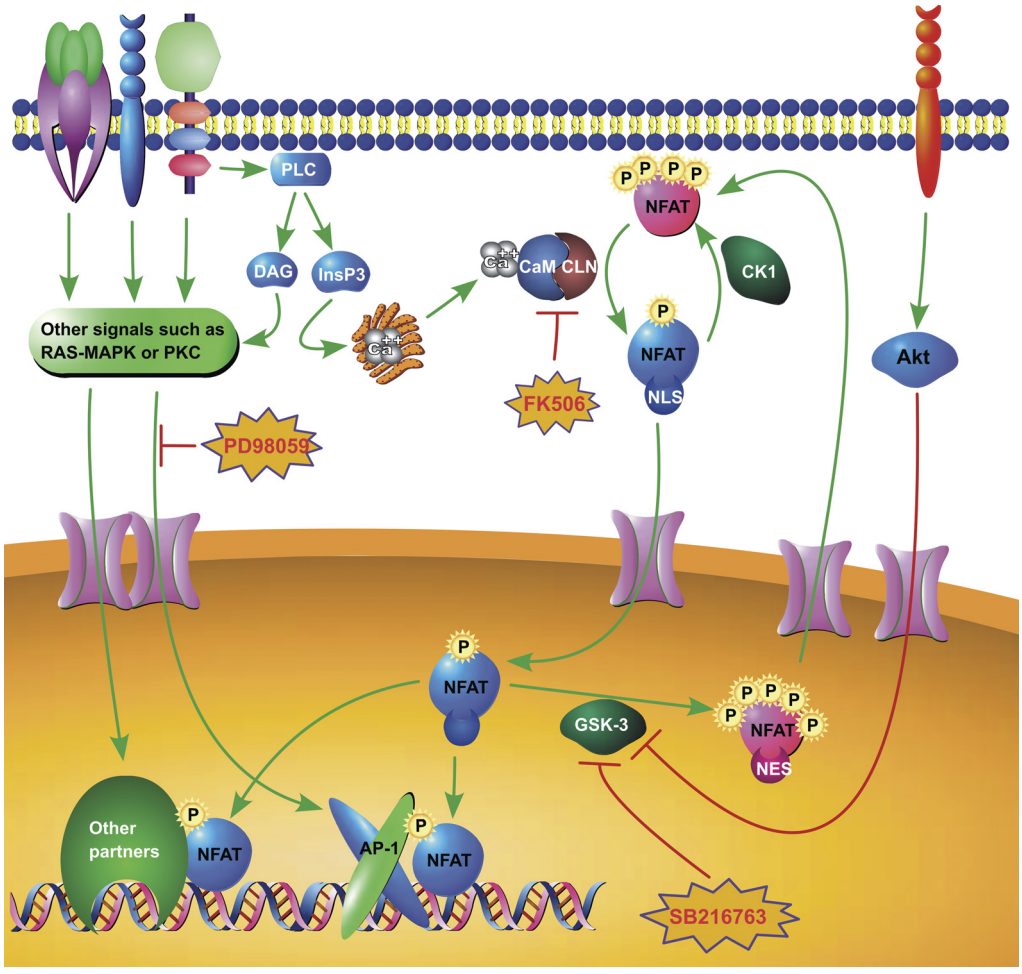


Figure 4 A proposed model of IL-4 transcriptional activity regulation under hypoxia and Hg++ exposure

IL-4 induction in mast cells by mercuric ions

Treatment with mercuric ions has long been used as a model system for systemic autoimmune inflammation (106) including arthritis, vasculitis and glomerulonephritis (107-109). Such treatment causes polyclonal B cells activation (110), enhanced IgE and IL-4 production (111, 112). The involvement of mast cells and mast cell-derived IL-4 in full elicitation of these responses has been suggested (113, 114). Immune response accountable for this systemic inflammation is predominantly of the Th2 type, although its initiation critically depends on IFN-γ (115). Shifting the balance towards Th1 by administration of IL-12 and anti-IL-4 antibodies reduced some disease manifestations, such as IgE level or renal vessel wall immune complexes deposition, while other remained unchanged (115). The established role of IL-4 in elicitation of Hg-induced autoimmune inflammation prompted us to analyse the mechanisms responsible for Hg-dependent IL-4 induction.

Involvement of reactive oxygen species in Hg-induced IL-4 induction has been implicated as augmenting the response (116, 117). Superoxide, however, was reported to inhibit calcineurin activity (118). Therefore we attempted dissecting the molecular mechanism underlying IL-4 induction by Hg (**chapter 5**). We have shown that in murine C57.1 cells (33) Hg activates IL-4 production and this effect is both dose-dependent and additive to IgE/antigen stimulation. In a chloramphenicol acetyltransferase (CAT) reporter system we demonstrated that mutation of P1 NFAT binding site in IL-4 promoter abrogates production of this cytokine in response, not only to Hg, but also to much more powerful combination of stimuli such as PMA and A23187 calcium ionophore. Similar, although not as potent, this effect was also obtained by treatment of cells with the calcineurin inhibitor FK506. This pointed to calcineurin/NFAT system as responsible for Hg-induced enhancement of IL-4 production. This was further confirmed by the similar response pattern of a reporter gene (SEAP) driven by NFAT-responsive element. Additionally, transcriptional activity of this promoter was effectively blocked by coexpression of dominant-negative form of calcineurin. To assess whether Hg effect on calcineurin activity was direct, we assayed calcineurin activity *in vitro* in the presence of mercuric ions. We observed dose-response positive effect of Hg on calcineurin activity. These results are seemingly contradicting the reports stating that IL-4 expression in such conditions is reactive oxygen species (ROS)-dependent (116, 117), but the increase of calcineurin activity in an *in vitro* assay in the presence of Hg may represent a ROS-independent component.

In conclusion, our data support the hypothesis that mercuric ions directly stimulate calcineurin activity that in turn activates NFAT and induces IL-4 transcription. This molecular mechanism, possibly in cooperation with other signal transduction pathways (119), may be involved in the initiation of Th2 immune responses induced in humans (120) and experimental animals exposed to mercuric compounds (112).

General conclusions

Mast cells, as discussed above, are cells that in some situations are regulated by unique signal transduction pathways. They also are tissue resident cells that places them close to the interface of the organism and the exterior. This, in conjunction with the fact that mast cells store large amounts of highly bioactive compounds in their granules makes them perfect candidates for initiating immune or non-immune response in case of microbial infection, parasite infestation or an exposure to biophysical stimulus such as UV, hypoxia or environmental pollutions (exemplified by mercuric compounds). It has been demonstrated in several setups that mast cells have the potential of setting the course of the immune response they initiate. Considering the increasing body of evidence that these cell may act in both pro- and anti-inflammatory manner, mast cells should be regarded an important point in immunoregulation and target in therapy of inflammatory diseases and immunodeficiencies.

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