Regulation of cytokine expression in mast cells:

pro- and antiinflammatory potential

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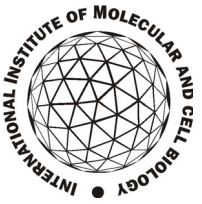

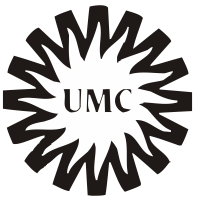

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Regulation of cytokine expression in mast cells:

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Regulatie van cytokine expressie in mestcellen: pro- en anti-inflammatoir potentieel

(met een samenvatting in het Nederlands)

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Success does not consist in never making mistakes but in never making the same one a second time

George Bernard Shaw

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Chapter 1

General introduction

The inflammatory process

Immune responses are often accompanied by other cellular, physiological and biochemical processes. Many of these are evolutionarily primitive processes that can be induced with or without immune activation. One example is acute injury that induces response in local vasculature resulting in redness, warmth and swelling. Many types of host immune cells can migrate into the affected area forming what is microscopically visible as an infiltrate. Tissue reactions may include activation of complement or kinin cascades; systemic manifestations may include fever, malaise or aching. If injury is severe, fibroblasts and endothelial cells may proliferate locally and form a scar.

The entire host reaction to pathophysiological stimuli, including bacterial infections, parasite infestation or exposure to irritant environmental substances and conditions, is named inflammation. Many distinct inflammatory pathways exist, each of them involving a different set of cells and mediators. Individual events in course of the inflammatory process are controlled by cytokines and other small regulatory molecules which in this context are named inflammatory mediators. A given mediator may exert its effect directly or indirectly by regulating the activity of other mediators. This complicated interplay of the network mediators gives rise to an integrated response. The outcome of an inflammatory process depends on many factors, including the characteristics of the host and the nature of the stimulus. This outcome may be regarded as beneficial, detrimental or both. Although the mechanisms constituting inflammation evolved to eliminate injurious substances or limit their spreading through the organism, they may be the cause of excessive damage in inflamed tissue when injury is severe or when they become misregulated. In the course of sepsis, it is not the inducing pathogens, but the excessive immune inflammatory responses that are responsible for severe health problems. Allergic reactions are examples of excessive immune inflammatory response against innocuous substances such as dust, pollen, food or drugs. The same mechanisms of immunological inflammation are partly responsible for pathogenic consequences of autoimmune diseases.

TNF ligands superfamily

TNF is a prototype member of a group of proteins known as TNF ligand superfamily. This group is comprised of at least 18 type II (lumenal N-terminus) transmembrane proteins, including such well-known members as TNF (formerly known as cachectin), LT-α (lymphotoxin-α), Fas ligand (FasL) and CD40 ligand, as well as increasing number of newly discovered members, including APRIL (a proliferation-inducing ligand), TRAIL (TNF-related apoptosis inducing ligand), TWEAK (TNF-like and weak inducer of apoptosis), RANKL (RANK ligand) and BLyS (B lymphocyte stimulator). The biological activities of TNF were first described in the 1960s and 70s with the identification of a macrophage- and lymphocyte-derived factor that caused hemorrhagic necrosis in solid tumors (1). Since then a plethora of biological functions of TNF have been identified, many of them extending beyond the immune system. The functions of

the members of TNF superfamily often overlap, but they also display considerable diversity. In fact many members of the family are not so much involved in immune response, but rather in development and organogenesis (2-5). Not surprisingly, also the receptors for numerous TNF superfamily ligands comprise a superfamily of related genes with distinct and common structure and signal transduction pathways (summarized in Table 1).

TNF SUPERFAMILY			**TNF RECEPTOR SUPERFAMILY**		
Systematic Name	Alternative Names	Cells	Systematic name	Alternative names	Cells
TNFSF1	LT, LTα, TNFB	NK, T, B	TNFRSF1A	TNF-R, TNFAR, TNFR60, TNF-R-I, CD120a, TNF-R55, TNFR1	All cells
			TNFRSF1B	TNFBR, TNFR80, TNF-R75, TNF-R-II, p75, CD120b, TNFR2	E, Immune cells
TNFSF2	TNF, TNFA, DIF	NK, T, B	TNFRSF1A		
			TNFRSF1B		
TNFSF3	LTβ, TNFC, p33	NK, T, B, DC, M	TNFRSF3	TNFCR, TNFR-RP, TNFR2-RP, TNF-R-III, LTBR	NK, T CD4/8+
TNFSF4	OX-40L, gp34, CD252,TXGP1	T, B	TNFRSF4	ACT35, OX40, CD134, TXGP1L	T
TNFSF5	CD40L, TRAP, gp39, hCD40L, CD154, HIGM1, IMD3	T, B	TNFRSF5	p50, Bp50, CD40	B
TNFSF6	FasL, CD178, APT1LG1, FASLG	T*	TNFRSF6	CD95, APO-1, FAS1, APT1, FAS	Nucl. Cells
			TNFRSF6B	DcR3, DCR3, TR6, M68	Lung, colon
TNFSF7	CD70, CD27LG, CD27L	NK, T, B	TNFRSF7	S152, Tp55, CD27	
TNFSF8	CD153, CD30LG	T, Mo	TNFRSF8	CD30, D1S166E, Kl-1	
TNFSF9	4-1BB-L	B, DC, M	TNFRSF9	CD137, 4-1BB, ILA	T*, Mo, NK
TNFSF10	TRAIL, Apo-2L, TL2, CD253	Lc, DC	TNFRSF10A	DR4, Apo2, TRAILR-1, CD261	Most cells
			TNFRSF10B	DR5, KILLER, TRICK2A, TRAIL-R2, TRICKB, CD262	Most cells
			TNFRSF10C	DcR1, TRAILR3, LIT, TRID, CD263	Most cells
			TNFRSF10D	DcR2, TRUNDD, TRAILR4, CD264	Most cells
TNFSF11	TRANCE, RANKL, OPGL, ODF, CD254	T*, Ob	TNFRSF11A	RANK, CD265	preOc
			TNFRSF11B	OCIF, TR1, OPG	preOc, E
TNFSF12	TWEAK, DR3LG, APO3L	Mo	TNFRSF12	DR3, TRAMP, WSL-1, LARD, WSL-LR, DDR3, TR3, APO-3	T*
			TNFRSF12A	FN14, TweakR, CD266	E, F

TNFSF13	APRIL, CD256	SLO	TNFRSF13B	TACI, CD267	T, B
TNFS-F13B	BAFF, THANK, BLYS, TALL-1, TALL1, CD257	T, DC, M, Mo	TNFRSF13C	BAFFR, CD268	B
TNFSF14	LIGHT, LTg, HVEM-L, CD258	T, DC, Mo, Gr	TNFRSF14	HVEM, ATAR, TR2, LIGHTR, HVEA	T, B, Mo
TNFSF15	TL1, VEGI	E			
			TNFRSF16	NGFR, CD271	
			TNFRSF17	BCM, CD269, BCMA	
TNFSF18	AITRL, TL6, hGITRL	E, T*	TNFRSF18	AITR, GITR	E, T*
			TNFRSF19	TAJ-alpha, TROY, TAJ, TRADE	Ep, ES, Hair, Brain
			TNFRSF19L	RELT	LyT
			TNFRSF21	DR6	T
			TNFRSF27	XEDAR, EDA-A2R, EDAA2R, EDA2R	Ec

Table 1 Members of TNF ligand and receptor superfamilies and their occurrence. T-T cells (*-activated), B-B cells, Lc-lymphocytes, DC-dendritic cells, NK-NK cells, M-macrophages, Mo-monocytes, Ob-osteoblasts, preOc-osteoclast precursors, SLO-secondary lymphoid organs, Gr-granulocytes, E-endothelial cells, F-fibroblasts. Based on HUGO Gene Nomenclature Committee recommendations at http://www.gene.ucl.ac.uk/nomenclature/

The diversity of functions of TNF ligand superfamily members has only begun to be appreciated, and already there is general recognition that they control and regulate inflammatory and immune responses. For instance lymphotoxin-α, RANKL and TNF are critical for the development of peripheral lymphoid organs (6). The differentiation of several lymphocyte and myelocyte cell lineages is also dependent on regulated and timed release of TNF superfamily ligands, such as BLyS (7), CD40 ligand (8), 4-1BB ligand (9), OX40 ligand (10) or CD27 ligand (11). Equally important is the role that several ligands of the superfamily, including TNF, FasL and TRAIL play in cytotoxic activity of effector cells or the removal, via activation-induced apoptosis, of lymphocyte populations and homeostasis restoration (12-14). Some of the ligands, notably lymphotoxins, TNF and LIGHT, provide communication between immune cells and the surrounding stromal and parenchymal cells, creating the signalling network that is necessary for innate and adaptive immune response (15).

Involvement of TNF ligand superfamily members in many disorders, especially those connected to inflammation has been shown. Rheumatoid arthritis and inflammatory bowel disease are caused by excessive local production of TNF and three specific TNF inhibitors (etanercept, infliximab and adalimumab) are used in therapy of RA. Misregulated TNF expression has also 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. Several hereditary diseases of the immune system are also associated with mutations in genes or receptors of TNF superfamily, including hyper IgM syndrome (CD40L) (16),

autoimmune lymphoproliferative syndrome (FasL/FAS) (17), and the TNF receptor associated periodic fever syndrome (TNF) (18). Mutations in ED1 or EDAR lead to severe impairment of skin, hair and teeth development associated with ectodermal dysplasia syndrome (19).

Mast cells and their granules

Mast cells are tissue-dwelling cells that are predominantly located at the interfaces of the organism and the exterior, such as skin, gut mucosal membranes and lung. They are evolutionarily old cells that play multiple roles in many modes of immune response, including innate and antibody-dependent reactions. Mast cells derive their name from the original name given by Paul Ehrlich, *Mastzellen* (well-fed cells) reflecting the fact that a mature mast cell contains large number of cytoplasmic granules. These granules are specialized organelles, found primarily in granulocytes, which possess the unique capability of rapid release. Ultrastructural analysis of mast cell granules reveals several subtypes such as scroll-containing, crystal-containing, particle-containing and homogeneously electron-dense content-containing granules although the functional importance of this heterogeneity is not clear. Many granules contain a mixture of these patterns. The granules were initially considered a storage organelle for the products of a cell; these products could be rapidly released upon appropriate stimulation. Initial studies of human mast cell granule composition by enzyme-affinity labelling and ultrastructural immunocytochemical techniques allowed for identification of proteases chymase and tryptase (20), a proinflammatory biogenic amine histamine (21) and the proteoglycan heparin (22). Subsequently, new techniques allowed for establishing granular localization of many more mediators, among them several cytokines such as bFGF (23), SCF (24), VEGF (25), IL-4 (26) and TNF (27). The presence of cytokines in granules adds a new dimension to a role of mast cells in cytokine biology. Perhaps the best example of novel mechanisms in cytokine biology is the role of mast cell granule-derived TNF. This pool of the cytokine plays critical role in host defence against bacterial infections (28, 29) and its lack results in drastically reduced neutrophil influx and significantly increased mortality of experimental animals. This unique capability of fast releasing of a considerable amount of preformed cytokines might enable mast cells to influence the course of the immune processes being initiated, directing it towards inflammatory or allergic response, depending on the profile of cytokines released.

It is often observed that mast cells secrete bioactive compounds, including cytokines, without full degranulation (30, 31). This process is interchangeably named "differential" or "selective" release; ultrastructural analysis of the process resulted with a term "piecemeal degranulation" as opposed to "anaphylactic degranulation" meaning full release of the granules. This mode of granule cargo release was shown to be mediated by vesicular transport (32) and apparently involves additional regulatory checkpoints. On the functional level this has been demonstrated by differential sensitivity of degranulation and TNF release to a H1 histamine inhibitor azelastine (33). The mechanisms responsible for selective release of granule cargo without the full degranulation remain largely unclear.

Another interesting aspect of granule biology concerns its potential role in protein biosynthesis. It has been reported that in mast cells undergoing piecemeal degranulation or recovering from anaphylactic degranulation, when the cargo rebuilding process requires high protein synthesis, many ribosomes are observed in perigranular space, on the surface and in the lumen of granules (34). In addition mRNA has been detected in the lumen of granules (35). The accumulating evidence of the synthesizing machinery in secretory granules may suggest more complex role for these organelles than previously recognized, possibly including protein synthesis and sequestration of excessive mRNA.

Mast cells in immune response

General remarks

Mast cells are an important element of both innate and acquired immunity. They derive from a distinct precursor in bone marrow (36, 37) and differentiate under the influence of SCF (38). They express numerous receptors that, when stimulated, may induce production of plethora of mediators. These receptors include IgE and IgG, complement, IL-1, TNF and several Toll-like receptors, to name just a few most important (39). Upon stimulation mast cells undergo activation and, in some cases, degranulation, releasing and synthesizing highly bioactive, proinflammatory, vasodilative, chemotactic, and cytotoxic substances. These cells are crucial for the function of several biologically and clinically important mechanisms of immune response such as allergy, inflammation and, as shown recently, also immune tolerance (40). Some of the more important mediators prestored and synthesized by mast cells and their major patophysiological effects are summarized in Table 2. Mast cells play multiple roles without inducing anaphylactic shock and this requires mechanisms of differential activation; indeed mast cells are rarely seen to degranulate during autoimmune (30) or inflammatory responses (31). The mechanism of selective stimulated release was first observed in case of serotonin and histamine and named "differential" or "selective" release (41). The profile of mediators released depends on the type of mast cell and the stimulus which clearly shows there is a complicated network of signalling pathways regulating secretion; several examples of differential release and their physiological importance are presented in Table 3.

Innate immunity

Looking from a broader perspective of host defence, mast cells play several roles in innate and acquired immunity. Although there is some evidence of mast cells exhibiting directly germicidal activity by phagocytosis (42) or bactericidal peptide release (43), several lines of evidence suggest that the most important way by which mast cells contribute to innate immune response is initiation and regulation of the magnitude of leukocyte infiltration into the site of inflammation. It has been demonstrated using mast-cell deficient mice that at least TNF and leukotrienes are important factors in neutrophil recruitment towards sites of bacterial infection and mast cells deficiency correlates with much worse prognosis in experimentally infected mice (28, 44). Experiments in other model systems have shown that secretion of TNF (45) and leukotrienes (46) in acute phase of inflammatory process may also

promote influx of leukocytes other than neutrophils, such as T cells or macrophages which are typical for chronic inflammatory state. Such leukocytes can then initiate and maintain features characteristic of chronic inflammatory state.

MEDIATOR	MAJOR PATHOPHYSIOLOGIC EFFECT
Prestored	
Biogenic amines	
Histamine	Vasodilation, angiogenesis, mitogenesis, suppressor T-cell activation
5-HT	Leukocyte regulation, vasoconstriction, pain
Chemokines (IL-8, MCP-1, MCP-3, MCP-4, RANTES)	Chemoattraction and tissue infiltration of leukocytes
Enzymes	
Chymase	Tissue damage, pain, angiotensin II synthesis
Tryptase	Activation of PAR, inflammation, pain, tissue damage, degradation of antigens and peptides
Kinogenases	Synthesis of kinins, pain
Nitric oxide synthase	NO production
Polypeptides	
CRH	Inflammation, vasodilation, mast cell VEGF release
Endothelin	Sepsis
Kinins	Inflammation, pain, vasodilation, mast cell trigger
Somatostatin (SRIF)	Anti-inflammatory (?), mast cell trigger
VEGF	Neovascularization, vasodilation
Proteoglycans	
Chondroitin sulfate	Connective tissue component, anti-inflammatory, mast cell inhibitor
De novo synthesized	
Cytokines	
IL-1, -3, -4, -5, -6, -9, -10, -13, -16, IFN-γ, MIF, TNF	Multiple roles
Growth Factors	
SCF, GM-CSF, GnRH-I β-FGF, NGF, VEGF	Growth of a variety of cells, mast cell proliferation
Phospholipid metabolites	
LTB4	Leukocyte chemotaxis
LTC4	Vasoconstriction, pain
PAF	Platelet activation, vasodilation, inflammation
PGD2	Bronchoconstriction, pain
NO	Vasodilation, neuromodulation

Table 2 Selected mast cell mediators and their major pathophysiologic activities. β-FGF, fibroblast growth factor; GnRH, gonadotropin-releasing hormone-I; LTB4, leukotriene B4; MIF, macrophage inflammatory factor; NO, nitric oxide; PAF, platelet-activating factor; PGD2, prostaglandin D2; SRIF, somatomedin release inhibitory factor, somatostatin; TGF-β, transforming growth factor-β. Adapted from (88)

Another function mast cell play in innate response is limiting the toxicity of certain substances generated by the host which have adverse effects when present in high concentrations. An example of such activity is degradation of endothelin-1, a peptide that is involved in sepsis (47, 48), by the proteases released from mast cell granules (47). Mast cells are also capable of releasing mediators influencing (positively or negatively) the transition from innate to acquired immunity (38). It has been

reported that mast-cell derived TNF plays a role in draining lymph node hypertrophy and T cell recruitment to these nodes in a model of *E. coli* infection in mouse (45). In a model of FITC challenge in the skin of mast cell-deficient mice dendritic cell migration to lymph nodes was significantly decreased 24 hours after the challenge, but no defect in migration was detectable 48 hours after the challenge (49). This indicates that while there is a mast cell-dependent component in the development of adaptive immune response, the mechanisms are likely to be more redundant as compared to innate response. It is of note, however, that in certain circumstances mast cells can have suppressive effect on antigen sensitization and this effect seems to be attributable, at least in part, to histamine (50-52).

Stimulus	Mast cell type	Mediators released	Mediators not released	Physiological importance
Endogenous				
CD8 ligands	RPMC	TNF, NO	H	T cell interactions
Endothelin-1-3	RMMC	TNF, IL-12↑	IL-4, IL-10, IL-13↓	Th1 immunity
IL-1	hCBMC	IL-6, IL-8, TNF	H, tryptase	Inflammation
IL-12	RPMC	IFN-γ	H	Th1 immunity
Monomeric IgE	BMMC	IL-6	H, LTC4	Mast cell survival
PGE2	hCBMC	MCP-1	No degranulation	Angiogenesis
Suboptimal FcεRI stimulation	BMMC	MCP-1, low H	IL-10, H	Chemokines >> Cytokines
Exogenous				
Cholera toxin	RPMC	IL-6	TNF	Inflammation
CpG DNA	BMMC	TNF, IL-6	H, IL-4, IL-12, GM-CSF, IFN-γ	Host response to bacteria
H. pylori VacA toxin	BMMC	IL-6, IL-8, TNF	H	Gastric injury
LPS	RPMC	IL-6	H	Bacterial infection

Table 3 Selected examples of selective release of mediators from mast cells. Adapted from (88)

IgE-associated adaptive responses

Another aspect of mast cells contribution to immune response is their involvement in adaptive immunity. Originally, these activities were connected to antigen-specific IgE that, when bound to FcεRI and crosslinked by an antigen, activate multiple pathways in the mast cell. Recent findings demonstrate, that IgE at high concentrations have more than just passively sensitizing activity. Some antibodies are able to elicit full response in the absence of antigen while other only upregulate FcεRI and enhance mast cells survival (53). This survival enhancement is mediated by autocrine IL-3 stimulation and activation of Bcl-xL/Bcl-2 (54). The extent of mast cell activation in absence of antigen depends on a particular antibody, although the molecular determinants of this anti-apoptotic activities are not defined (55). Additionally, the increased survival after FcεRI stimulation differs between mast cell subpopulations (56).

It's been widely accepted that mast cells contribute significantly to acute inflammatory reactions to antigens/allergens against which the host bears antibodies of the IgE class. Mast cells are responsible for virtually all of the increased

vascular permeability and tissue swelling during IgE-dependent passive cutaneous anaphylactic response (57). If the stimulation is of more persistent or more severe nature, acute response may undergo transition into late-phase reaction (LPR) which, except for the time scale ranging from few to several hours from initial antigen challenge, is characterized by recruitment of leukocytes to the site of inflammation. Apart from the role played by mast cells in recruitment of other immune cells to the site of inflammation (58), they can also influence the phenotype and function of T cells and other leukocytes; these regulatory activities may be attributed to both soluble mediators, such as TNF, and cell-to-cell interactions involving costimulatory molecules, e.g. OX40 ligand (59, 60). Many of the clinical symptoms of LPR are thought to result from the actions of the cells recruited to these sites rather than from direct activity of the mediators released by mast cells during the acute phase (30, 61). It has to be noted, however, that not in all cases LPR are preceded by the acute allergic reactions *in situ* (62). Such sequence of events corresponds well with many reports concerning differential release of mast cell mediators, an example being Bcl10-MALT-1 adapter complex-dependent specific release of IL-6 and TNF without degranulation following FcεRI stimulation (63). This is consistent with the fact that of many mast cell products, TNF has been most clearly implicated in leukocyte recruitment and the development of other LPR features (58, 64). Mast cells are also capable of providing mediators and direct intercellular contact-mediated functions at sites of long-term, IgE-associated chronic allergic inflammation characteristic for disorders like asthma, allergic rhinitis and atopic dermatitis (60, 65, 66). It has been shown recently, that optimal expression of ovoalbumin-induced asthma in mice is mast cell-dependent (67). In this model many features of the disorder were markedly reduced in mice whose mast cells lacked FcRγ and thus could not be activated by IgE or IgG1. Interestingly, certain features of this disorder were found mast cell-dependent, but FcRγ-independent. In this view, a wide range of innate and IgE-associated immune responses appears to represent a situation in which mast cells activity, depending on particular circumstances, may be either beneficial or detrimental to the host.

IgE-independent responses

Apart from IgE-dependent responses, mast cells have been implicated in pathogenesis of several autoimmune diseases (30), including multiple sclerosis (68) and rheumatoid arthritis (69) in humans and experimental autoimmune encephalomyelitis (EAE) (70) and IgG1 antibody-dependent autoimmune arthritis in mice (71). Moreover, under some experimental conditions mast cells are necessary for complete elicitation of inflammation associated with hapten-induced contact hypersensitivity (CHS) (72) or asthma (73) and inflammatory bowel disease (74). In addition, an effector role for mast cells has been proposed in murine models of fibrosis associated with chronic inflammatory states e.g. scleroderma. In a model of chronic graft versus host disease (CGVHD) based on injection of spleen cells from B10.D2 mice into irradiated BALB/c recipients, mast cells present in affected skin were found depleted of cytoplasmic granules which leaded to hypothesis defining mast cells activation as an important factor for fibrosis that is a major feature of

both CGVHD and scleroderma (75). Injecting bleomycin into skin of wild type and mast cell-deficient mice for 1 week elicits dermal sclerosis only in the former; after 4 weeks however this state is equally developed in both populations, indicating that while mast cells are not necessary for the development of skin fibrosis they may accelerate its development (76).

Mast cell-T cell interactions

As mentioned above mast cells can exert their activities by being effector cells or response initiators. There is, however, yet another important way for these cells to shape immune response: regulation of T cells function. These interactions may be of both direct and indirect nature. Due to relatively low numbers of mast cells in secondary lymphoid organs their presence was not considered important for T cell differentiation. However, under inflammatory conditions mast cells can additionally migrate to spleen and lymph nodes and modulate the immune response (77, 78). The proximity of mast cells and naïve T cells in these organs allows for cytokines released by the former influence priming of the latter for polarized differentiation. In some settings mast cell-derived IL-4 may directly skew Th2 responses. Mast cells are also good sources of TGF-β and IL-6, cytokines known to favour Th17 development. The expression of surface proteins that display stimulatory or inhibitory activity towards T cells may also be relevant in these settings (38). Under some conditions, mast cells express the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) and the adhesion molecules CD54 (ICAM-1) and CD18 (β2 integrin chain of leukocyte function-associated antigen-1), all of which are involved in T-cell activation. More recently expression of members of the TNF/TNF receptor superfamily, including OX40L, CD153, Fas, CD137, and glucocorticoid-induced TNF receptor, by mast cells and the importance of these molecules for T cell activation has been demonstrated (60). Although the range of mediators expressed by mast cells suggests a broad ability of mast cells to direct T cell differentiation, only limited evidence for such activity was demonstrated in *in vitro* co-culture system (59, 60, 79). The development of *in vivo* models to evaluate the importance of these interactions will be a major progress in mast cell and T cell biology.

Another feature of mast cell enabling them to influence T cell fate is their ability to act as antigen-presenting cells. They express both MHC-I and MHC-II and have been shown to present antigen to T cells in an *in vitro* system (42, 80). Mast cell can also influence trafficking of T cells into the site of infection or inflammation. Leukotriene B4, that is essential chemotactic factor for CD4+ Th1 and Th2 cells and for CD8+ effector T cells, is produced by mast cells upon activation (46). Released mediators, including TNF, upregulate expression of endothelial adhesion markers such as ICAM-1 and vascular adhesion molecule-1 (81) and vasoactive amines, such as histamine, increase endothelial permeability as well as P selectin expression on endothelial cells, allowing for an influx of activated T cells.

Mast cells in limitation of inflammation

Although there is a solid body of evidence that mast cells exert predominantly proinflammatory activities, there are a few reports stating otherwise. Examination of biological activity of mast cell mediators, indicates that some of them, including TGF-β (82), IL-4 (83), IL-10 (84) and histamine (85) have potentially anti-inflammatory activity, although until now very few studies have demonstrated this *in vivo*. The first report of such activity of mast cells in knockin mouse concerned UV-induced suppression of contact hypersensivity to TNCB that was, at least partly, mediated by histamine (86). It has also been demonstrated, that mice that were bitten by a mosquito display lowered antigen-specific T cell responses in the model of delayed hypersensitivity to OVA and that this phenomenon requires mast cells at the site of the bite (87). The mechanism of this regulatory activity remains unknown. The results of yet another study show, that mast cells are necessary for peripheral tolerance in skin allografts (40). In tolerant mice considerable increase of mast cell-specific transcripts and number of mast cells was observed. This increase correlates with the influx to the graft of IL-9-producing CD4+Foxp3+ T cells. Mast cell-deficient mice cannot be tolerized and experience rapid graft rejection, which can be prevented by local skin reconstitution with mast cells. IL-9 released by Tregs is the major mediator of mast cells recruitment and activation in the dermis of these tolerant grafts. Mast cells may then act by limiting the influx of inflammatory T cells or cooperating with dermal Tregs. Unexpected as it sounds, mast cells do contain TGF-β that is a major Tregs inducing factor. In conclusion, mast cell activators may yield pro- or anti-inflammatory responses; under IL-9 stimulation mast cells seem to rather suppress than induce the inflammatory processes.

Conclusions on mast cell role in regulation of inflammatory process

The regulatory activities of mast cells related to induction and resolution of the inflammatory process seem to favour defining them as proinflammatory cells. While in many settings such activity is detrimental to the host it has to be remembered, that in the view of host defence inflammatory process is often crucial to restoring homeostasis. Additionally, there is gradually accumulating evidence, that in some settings mast cells may exhibit anti-inflammatory activity which makes them potentially attractive therapy target in disorders involving chronic inflammation. As far as the perspective of inventing a chemical compound specifically targeting selected mast cell functions is rather improbable, this kind of precise manipulation seems to be feasible with the use of biologicals. The new track of research concerning mast cells as anti-inflammatory regulatory cells is certainly worth pursuing.

Outline of this thesis

Mast cells as an important regulator of immune response have long been underestimated. There is, however, growing body of evidence indicating, that these cells are crucial for induction and perhaps attenuation of inflammatory response. This thesis focuses on analysis of expression of TNF and IL-4, cytokines regarded pro- and anti-inflammatory, respectively, by mast cells.

Given the unique physiological properties of the pool of TNF that is stored in mast cell granules, we analyzed intracellular trafficking pathways leading to the storage of this cytokine in mast cell secretory compartment. We examined amino acid motifs and posttranslational modifications necessary for proper targeting of TNF. The differences between mouse and human TNFs were analyzed revealing potential weaknesses of murine model when TNF biology is considered (chapters 2 and 3)

Inflammatory processes elicited by non-immune stimuli may take a course that is detrimental to the host. We analyzed potential involvement of mast cells in the regulation of immune response in such situations. Models of hypoxia and heavy metal ions exposure were utilized in order to find out how IL-4, that is a potential inflammation limiting factor, is regulated in mast cells under these conditions (chapters 4 and 5).

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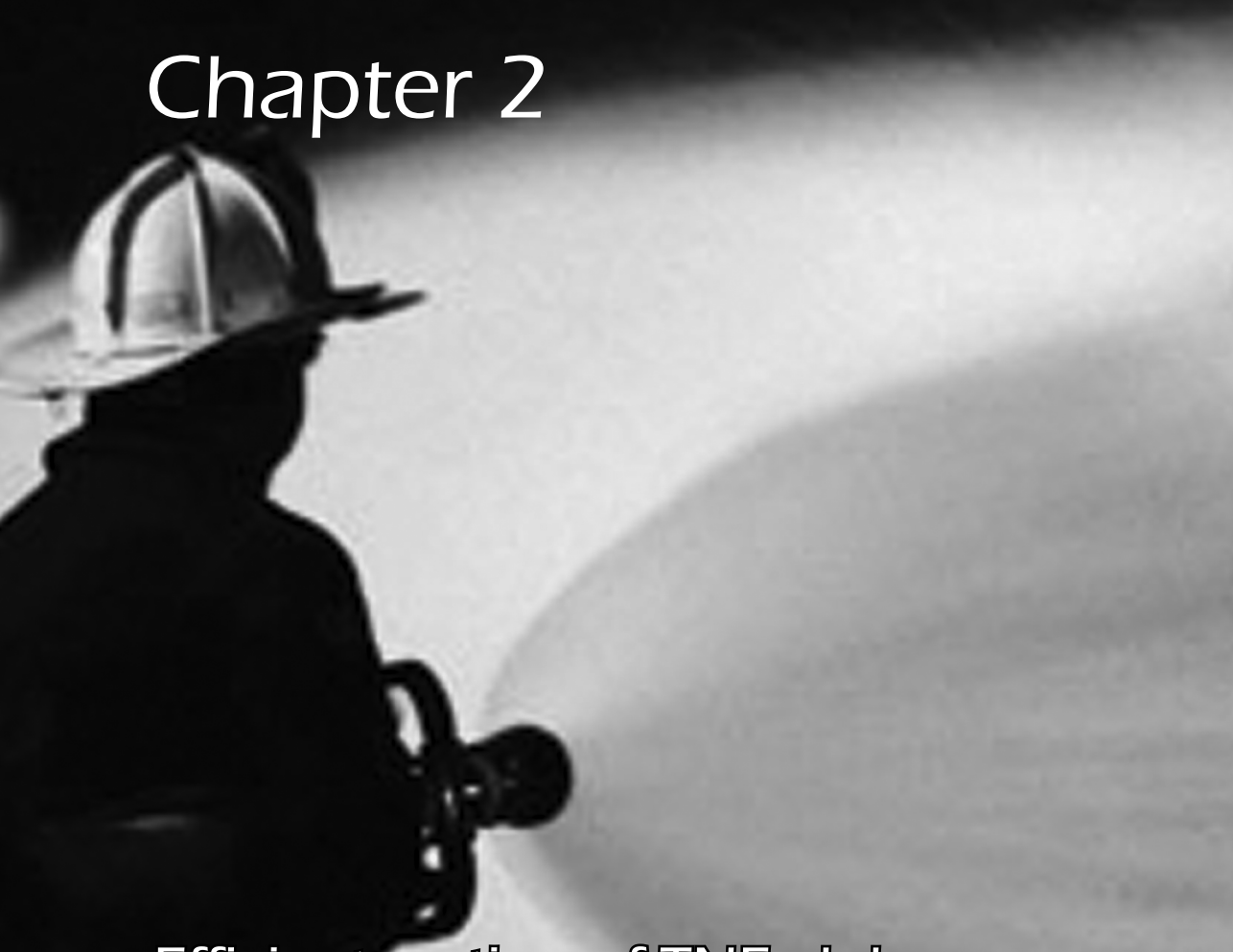
Chapter 2



Efficient sorting of TNF-alpha to rodent mast cell granules is dependent on N-linked glycosylation

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Abstract

Mast cells play an important role at the early stages of immunological response to bacterial infections and parasite infestations. One of the major mast cell pro-inflammatory mediators is TNF-alpha. Mast cells are considered the only cells capable of storing TNF-alpha in cytoplasmic granules and rapidly releasing it upon activation. To determine what pathway is utilized to direct TNF-alpha to cytoplasmic granules and what motifs are responsible for the sorting process, we constructed a fusion protein covering the full sequence of TNF-alpha, N-terminally fused to enhanced green fluorescent protein (EGFP). In rodent mast cells, such protein was sorted to secretory granules, and this process was inhibited by both brefeldin A and monensin. Considering the relationship between lysosomes and secretory granules and following TNF-alpha sequence analysis, it was determined whether TNF-alpha is sorted through the mannose-6-phosphate receptor (MPR)-dependent pathway. We observed that ammonium chloride and tunicamycin blocked TNF-alpha-EGFP fusion protein delivery to secretory granules. In situ mutagenesis experiments confirmed the necessity of N-linked glycosylation for efficient sorting of TNF-alpha into rodent mast cell granules. In this work we established that TNF-alpha travels from the ER to mast cell granules via a brefeldin A- and monensin-sensitive route, utilizing the MPR-dependent pathway, although this dependency does not seem to be absolute.

Introduction

Mast cells play an important role in both innate and acquired immunity. Antigen cross-links IgE bound to its receptors, leading to cell activation and subsequent degranulation and induction of synthesis of many chemotactic, proinflammatory, vasodilatative and cytotoxic substances. Some of the mediators released are cytokines, amongst them TNF-alpha. In a model of bacteria-induced inflammation the lack of mast cell-derived TNF-alpha was associated with drastically reduced neutrofil influx, and significantly higher mortality (1, 2). Similar data suggesting the critical role played by mast cell-derived TNF-alpha in certain types of inflammatory response were obtained using reverse Arthus reaction as an animal model of inflammatory state (3, 4). The special role for mast cells as a source of TNF-alpha might be explained by the presence of preformed cytokine protein in cytoplasmic granules that allows for its immediate release upon stimulation (5). This is supported by the two-stage kinetics of TNF-alpha release from murine mast cells (6). Despite temporal coordination of degranulation and preformed TNF-alpha release, these processes are differentially sensitive to some inhibitors, *e.g.,* azelastin (7). The presence of preformed cytokine protein in cytoplasmic granules is also confirmed by the observation that mouse mast cells are capable of TNF-alpha release following treatment with the transcription inhibitor actinomycin D (6). Within few hours from the release of preformed TNF-alpha, mast cells synthesize and release additional amount of this cytokine in a process that, as in other TNF-alpha-producing cell types, involves direct vesicular transport from the Golgi apparatus to the cell membrane (8, 9). This process was extensively studied in macrophages, where TNF-alpha is synthesized as a transmembrane protein (26-kDa, tmTNF-alpha) and cleaved to soluble form (17 kDa, sTNF-alpha). The majority of TNF-alpha exposed to the environment exists in the sTNF-alpha form, and the conversion of tmTNF-alpha into sTNF-alpha begins at some post-ER stage and is closely associated with TNF-alpha movement to the cell surface (10). The enzyme catalyzing this reaction, TNF-alpha converting enzyme (TACE, ADAM17) belongs to the metalloprotease family and is mainly found in a perinuclear compartment morphologically resembling the Golgi apparatus, and on the surface of the cell (11, 12).

Unlike that seen in macrophages, TNF-alpha trafficking and processing in mast cells has not been extensively investigated. One of the unanswered questions is what processes are responsible for the unique trafficking of TNF-alpha to cytoplasmic granules. Analysis of composition of mast cell granules suggests they are related to lysosomes (13, 14), although unlike lysosomes they are released following stimulation. Mechanisms directing proteins to lysosomes are diverse, based on both amino acid sequence and glycosylation of sorted proteins (15-17). Sequences present in cytoplasmic tails of type I transmembrane lysosomal proteins have been identified (reviewed in(18)) and used to direct fusion proteins to mast cell granules (19, 20). TNF-alpha, however, is a type II transmembrane protein and none of the aforementioned signals is found in its cytoplasmic tail. Alternative sorting of lysosomal proteins depends on recognition of the mannose-6-phosphate residues of

the glycan chain of the glycoprotein being sorted by one of the two distinct receptors, cation-dependent and cation-independent mannose-6-phosphate receptor (CD-MPR or CI-MPR, respectively). The cytoplasmic tails of these receptors contain sorting signals similar to those present in transmembrane lysosomal proteins (reviewed in (21, 22)). It has been reported that a MPR-dependent pathway is engaged in sorting of cathepsin D into cytoplasmic granules in mast cells (16). It is possible that TNF-alpha sorting to cytoplasmic granules might also utilize one of the MPR-dependent pathways. This possibility is supported by the fact that TNF-alpha contains a putative N-glycosylation site (N86 in murine TNF-alpha) and exhibits some glycosylation pattern (23). However, at present it has not been established whether TNF-alpha is N-glycosylated on N86 and whether this glycosylation is of any importance for the sorting process.

We employed transfected rodent mast cells expressing different TNF-alpha-enhanced green fluorescent protein (EGFP) fusion proteins to study mechanisms trafficking TNF-alpha into cytoplasmic granules. We report here that the full TNF-alpha sequence efficiently directs EGFP to cytoplasmic granules. This process is sensitive to brefeldin A (BFA) and monensin, and requires MPR and N-linked glycosylation of TNF-alpha, although this dependency does not seem to be absolute.

Results

Expression of 1-235TNF-EYFP

Microscopic observations were performed in RBL-2H3 cells transiently cotransfected with the following pair of plasmids: p1-235TNF-EYFP-N1 and pECFP-Golgi. Fluorescence pattern in cells was observed after 2, 6 and 16 h. In cells cotransfected with the pECFP-Golgi/ p1-235TNF-EYFP-N1 plasmids pair, both ECFP fluorescence and EYFP fluorescence were visible in the Golgi apparatus 2 h after transfection. While the pattern of ECFP fluorescence remained mostly unchanged over the following 14 h, EYFP fluorescence gradually diminished in the Golgi apparatus and accumulated in peripheral vesicular structures, which, in their number, size and distribution, resembled mast cell granules. After 16 h, this process was apparently complete (Fig. 1A-I). A similar fluorescence pattern was observed in BM-derived mast cells (BMMC) and C57.1 cells transfected with p1-235TNF-EYFP-N1 (data not shown). In cells cotransfected with pEYFP-N1/p1-235TNF-ECFP-N1 plasmids, EYFP fluorescence was present in cytoplasmic and nuclear compartments, but not in apparently vesicular structures at all time points tested. ECFP fluorescence was first observed in ER/ Golgi compartment (2-h time point), and over following 14 h localized in vesicular structures negative for EYFP (data not shown).

1-235TNF-EYFP fusion protein is localized in mast cell secretory granules

To test whether ECFP/EYFP-positive vesicular structures appearing in the cells transfected with p1-235TNF-ECFP/EYFP-N1 are indeed mast cell granules, we compared intracellular localization of the 1-235TNF-EGFP fusion protein with the localization of some established mast cell granule markers. We have stained the cells for 5-hydroxytryptamin (5-HT, serotonin), which is found selectively in mast

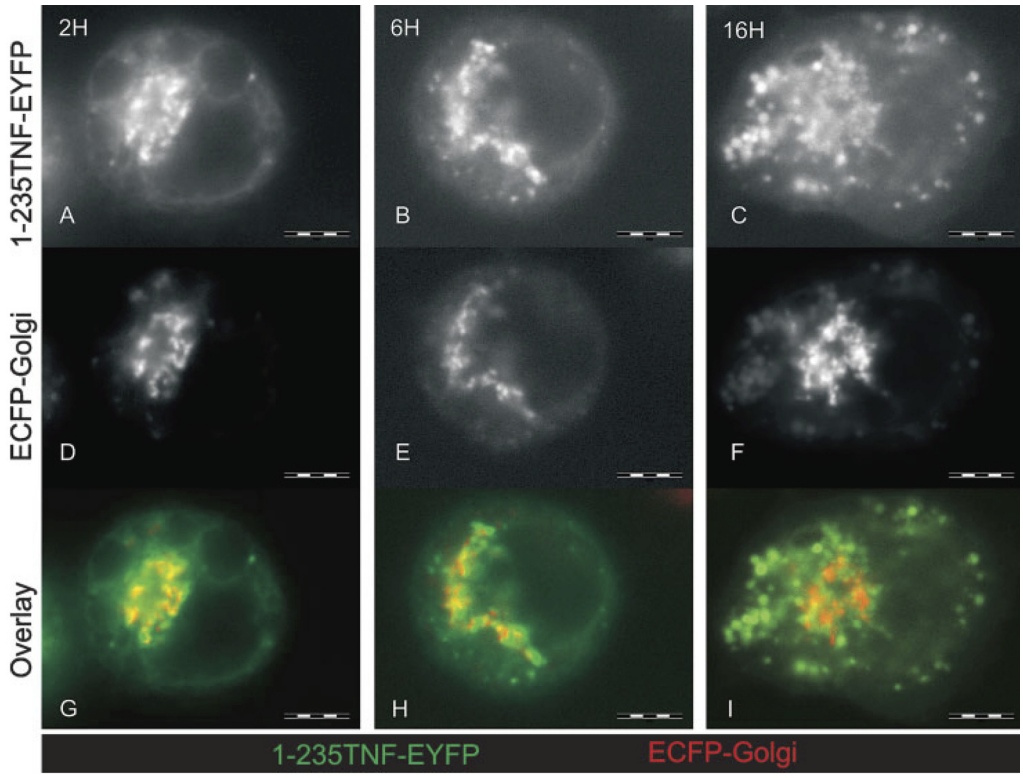

Figure 1. TNF-EYFP fusion protein undergoes intracellular sorting following transfection into rat mast cells. (A–I) RBL-2H3 cells were cotransfected with p1–235TNF-EYFP-N1 and pECFP-Golgi plasmids and subjected to microscopy at the indicated time points. Images A–C depict localization of 1–235TNF-EYFP fusion protein 2, 6 and 16 h post-transfection, images D–F depict localization of ECFP-Golgi protein 2, 6 and 16 h post-transfection. In overlay images (G–I), EYFP and ECFP are represented by false colours green and red, respectively. Bars represent 5 μm on all images

cell secretory granules after its uptake from the medium (24), and for RMCP-II, which is rat mast cell granule-specific protease. We have also used a fluid-phase endocytic marker Texas Red-Dextran conjugate (TR-D), which is known to accumulate in lysosomes and mast cell granules (16). For 5-HT and TR-D studies, cells were allowed to internalize the respective compound overnight; they were then thoroughly washed, transfected with p1-235TNF-EGFP-N1 (5-HT and TR-D-fed cells) and left overnight to attach. For RMCP-II staining, cells were transfected with p1-235TNF-EGFP-N1 and left overnight to attach. Staining revealed a vesicular pattern for both 5-HT and RMCP-II, significantly overlapping with fusion protein fluorescence (Fig. 2A-C and D-F, respectively). Colocalization of 5-HT and 1-235TNF-EGFP was also significant in C57.1 murine mast cells (data not shown). For TR-D and fusion protein colocalization, overlap was even greater, probably due to live imaging facilitating protein fluorochrome fluorescence (Fig. 2G-I). To establish what fraction of endocytosed TR-D is localized in mast cell granules, cells were allowed to internalize the compound overnight and stained for RMCP-II. As shown in Fig. 2J-L, a major fraction of TR-D was localized in a RMCP-II-containing compartment, thus validating its use as a mast cell granule marker.

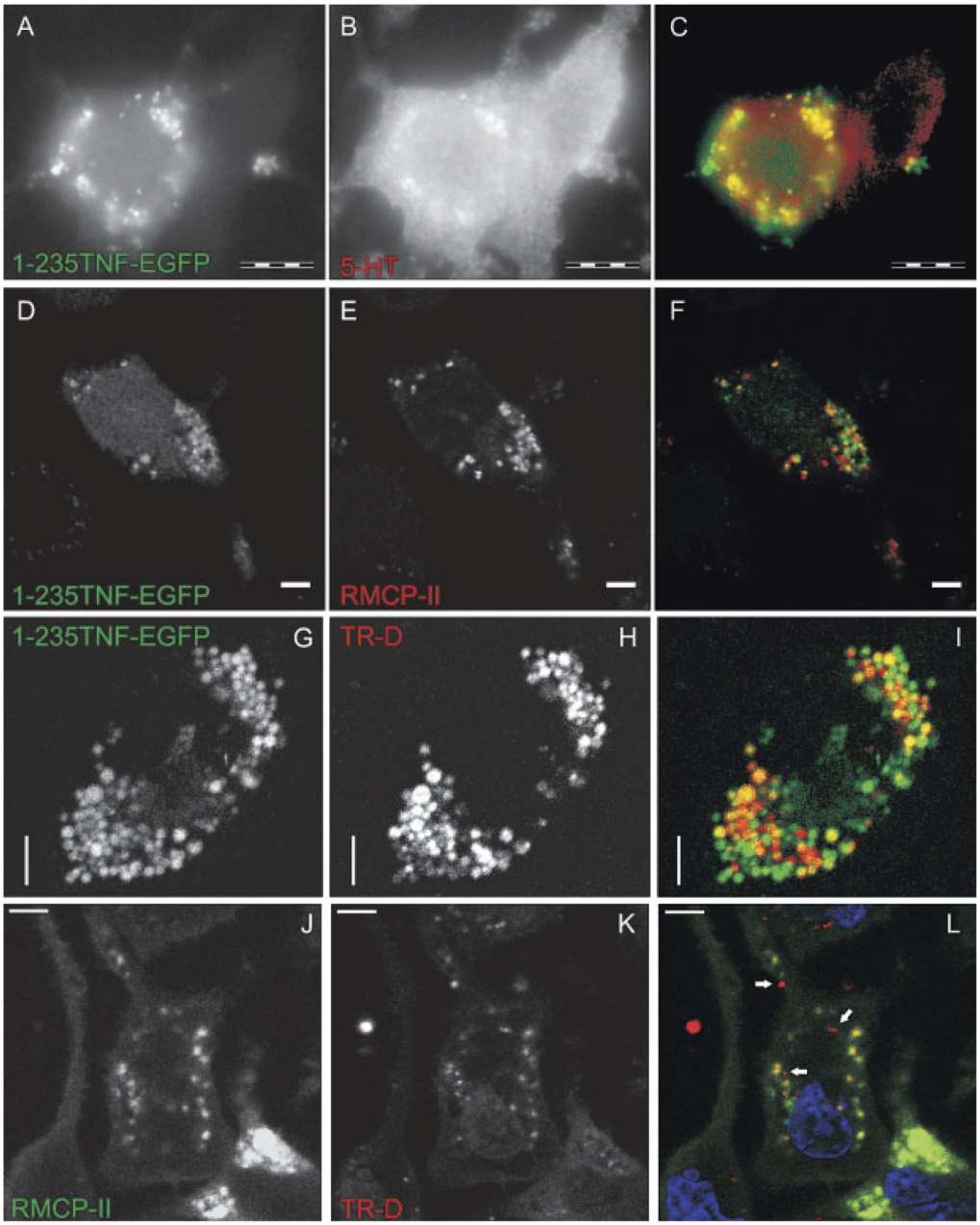

Figure 2. TNF-EGFP fusion protein localizes to mast cell granules. (A–F). RBL-2H3 mast cells were transfected with p1–235TNFEGFP-N1 (A, D) and stained against 5-HT (B) or RMCP-II (E). Images C and F represent colocalization of TNF-EGFP (false colour green) and 5-HT (C) or RMCP-II (F) (both false colour red). (G–I). RBL-2H3 cells were allowed to internalize TR-D and transfected with p1–235TNF-EGFP-N1. Colocalization of TNF-EGFP (G, false colour green) and TR-D (H, false colour red) is shown on image I. TNF-EGFP is represented by false colour green, and TR-D is represented by false colour red. (J–L) RBL-2H3 cells were allowed to internalize TR-D and stained for RMCP-II. Image L represents colocalization of RMCP-II (J, false colour green) and TR-D (K, false colour red). Nuclear stain (TO-PRO-3) is represented in blue. Cells were imaged under a fluorescence (A–C) or confocal (D–L) microscope. In the latter case, representative confocal sections are presented. In (L), arrows indicate lysosomes, which are RMCP-II negative, TR-D-positive structures. Bars represent 5 μm on all images.

TNF-EYFP is properly expressed in and can be released from mast cells

To confirm proper expression of 1-235TNF-EGFP under the experimental conditions employed, lysates from RBL-2H3 cells transfected with 1-235TNF-EGFP were analyzed by Western blot (Fig. 3B). Using both anti-TNF-alpha and anti-GFP antibodies, protein of an apparent molecular mass of 59 kDa was detected with additional band of 49 kDa visible on anti-GFP-probed membrane, probably due to stronger overall signal. Thus, double immunogenicity of 1-235TNF-EGFP protein has been shown, confirming its identity and revealing possible proteolytic processing.

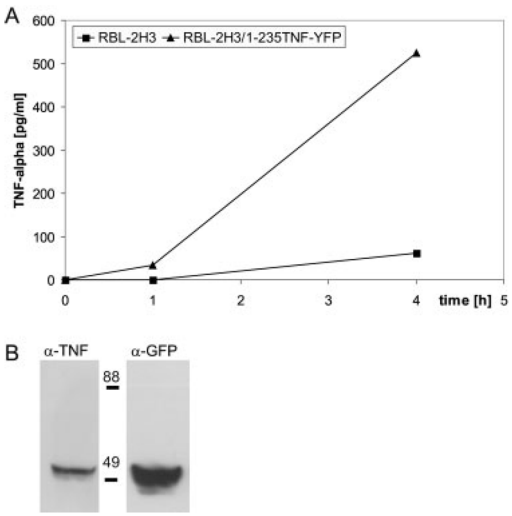

Figure 3. TNF-EYFP is properly expressed in, and can be released from, mast cells. (A) RBL-2H3 cells and RBL-2H3 cells transfected with p1-235TNF-EYFP-N1 were stimulated with 100 ng/mL PMA and 1 μM ionomycin. Concentration of TNF-alpha in culture supernatants was determined by ELISA at the indicated time points. Results of a representative experiment are shown. (B) 1–235TNF-EYFP expression pattern in RBL-2H3 cells was analyzed byWestern blot. Lanes represent analysis of lysates from RBL-2H3 transfected with p1–235TNF-EYFP-N1. Membrane was probed with anti-TNF antibodies, stripped and reprobed with anti-GFP antibodies.

To establish whether 1-235TNF-EYFP fusion protein can be released from mast cells in response to stimuli triggering release of endogenous TNF-alpha, RBL-2H3 cells were transfected with p1-235TNF-EYFP and left overnight to attach. For TNF-alpha release, cells were incubated in culture medium alone or culture medium with 100 ng/ml PMA and 1 μM ionomycin. In supernatants from non-stimulated cultures, no TNF-alpha was detected, regardless of transfection (data not shown). After 1 h of stimulation, 34 pg/ml TNF-alpha was detected in supernatants from transfected cells, but none in the case of non-transfected cells. After 4 h of stimulation, 62 pg/ml TNF-alpha was detected in supernatants from non-transfected cells, and 525 pg/ml in supernatants from transfected cells. These results showed that fusion protein covering full sequence of TNF-alpha is properly expressed, targeted specifically to mast cell secretory granules and can be released from cells upon stimulation (Fig. 3A).

1-235TNF-EYFP is retrieved from secretory pathway at the stage of late Golgi/ trans-Golgi network

Since it was confirmed that 1-235TNF-ECFP/EYFP fusion protein is sorted to secretory granules, the question arose at what stage of the secretory pathway this sorting occurs. To resolve this, RBL-2H3 cells were transfected with p1-235TNF-EYFP-N1 and incubated with 1 μg/ml BFA (anterograde ER-to-Golgi transport inhibitor) or 1 mM monensin [trans-Golgi network (TGN) export inhibitor] from the time of transfection. At 2 h post transfection, cells incubated with BFA exhibited a reticular pattern of EYFP fluorescence with a concentration of EYFP fluorescence in perinuclear

area, and this did not change over after 24 h (Fig. 4A-C). In cells incubated with monensin EYFP fluorescence at first (after 2 h) concentrated in vesicular structures in central area of the cell. These vesicular structures increased over time, closely resembling swollen TGN observed in light microscopy (data not shown). During this time EYFP fluorescence localization changed from mostly membrane bound to mostly luminal, suggesting possible proteolysis of the fusion protein (Fig. 4D-F). When cells were fed with TR-D, transfected with p1-235TNF-EYFP-N1 and incubated with BFA or monensin, there was no colocalization of EYFP and Texas Red fluorescence (Fig. 4H, I). Contrary to BFA and monensin-treated cells, control cells at 24 h after transfection exhibited concentration of EYFP in mast cell secretory granules (Fig. 4G). To exclude the possibility that the change in fluorescence pattern observed in cells incubated with BFA or monensin is an effect of improper protein processing or glycosylation, cell lysates were analyzed by Western blot. The majority of the fusion protein appears as a band of 59 kDa with minor band at 49 kDa. Following peptide: N-glycosidase F (PNGase F) treatment bands of 55 and 45 kDa were observed (Fig. 4J). This is in agreement with the calculated molecular masses of transmembrane and soluble forms of the 1-235TNF-EYFP fusion protein, respectively. This also indicates that 1-235TNF-EYFP undergoes N-glycosylation, and that a fraction of this protein is proteolytically processed, in a way similar to endogenous TNF-alpha. Thus, we observed that 1-235TNF-EYFP is expressed as a transmembrane glycoprotein and that BFA and monensin interfere with its trafficking, but not its post-translational modifications.

1-235TNF-EGFP sorting to mast cell granules is sensitive to NH_4Cl and tunicamycin

Since it was established that 1-235TNF-EYFP is a glycoprotein we decided to elucidate whether MPR-dependent pathway is used for its sorting to mast cell granules. RBL-2H3 cells were allowed to internalize TR-D overnight and were then incubated for 4 h with 10 mM NH_4C1, which is known to inhibit the MPR-mediated transport. Following incubation, cells were transfected with 1-235TNF-EYFP and examined under a fluorescent microscope. At early stages post-transfection, similar reticular fluorescence patterns were observed in cells incubated with NH_4Cl and control cells (data not shown), but after 16 h NH_4Cl-treated cells, but not control cells, exhibited EYFP fluorescence in large vesicular structures located near the center of the cell. At the same time, there was no significant overlap between 1-235TNF-EYFP and TR-D, while the morphology of the structures containing TR-D (lysosomes and mast cell granules) was not seriously disturbed (Fig. 5A-C).

Thus, NH_4Cl causes retention of the fusion protein in some compartment other than granules. To determine whether 1-235TNF-EYFP sorting to mast cell granules requires N-linked glycosylation, TR-D-fed RBL-2H3 cells were incubated with 2 μg/ml tunicamycin for 4 h prior to transfection with p1-235TNF-EYFP-N1. At 16 h post-transfection, cells exhibited pattern comparable to that of NH_4Cl-treated cells in the green fluorescence channel, and the vesicular pattern in the red fluorescence channel, indicating that whereas granule morphology was not disturbed, fusion protein was absent from secretory granules (Fig. 5D-F).

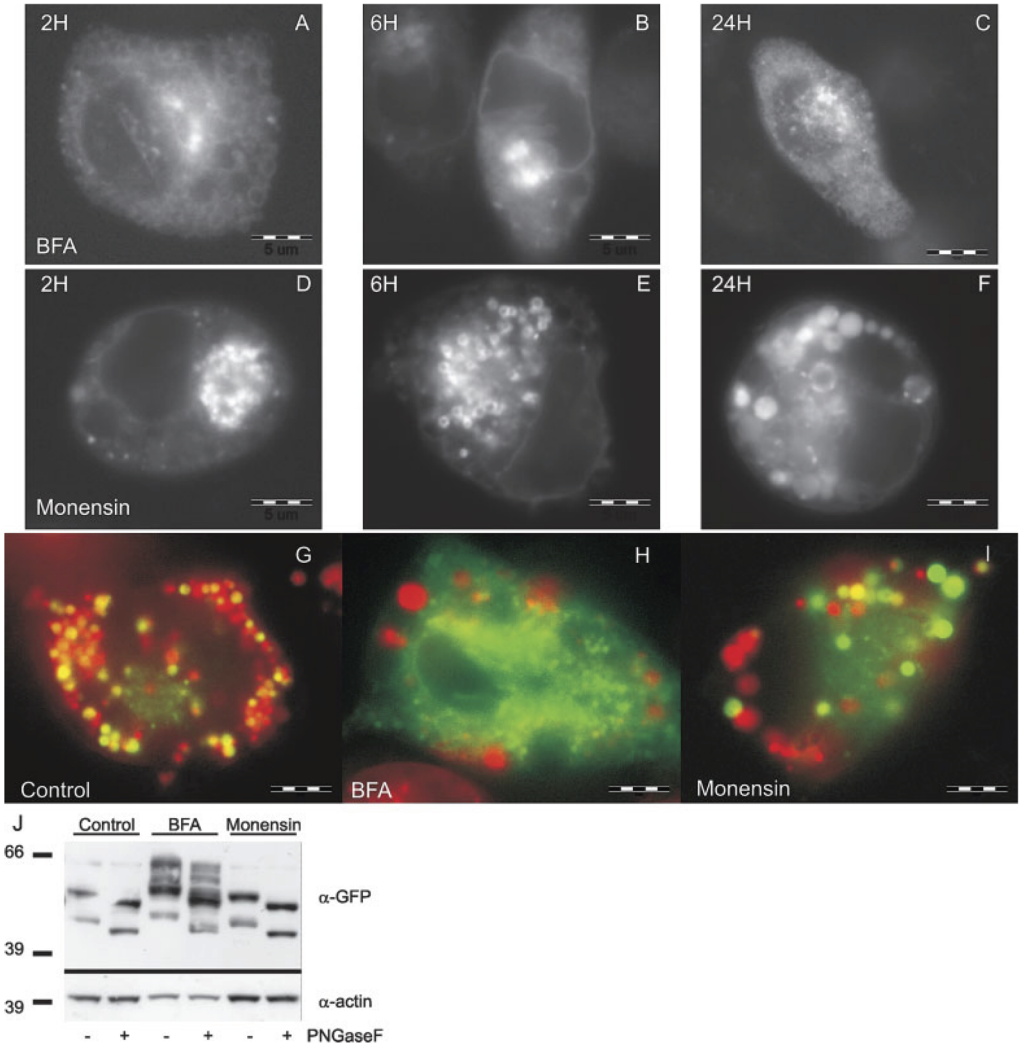

Figure 4. TNF-EYFP sorting is a BFA- and monensin-sensitive process. (A-F) RBL-2H3 mast cells were transfected with p1-235TNF-EYFP-N1, incubated with 1 µg/ml BFA (A-C) or 1 µM monensin (D–F) from the time of transfection, and imaged at the indicated time points.(G–I) RBL-2H3 cells were allowed to internalize TR-D and transfected with p1-235TNF-EYFP-N1. After transfection cells were incubated in culture medium alone (G) or medium containing 1 µg/ml BFA (H) or 1 µM monensin (I). Following attachment, cells were imaged. In all overlay images EYFP and Texas Red are represented by false colors green and red, respectively. Cells were imaged under fluorescence microscope. Bars represent 5 µm on all images. (J) 1–235TNF-EYFP expression pattern in cells imaged above was analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1 and treated with medium alone (Control), BFA or monensin; lysates were not treated (-) or treated (+) with PNGase F. Proteins were detected with anti-GFP antibodies (upper panel) or anti-actin antibodies (lower panel).

To investigate the effect of NH_4Cl and tunicamycin on expression of 1-235TNF-EGFP fusion protein, cell lysates from cells treated with NH_4Cl or tunicamycin for 16 h from transfection were analyzed by Western blot. As shown in Fig. 5G, the pattern of 1-235TNF-EGFP fusion protein expression in NH_4Cl-treated cells was comparable to controls, while incubation with tunicamycin resulted in appearance of additional protein bands of 55 and 45 kDa that were resistant to PNGase F treatment. This

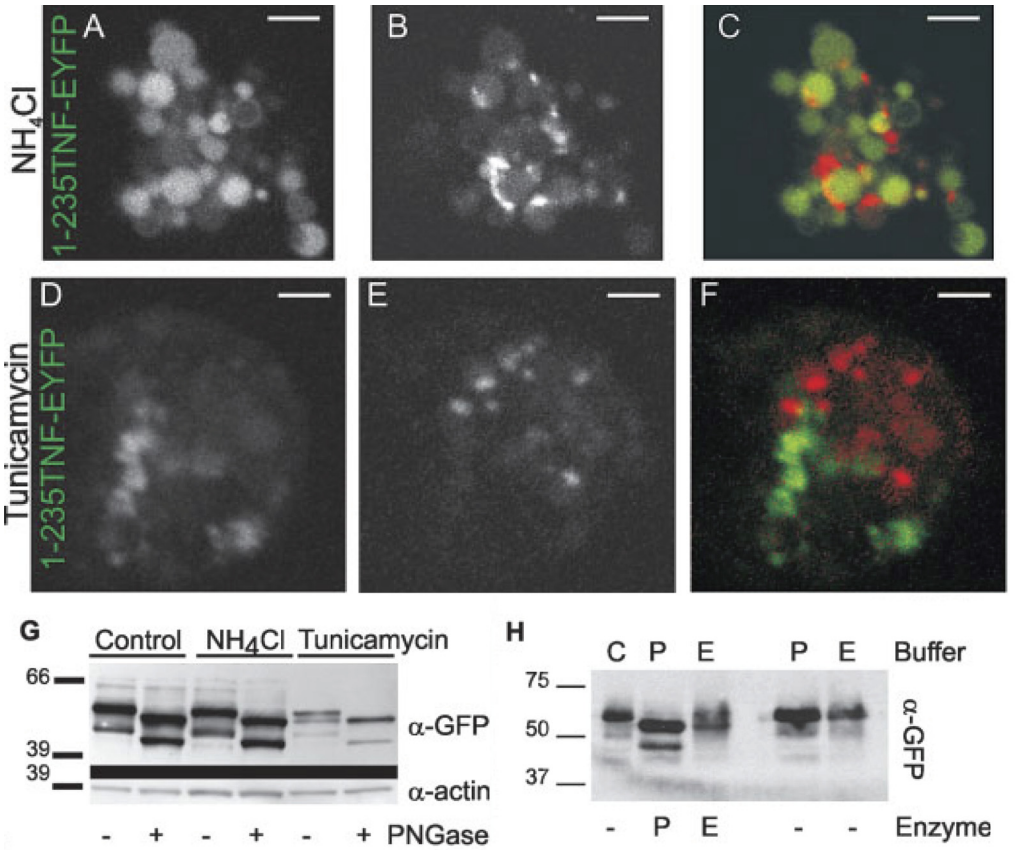

Figure 5. TNF-EYFP sorting to mast cell granules is an NH4Cl- and tunicamycin-sensitive process. (A–F) RBL-2H3 cells were allowed to internalize TR-D and incubated in culture medium containing 10 mM NH4Cl (A-C) or 2 μg/mL tunicamycin (D–F) for 4 h before transfection and following transfection. Cells were imaged at 16 h after transfection with p1–235TNF-EYFP-N1. Images A and D represent fusion protein fluorescence, images B and E represent TR-D fluorescence. In overlay images (C and F), EYFP and Texas Red are represented by false colours green and red, respectively. Cells were imaged under confocal microscope and representative confocal sections are presented. Bars represent 5 μm on all images. (G) 1–235TNF-EYFP expression pattern in cells imaged above was analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1 and treated with medium alone (Control), NH4Cl or tunicamycin; lysates were not treated (-) or treated (+) with PNGase F. Proteins were detected with anti-GFP antibodies (upper panel) or anti-actin antibodies (lower panel). (H) Composition of a N-linked glycan was analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1; lysates were incubated with indicated buffers and enzymes. C, PBS; P, PNGaseF; E, endoglycosidase H.

indicates that tunicamycin inhibited N-linked glycosylation of 1-235TNF-EGFP fusion protein. To further confirm the possibility of involvement of MPR-based system in trafficking of TNF-alpha, lysates from cells transfected with 1-235TNF-EYFP were subject to endoglycosidase H treatment. As shown in Fig. 5H, treatment with endoglycosidase H resulted in appearance of a protein band of 55 kDa in addition to the original 59 kDa. This shows that a fraction of 1-235TNF-EYFP is modified with mannose-rich glycan. The lack of the putative processed form can probably be attributed to generally lower signal in samples incubated in endoglycosidase H buffer (as seen in control lane). This could be a result of general instability of proteins incubated in relatively low pH buffer over prolonged period of time.

N86 is critical for efficient sorting of 1-235TNF-EYFP to mast cell granules

To further elucidate features of 1-235TNF-EYFP fusion protein required for its efficient sorting to secretory granules, a point mutation was introduced into the sequence of p1-235TNF-EYFP-N1, resulting in N86S substitution disrupting the N-glycosylation site in 1-235TNF-EYFP RBL-2H3 cells were transfected with p1-235TNF-EYFP-N1 or p1-235-N86S-TNF-EYFP-N1 and left overnight to adhere. At 24 h after transfection, cells transfected with p1-235TNF-EYFP-N1 developed a vesicular pattern of fluorescence with no fluorescence in cytoplasm. At the same time point, in cells transfected with p1-235-N86S-TNF-EYFP-N1 some fluorescence in vesicular structures was also observed, but a significant fraction of the mutant fusion protein was observed in cytoplasm (data not shown). To examine spatial and temporal relations of control and mutant fusion proteins more closely, RBL-2H3 cells were fed with TR-D, co-transfected with plasmids encoding for the 1-235-N86S-TNF-EYFP and 1-235TNF-ECFP proteins and subjected to microscopy After 48 h 1-235-N86S-TNF-EYFP fluorescence in granules increased, with majority still detected in cytoplasm, whereas 1-235TNF-ECFP and TR-D fluorescence patterns remained unchanged (Fig. 6A-F). However, the fraction of fluorescence of 1-235-N86S-TNF-EYFP in mast cell granules was lower than that of 1-235TNF-ECFP, suggesting that N86 is important for the transport of fusion protein to mast cell granules. Western blot analysis of the expression pattern of 1-235-N86S-TNF-EYFP revealed a pair of PNGase-resistant proteins of 55 and 45 kDa, which is equivalent to PNGase-treated 1-235TNF-EYFP expressed in control cells and 1-235TNF-EYFP expressed in tunicamycin-treated cells (Fig. 6G). In addition, ratiometric analysis of the given fusion protein expression level *versus* beta-actin performed on Western blots showed that in cells transfected with equal amounts of p1-235TNF-EYFP-N1 and p1-235-N86S-TNF-EYFP-N1, DNA mutant and control fusion proteins expression levels were comparable (data not shown). Thus, the mutant fusion protein deficient in N-linked glycosylation expressed in mast cells was not efficiently sorted to mast cell granules. It is of note, however, that even in the absence of N-linked glycans, a fraction of the mutant protein was sorted to secretory granules, indicating that another sorting system partly compensates for non-functional MPR-dependent sorting.

Discussion

The unique feature of mast cells among other TNF-alpha-expressing cells is their ability to store preformed cytokine protein and release it upon activation. While the mechanism of intracellular trafficking of TNF-alpha engaged in the process of secretion of *de novo* synthesized cytokine in macrophages has been partially delineated (25), the mechanism of TNF-alpha storage in mast cell cytoplasmic granules is still mostly unknown. EGFP-containing fusion proteins were successfully applied to decipher a variety of intracellular protein trafficking pathways. This approach was also effective in visualizing the trafficking of mast cell secretory granule proteins, including CD63, phospholipase D, VAMP-7 and syntaxin-3 (26-29). We have employed a similar strategy and transiently transfected RBL-2H3 mast cells with DNA constructs coding for TNF-alpha-EGFP fusion protein to gain insight into the process of intracellular

TNF-alpha trafficking in mast cells. One limitation of such approach is the fact that this mast cell line differs in the phenotype from peritoneal mast cells, which are known to store endogenous TNF in the granules *in vivo* (6).

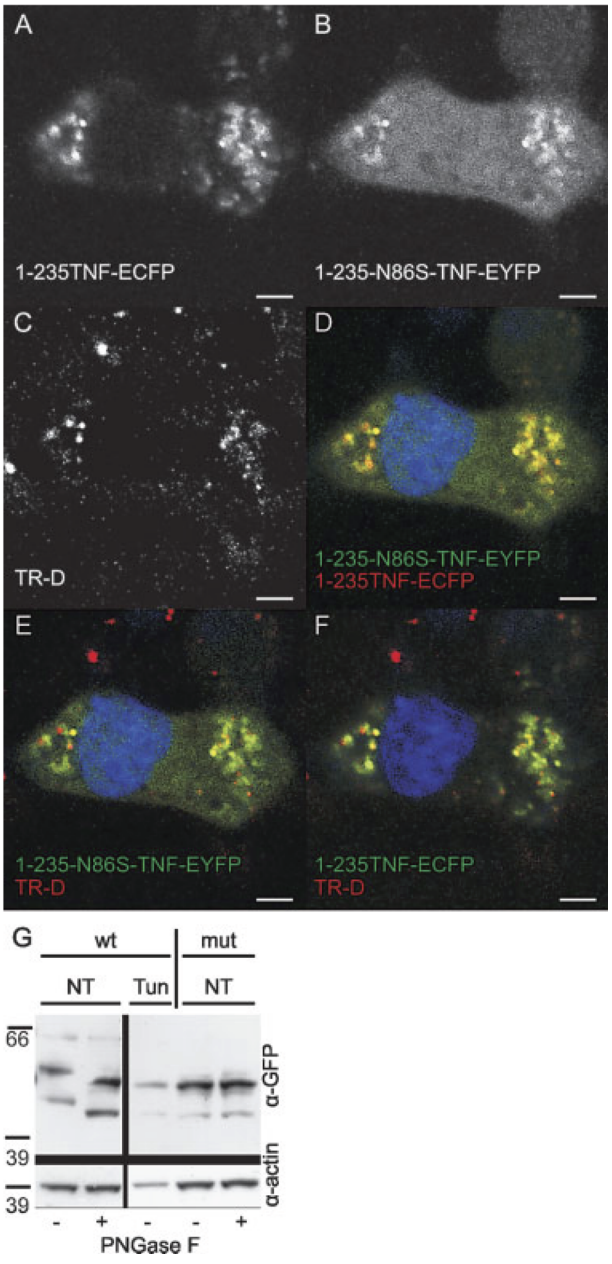

Figure 6. N86 is critical for TNF sorting into mast cell granules. RBL-2H3 cells were allowed to internalize TR-D and cotransfected with p1–235TNF-ECFP-N1 and 1–235-N86S-TNF-EYFP-N1 plasmids. Cells were imaged at 48 h after transfection. Representative confocal sections for fluorescence in ECFP, EYFP and Texas Red channels are shown on images A–C. Overlay images show colocalization of the following fluorochrome pairs (and their respective false colours): EYFP (green)/ECFP (red) (D), EYFP (green)/Texas Red (red) (E) and ECFP (green)/Texas Red (red) (F). Nuclear stain (TO-PRO-3) is represented by blue colour. Bars represent 5 μm on all images. (G) 1–235TNFEYFP and 1–235-N86S-TNF-EYFP expression patterns in cells imaged as above were analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1 (wt) or p1–235-N86S-TNF-EYFP-N1 (mut), treated with medium alone (NT) or tunicamycin (Tun); lysates were not treated (-) or treated (+) with PNGase F. Proteins were detected with anti-GFP (upper panel) or anti-actin antibodies (lower panel).

The EGFP fusion protein containing the entire sequence of the tmTNF-alpha, when expressed in mast cells, accumulated predominantly in cytoplasmic secretory granules (Fig. 1). The unequivocal identification of the compartment in which TNF-alpha fusion protein accumulated is supported by colocalization with three independent markers for secretory granules (Fig. 2). Accumulation of ectopically expressed TNF-alpha fusion protein is consistent with reported observation of immunogold detection TNF-alpha in this compartment (5). The kinetics of appearance of 1-235TNF-ECFP/EGFP fusion protein in transfected mast cells supports the hypothesis that it is sorted into cytoplasmic granules via a ER/Golgi secretory pathway (Fig. 1).

Observed differences in amounts of TNF-alpha released from non-transfected and p1-235TNF-EYFP-transfected mast cells (Fig. 3A) suggest that ectopically expressed 1-235TNF-EYFP fusion protein is not only sorted to mast cell granules, but also efficiently released. Biphasic kinetics of appearance of TNF-alpha in supernatants of stimulated cultures may suggest that relatively lower amounts of this cytokine are released from granule-stored pool within 1 h, while increased release in the following hours is a result of *de novo* synthesis. The apparent lack of release of TNF-alpha by non-stimulated cells, regardless of transfection, is a feature of mast cells, including this cell line(30).

This hypothesis is further supported by the retention of fusion protein in reticular structures or in tubulo-vesicular structures following treatment of transfected cells with BFA or monensin, respectively (Fig. 4). These observations are consistent with reported inhibition of TNF-alpha secretion by these compounds in activated macrophages and T cells (30-32). It is also consistent with current knowledge of the mechanisms of expression and secretion of cytokine proteins in immune cells (33, 34). One of the possible routes that allow for transport of proteins from the TGN to cytoplasmic granules is the MPR-dependent system. The observation that accumulation of TNF-alpha fusion protein in cytoplasmic granules is prevented by NH_4Cl treatment (Fig. 5) suggests that this process is largely mediated by a receptor-dependent route. Involvement of the MPR in this process is consistent with the notion that mast cell cytoplasmic granules are phylogenetically and functionally related to lysosomes (14, 35), as MPR-dependent pathways deliver proteins to lysosomes (36). This is also in agreement with previous reports that trafficking of cathepsin D to mast cell secretory granules partially depends on the MPR system (16). Interestingly, certain structural features of murine TNF-alpha are consistent with the hypothesis that this cytokine may be sorted via an MPR-dependent pathway. Whereas analysis of amino acid sequence of murine TNF-alpha did not reveal any known sorting signals, it did reveal N-linked glycosylation motif in position 86, and glycosylation of TNF-alpha protein has been reported (23). We have shown that the TNF-EGFP fusion protein is indeed glycosylated on N86 residue (Fig. 5G, 6G), and a significant fraction of the glycan attached is the mannose-rich type (Fig. 5H). Thus, the TNF-alpha molecule provides carbohydrate moieties necessary for interaction with MPR. Furthermore, TNF-alpha is a type II transmembrane protein, which renders the N86 residue available for recognition by MPR. In agreement with the involvement of carbohydrate part of TNF-alpha molecule in the sorting process, inhibition of N-glycosylation with tunicamycin prevented sorting of TNF-alpha fusion protein to cytoplasmic granules (Fig. 5). The dependency of TNF-alpha trafficking to cytoplasmic granules on N-linked glycosylation is further supported by the observation that TNF-alpha fusion protein deficient in N-glycosylation motif (N86S substitution), and thus is not N-glycosylated (Fig. 6G), was sorted to cytoplasmic granules with lower efficiency as compared to control protein (Fig. 6). It is of note, however, that this sorting was not completely abolished, even in the absence of N-linked glycans, clearly indicating that another, complementary sorting mechanism is in operation. This is consistent with our observation that a fraction of the glycan attached to TNF-alpha fusion protein is not

of the mannose-rich type, which precludes MPR involvement. Data obtained with the mutated protein confirm that N86 is utilized as a signal for N-linked glycosylation of TNF-alpha and is important for efficient sorting to mast cell secretory granules.

Relatively higher concentration of 1-235-N86-TNF-EYFP protein in cytosol (as compared to 1-235TNF-EYFP) may result not only from deficiency in carbohydrate-dependent retrieval from TGN. It cannot be excluded that once the non-glycosylated protein reaches its target compartment it cannot be stably maintained within granules, and a slow leak into cytoplasm occurs. Another possibility is that N86S mutation impairs trafficking at the stage of ER entry, thus rendering it unavailable for sorting systems operating further downstream. Thus, N86 is important for TNF-alpha sorting to mast cell secretory granules, but its role in this process may extend beyond N-linked glycosylation.

We present here what we believe to be the first observation of cytokine protein being retrieved from secretory pathway by MPR and stored in secretory granules. The hypothesis that TNF-alpha is transported from TGN to cytoplasmic granules via MPR-dependent pathway emphasizes the importance of glycosylation motif in TNF-alpha protein for formation of specific granular pool of TNF-alpha, which is functionally different from *de novo* synthesized cytokine (4, 6, 37). One of the unanswered questions arising from this hypothesis is the nature of the regulatory mechanism directing the cytokine either to cell membrane or to secretory granules as mast cells secrete both preformed and *de novo* synthesized TNF-alpha (6).

Materials and methods

Plasmid construction

The DNA fragment coding for TNF-alpha was obtained by RT-PCR. Briefly, 5 µg total murine RNA (isolated from C57.1 cell line, (38)) was subject to RT reaction using MMLV reverse transcriptase (Gibco) and oligonucleotide 5'-CCTTCACAGAG-CAATGACTC-3' in a final volume of 20 µL. cDNAwas amplified using Taq polymerase (Fermentas MBI) and 5'-TCCAGAAAAGACACC**ATG**AG-3' and 5'-CCTTCACAGAGCAATGACTC-3' as sense and antisense primer, respectively (start codon in bold).

Product of PCR reaction was cloned into pT-Adv plasmid (Clontech) resulting in plasmid pTA-TNF. pTA-TNF was sequenced to verify the integrity of the TNF-alpha reading frame. To obtain plasmid encoding for fusion protein consisting of full TNF-alpha and EGFP, full TNF-alpha ORF was amplified from pTA-TNF plasmid using 5'-GTGATGGATATCTGCA**GAATTC**GGCTTTCC-3' and 5'-CATTGCTCTGTGAA GGAAGCC**GAATTC**CAGC-3' primer pair (EcoRI sites in bold). Reverse primer was used for concomitant disruption of TNF-alpha STOP codon. Amplified fragment was digested with EcoRI, gel-purified and ligated into EcoRI-digested pECFP-N1, pEGFP-N1 and pEYFP-N1 plasmids (Clontech), resulting in series of plasmids encoding for fusion proteins containing full TNF-alpha ORF, 20 random amino acid linker and full ECFP or EGFP or EYFP ORF, i.e., p1-235TNF-ECFP/EGFP/EYFP-N1. The resulting plasmids were sequenced to confirm the integrity of fusion proteins reading frames. Mutagenesis *in situ* was performed with the use of Quick-change XL kit (Stratagene)

using 5'-CAGATCATCTTCT-CAAAGTTCGAGTGACAAGCCTG-3' and 5'-CAGGCTTGT-CACTCGAACTTTGAGAAGATGATCTG-3' primer pair and p1-235TNF-EYFP-N1 plasmid as a template. Plasmids isolated from three independent bacterial clones were sequenced and verified for the expression of the protein of proper molecular weight in C57.1 and RBL-2H3 cells. Plasmid coding for fusion protein carrying N86S mutation (with respect to TNF-EYFP ORF) was called p1-235-N86S-TNF-EYFP-N1.

Plasmid pECFP-Golgi was purchased from Clontech and encodes for human beta 1,4-GT-ECFP fusion protein, containing N-terminal 81 amino acids of human beta 1,4-galactosyltransferase

Cell culture, transfection and ELISA

The rat mast cell line RBL-2H3 was a gift from Dr. U. Blank (Paris, France). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM gluta-mine, nonessential amino acids, 25 mM HEPES, 100 µg/ml penicillin/streptomycin and sodium pyruvate. At approximately 80% confluence, cells were trypsinized and subcultured at $4x10^4$ cells/cm^2. C57.1 cells were grown in DMEM medium supplemented with 10% heat-inactivated FBS, 25 mM HEPES, 100 µg/ml penicillin/streptomycin and 4 mM glutamine. Density of the cells was maintained between $2x10^5$ and $1x10^6$ cells/ml. Murine BMMC were obtained in 4-6-week culture of cells from bone marrow of BALB/c mice in RPMI 1640 supplemented with 20% WEHI-3-conditioned medium as a source of IL-3, 10% FCS, 4 mM L-glutamine, 25 mM HEPES, and 100 µg/ml penicillin/streptomycin. All cells were grown in 5% CO_2 at 37°C in fully humidified atmosphere. All media components were purchased from Sigma.

For transfection, 25-50 µg DNA was added to $2x10^6$-$10x10^6$ cells in 400 µl culture medium and electroporated in Gene Pulser II (Bio-Rad) at 250 V, 950 µF. Immediately following transfection, cells were diluted to $2.5x10^5$ cells/ml in fresh medium.

For inhibitor studies, cells were incubated with 1 µg/ml BFA or 1 µM monensin applied at the time of transfection, or with 10 mM NH_4Cl or 2 µM tunicamycin applied 4 h prior to transfection or at the time of transfection. The viability of the cells subject to concentrations of BFA, monensin, NH_4Cl and tunicamycin used in experiments was assessed by annexinV-PE/7-AADstaining, and established as 60%, 75%, 88% and 95%, respectively. Non-inhibited cells exhibited 95% viability. For microscopic analysis only the cells exhibiting viable morphology have been chosen.

For TNF-alpha-release determination, cells transfected with p1-235TNF-EYFP, following removal of non-viable floating cells, were seeded at $8x10^4$ cells/cm^2. Medium volume was adjusted to obtain final culture density of $2.5x10^5$ cells/ml. TNF-alpha ELISA was performed using commercial kit (Biosource, Nivelles, Belgium) according to manufacturer's instructions.

SDS-PAGE and Western blotting

For SDS-PAGE, cells were collected and heated for 10 min in 99° C in PNGase F glycoprotein denaturing buffer (New England Biolabs) or in 0.1 M acetate buffer pH 5.5 containing 0.2% SDS, and immediately processed or stored in -20°C. Total lysates prepared in PNGase F glycoprotein denaturing buffer were deglycosylated with PNGase F (NEB) according to manufacturer's instructions. Lysates prepared in 0.1 M acetate buffer pH 5.5 containing 0.2% SDS were diluted 1:1 with 0.1 M acetate buffer pH 5.5 containing 2% Triton X-100 and deglycosylated with endoglycosidase H (Roche Applied Science) according to manufacturer's instructions. Samples were resolved on 4-12% NuPage gels (Invitrogen) in MOPS or MES buffer system, under denaturing conditions. Following electrophoresis proteins were transferred to Hybond N membrane (Amersham) in semi-dry blotter (Bio-Rad) at 15 V for 30 min. Western blotting was conducted according to standard procedures using rabbit anti-GFP and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies or goat anti-actin and HRP-conjugated donkey anti-goat antibodies (Santa Cruz Biotechnology). Chemiluminescent detection was performed using ECL kit (Amersham) according to manufacturer's protocol.

Microscopy

RBL-2H3 cells were seeded in LabTek chambered coverslips (Nunc) at the density of $5x10^4$ cells/cm^2. For fixation medium was removed, and adhered cells were fixed in 4% formaldehyde/PBS. For anti-RMCP-II staining all steps were performed in 0.05% saponin/3% BSA/PBS. Briefly, cells were pre-permeabilized for 15 min at room temperature and incubated for 1 h at room temperature with 1:100 sheep anti-RMCP-II polyclonal antibodies (Moredun Scientific). Following thorough washing, cells were incubated for 1 h at room temperature with 1:200 Texas Red-conjugated rabbit anti-sheep $F(ab')_2$ (Jackson ImmunoResearch) and washed. For colocalization with TR-D cells were incubated for 1 h at room temperature with 1:200 goat anti-sheep HRP-conjugated antibodies followed by incubation with Alexa 488-coupled tyramide (Molecular Probes). For 5-HT staining, cells were incubated with 200 μM 5-HT for 16 h, fixed and stained in 0.1% Triton X-100/5% FBS/PBS, 1:1000 rabbit anti-serotonin (Sigma) and 1:5000 Texas Red-conjugated goat anti-rabbit $F(ab')_2$ (Jackson ImmunoResearch) and washed. For TR-D (Molecular Probes) live observations, cells were incubated in medium with dextran (100-500 ng/ml) for the indicated times, washed and resuspended in fresh phenol red-free medium. Nuclei were stained by 10-min incubation in 1 μM TO-PRO-3 (Molecular Probes).

C57.1 cells were stained in suspension following the above procedures, centrifuged, resuspended in PBS, transferred to LabTek chambers and subjected to microscopy. After final washing, cells were mounted in 5 mg/ml DABCO (Sigma)/ Fluoromount G (Southern Biotech). Fluorescence microscopy, image acquisition and analysis was performed using Olympus IX70 inverted microscope (UPLAPO 100x oil immersion objective), FView II cooled CCD monochrome camera (Soft Imaging System GmbH, Germany), bandpass filter sets for CFP, GFP, YFP and Texas Red (Chroma) and AnalySIS 3.2 software (Soft Imaging System). Multicolour reconstruction was also

performed in AnalySIS 3.2 software. Confocal microscopy was performed on Leica TCS SP2 AOBS microscope. Image processing was performed in Leica Confocal Software package. Additional processing was performed using NIH ImageJ software.

All monochrome images with exception of images in Fig. 6A-C are presented in unprocessed form. In processed images, linear transformation of brightness/contrast was performed to enhance details. For visualization purposes, colour restoration in multicolour images was accompanied by linear adjustments of brightness/contrast.

Sequence analysis

Predictions of N-linked glycosylation and O-linked glycosylation sites were performed using software hosted on Center of Biological Sequence Analysis, Technical University of Denmark DTU (http://www.cbs.dtu.dk/services).

Acknowledgements

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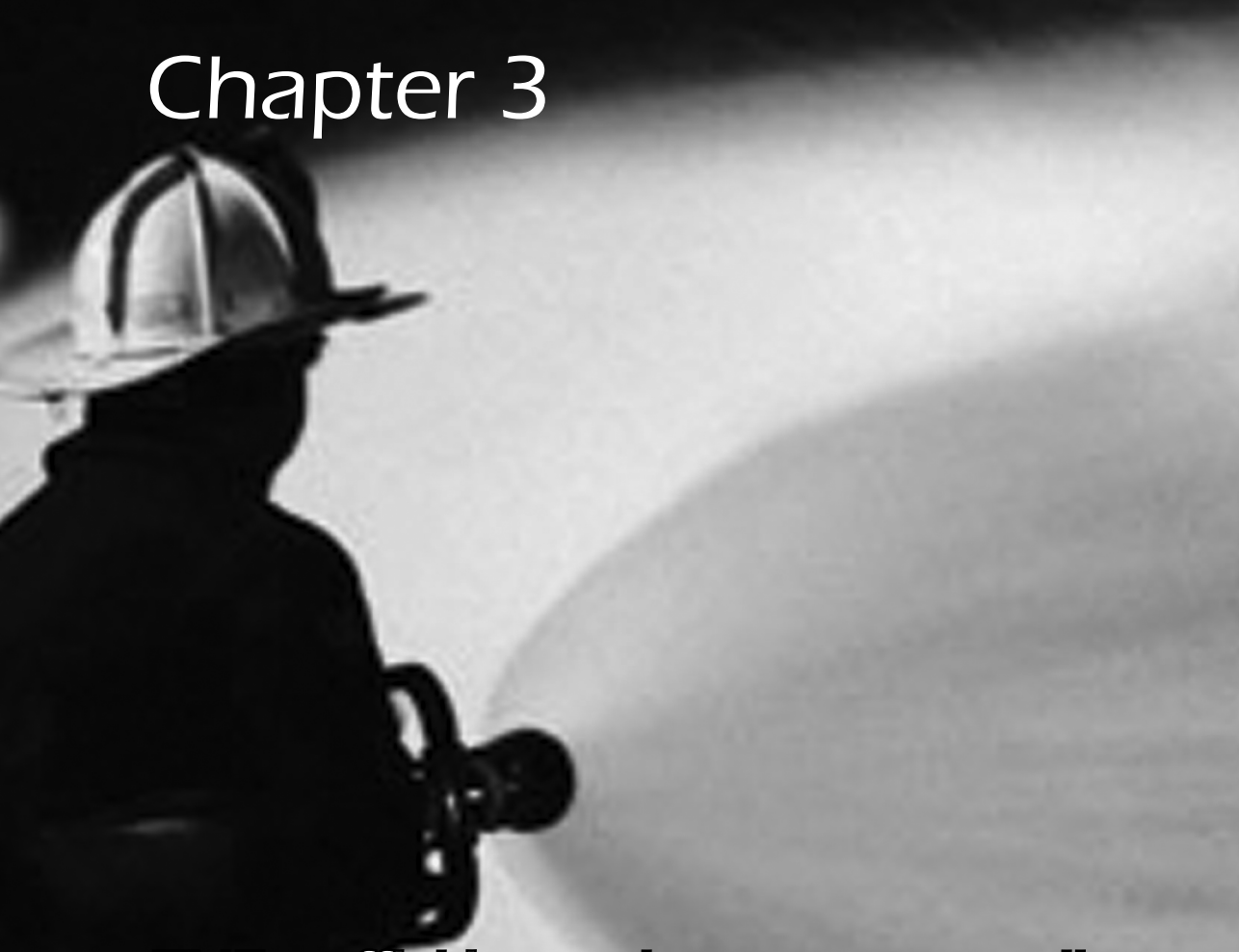
Chapter 3



TNF trafficking to human mast cell granules: mature chain-dependent endocytosis

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Abstract

Mast cells play crucial role at early stages of immune response against bacteria and parasites where their functionality is based on their capability of releasing highly bioactive compounds, amongst them TNF. Mast cells are considered the only cells storing preformed TNF which allows for immediate release of this cytokine upon contact with pathogens. We approached the question of mechanisms and amino acid motifs directing newly synthesized TNF for storage in cytoplasmic granules by analyzing trafficking of a series of TNF-EGFP fusion proteins in human mast cell lines HMC-1 and LAD2. Protein covering full TNF sequence was successfully sorted into secretory granules in a process involving transient exposure on the outer membrane and reendocytosis. In human cells, contrary to results obtained in a rodent model, TNF seems not to be glycosylated and thus trafficking is carbohydrate-independent.

In an effort to localize the amino acid motif responsible for granule targeting, we constructed additional fusion proteins and analyzed their trafficking, concluding that granule targeting sequences are localized in the mature chain of TNF and that the cytoplasmic tail is expendable for endocytotic sorting of this cytokine thus excluding direct interactions with intracellular adaptor proteins.

Introduction

Mast cells are an important element of both innate and acquired immunity. Upon appropriate stimulation cells undergo activation and subsequent degranulation, releasing and synthesizing highly bioactive, proinflammatory, vasodilatative, chemotactic and cytotoxic substances. One class of mast cell mediators are cytokines, amongst them TNF. As shown in a model of bacteria-induced inflammation, deprivation of the mast cell-derived pool of TNF leads to significantly reduced influx of neutrophils and increased mortality (1, 2). In addition, in the reverse Arthus reaction animal model the critical importance of mast cell-derived TNF was ascertained (3, 4). The important role of mast cells as a source of TNF may be explained by their unique capability of storing preformed cytokine in cytoplasmic granules, which allows for its immediate release upon stimulation (5, 6). Several hours after initial stimulation and degranulation-related release of TNF, mast cells synthesize and release additional amounts of TNF by direct vesicular transport from Golgi apparatus to the plasma membrane (7, 8). The mechanism of TNF trafficking has been studied in macrophages, revealing that TNF is synthesized as a 26 kDa transmembrane precursor protein (tmTNF) and later on proteolytically processed to 17 kDa soluble form (sTNF). Conversion of tmTNF into sTNF takes place en route from endoplasmatic reticulum to the plasma membrane (9) and is catalyzed by TNF converting enzyme (TACE/ADAM17).

The mechanisms regulating TNF trafficking in mast cells have not been studied extensively. Particularly little is known about the nature of the switch directing TNF protein either into cytoplasmic granules or directly to the cell membrane, which might depend on the cellular activation status.

We have shown previously (10) that in rodent mast cells TNF sorting to cytoplasmic granules largely depends on N-linked glycosylation and MPR (mannose-6-phosphate receptor)-based system. While major pieces of evidence for specific biological function of TNF stored in mast cell granules come from a rodent model the existence of similar pool of this cytokine in many subsets of human mast cells has been reported (11-13). There is growing body of evidence, that human mast cell-derived TNF plays a critical role in certain physiological and pathological processes including vascular diseases (14, 15), host defense (16), tissue remodeling (17, 18) and various chronic inflammatory states (19-21).

Both the pleiotropic nature of TNF and its potential as a drug target prompted us to explore the mechanisms of trafficking of this cytokine into cytoplasmic granules in human mast cells. Analysis of amino acid sequence of human TNF reveals that the N86 asparagine residue that is glycosylated in rodents is replaced by threonine and thus no N-linked glycosylation, important for trafficking of this molecule in rodent mast cells, is possible. In this work we provide evidence for existence of specific ER-entry and cytoplasmic granules targeting motifs in human TNF and show

that in human mast cells a fusion protein composed of full-length TNF fused to EGFP (Enhanced Green Fluorescent Protein) on its way to cytoplasmic granules is transiently exposed on the outer membrane.

Results

Human TNF is not modified with sialic acid

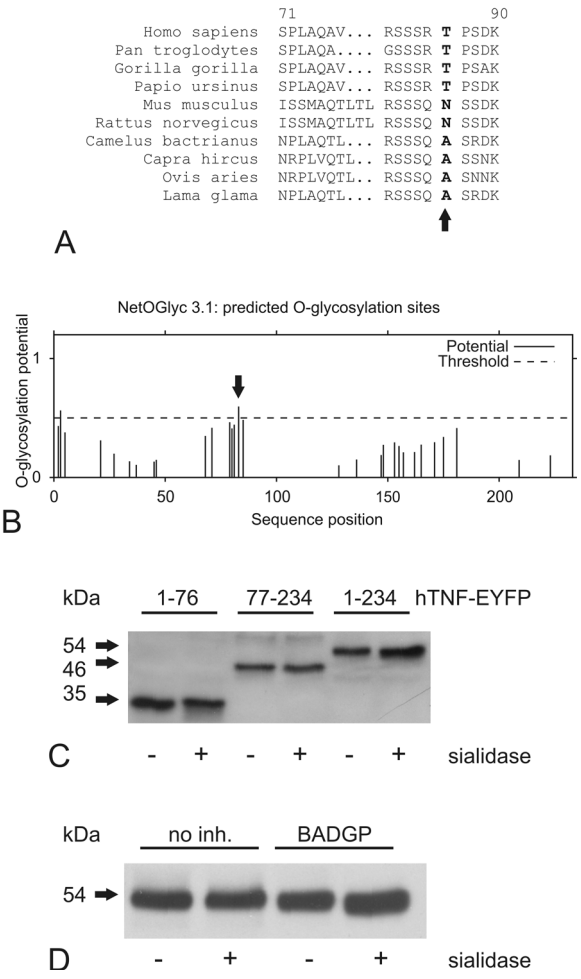


Figure 1 TNF glycosylation analysis. (A) TNF sequences were compared across species showing evolution from N-glycosylation (N86 residue in rodents) to potential O-glycosylation (T83 in primates, indicated by arrow) (B) Neural network prediction of O-glycosylation of human TNF. T83 residue indicated by arrow (C) Western blot analysis of lysates HMC-1 cells transfected with p1-76, p77-234 and p1-234hTNF-EGFP and treated with sialidase. (D) Western blot analysis of lysates HMC-1 cells transfected with p1-234hTNF-EGFP, cultured in the presence or absence of 2 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside and treated with sialidase

We have shown before that in rodent mast cells TNF delivery to mast cell granules largely depends on N-linked glycosylation (10). Phylogenetic protein sequence analysis showed, however, that the N-terminal region of sTNF is one of the least conserved and that the N-linked glycosylation motif NSS is unique to rodents and in primates is replaced by RTP motif (Fig. 1A). Sequence context-based prediction indicated that in human TNF T83 residue could be glycosylated (Fig. 1B). To test this prediction HMC-1 cells were transfected with p1-76hTNF-EGFP, p77-234hTNF-EGFP or p1-234hTNF-EGFP and lysed 24 hours after transfection .The former two were used as negative controls due to lack of the glycosylation target motif and improper subcellular localization, respectively. Lysates were treated with neuraminidase and analyzed by Western blot. Proteins of apparent molecular masses of 35, 46 and 54 kDa were detected in lysates of cells transfected with p1-76-, p77-234- and p1-234hTNF-EGFP. Regardless of the lysate tested, the protein molecular weight did not change following neuraminidase treatment which suggests the protein is not sialylated (Fig. 1C). To substantiate that result HMC-1 cells were transfected with p1-234hTNF-EGFP and following transfection cultured in the presence of 2 mM benzyl

2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP), which serves as a competitive substrate for N-acetyl-β-D-glucosaminyltransferase and inhibits O-linked glycosylation (25). 24 hours after transfection cells were lysed; lysates were treated with neuraminidase and analyzed by Western blot. Regardless of BADGP and neuraminidase treatment in all lysates a protein of apparent molecular mass of 54 kDa was detected (Fig. 1D). Since majority of O-linked carbohydrate chains contain sialic acid as a terminal residue, these results strongly suggest that in HMC-1 cells TNF is not O-glycosylated. This is further supported by a good agreement between calculated and observed molecular weight of TNF expressed in HMC-1 cells.

Intracellular localization TNF-EGFP fusion proteins in mast cells

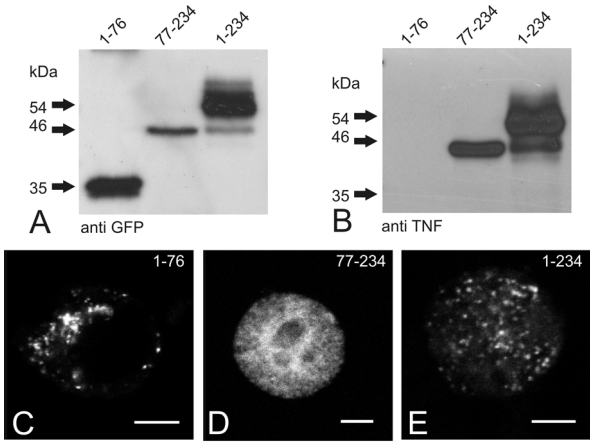
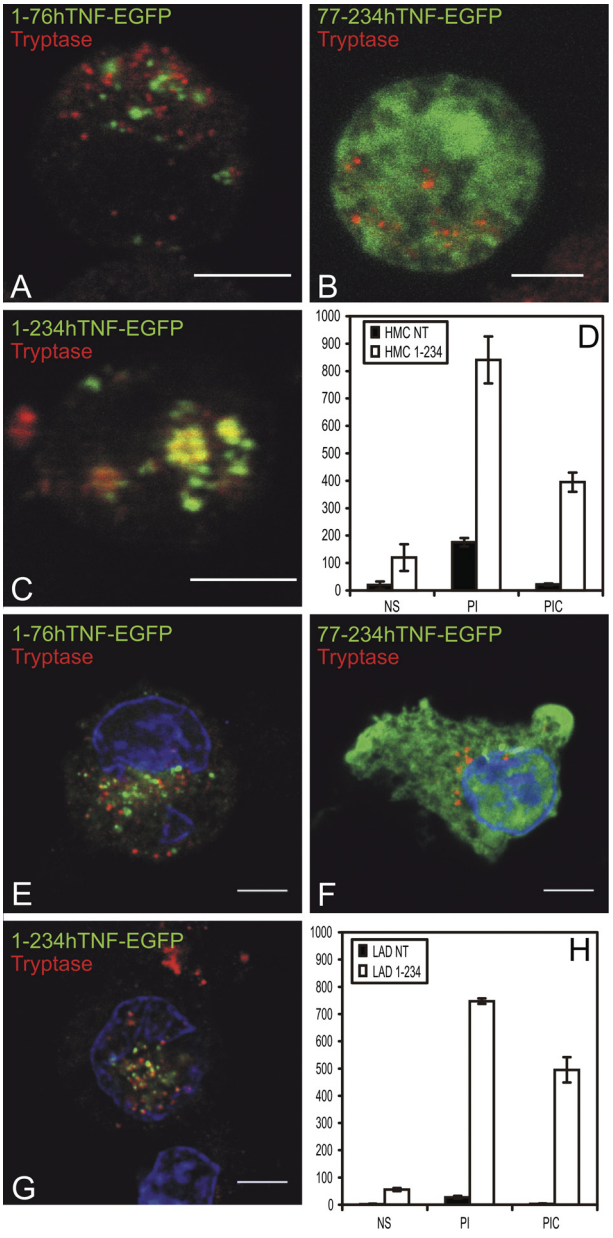


Figure 2 Expression and intracellular localization of TNF-EGFP fusion proteins. (A-B) HMC-1 cells were transfected with p1-76, p77-234 or p1-234hTNF-EGFP and lysed 24 hours post-transfection; lysates were analyzed by Western blot using anti-GFP and anti-TNF primary antibodies. (C-E) HMC-1 cells were transfected with p1-76, p77-234 or p1-234hTNF-EGFP and imaged under confocal microscope 24 hours post-transfection. Representative confocal planes are presented. Bars represent 5 μm.

HMC-1 cells were transiently transfected with p1-76hTNF-EGFP, p77-234hTNF-EGFP or p1-234hTNF-EGFP plasmids and 24 hours post-transfection cell lysates were analyzed by Western blot (Fig. 2A-B). In lysates of p1-76hTNF-EGFP-transfected cells a protein of apparent molecular mass of 35 kDa could be detected using anti-GFP antibodies whereas no bands were detected with antibodies directed against sTNF. In lysates of p77-234hTNF-EGFP-transfected cells a protein of apparent molecular mass of 46 kDa was detected with both anti-TNF and anti-GFP antibodies. In lysates of p1-234hTNF-EGFP-transfected cells a major band of apparent molecular mass of 54 kDa and minor bands of 46 kDa and 56 kDa were detected. Thus, proper expression of TNF-EGFP fusion proteins has been confirmed, additionally revealing possible proteolytic processing and posttranslational modification of 1-234hTNF-EGFP (46 and 56 kDa bands, respectively). In parallel, green fluorescence patterns in HMC-1 cells transiently transfected with p1-76hTNF-EGFP, p77-234hTNF-EGFP or p1-234hTNF-EGFP were analyzed by confocal microscopy at 3, 10 and 24 hours posttransfection. At early timepoint (3h) in both p1-76- and p1-234hTNF-EGFP-transfected cells fluorescence could be observed in a perinuclear organelle morphologically resembling Golgi apparatus, whereas in p77-234hTNF-EGFP-transfected cells fluorescence was distributed rather evenly (data not shown) and the distribution of 77-234hTNF-EGFP did not change for the whole observation period of 24 hours posttransfection (Fig. 2 D). In contrast 10 hours posttransfection in p1-76- and p1-234hTNF-EGFP-transfected cells vesicular fluorescent structures

could be observed (data not shown) and after 24 hours the number of these structures has increased while fluorescence in Golgi-resembling structure has diminished, probably due to transient transfection and decreasing expression of a transgene (Fig. 2C and E). Thus, both propeptide (1-76) and full-length (1-234) TNF sequences, as opposed to soluble TNF (77-234) were shown capable of sorting EGFP into vesicular intracellular structures.

1-234hTNF-EGFP is sorted to and released from mast cell granules



In order to establish whether fluorescent vesicular structures observed following transfection of mast cells with p1-76hTNF-EGFP and p1-234hTNF-EGFP indeed are mast cell granules, intracellular localization of the three fusion proteins and an established mast cell granule compound, tryptase, was compared. HMC-1 and LAD2 cells were transfected with p1-76hTNF-EGFP, p77-234 hTNF-EGFP or p1-234hTNF-EGFP and 24 hours after transfection stained for tryptase. Cells expressing 1-76hTNF-EGFP exhibited vesicular patterns in

Figure 3 1-234hTNF-EGFP is sorted to and released from mast cell granules. (A-C) HMC-1 cells and (E-G) LAD2 cells were transfected with p1-76, p77-234 or p1-234hTNF-EGFP (green channel) and 24 hours post-transfection fixed and stained for tryptase (red channel). Colocalization is represented by yellow color. For LAD2 cells nuclei are visualized by TO-PRO3 stain (blue channel). Representative confocal planes are shown. Bars represent 5μm. (D, H) ELISA analysis of TNF release from HMC-1 and LAD2 cells, respectively. Non-transfected cells (NT), cells transfected with p1-234hTNF-EGFP (1-234). Stimuli: non-stimulated (NS), PMA/ionomycin (PI), PMA/ionomycin/cycloheximide (PIC). Supernatants for analysis were collected 60 minutes following stimulation. For detailed description see text. The differences in TNF release resulting from transfection or treatments, measured at 1 hour time-point were found statistically significant.

both green and red fluorescence channels (EGFP and tryptase, respectively) but hardly any colocalization was observed (Fig. 3A and E for HMC-1 and LAD2, respectively). In cells transfected with p77-234hTNF-EGFP mostly a diffuse pattern of fluorescence was observed in EGFP channel whereas tryptase stain revealed vesicular pattern (Fig. 3B and F for HMC-1 and LAD2, respectively). No specific colocalization of 77-234hTNF-EGFP and tryptase was observed in any of the cell lines. Transfection of cells with p1-234hTNF-EGFP resulted in accumulation of EGFP fluorescence in vesicular structures most of which also stained positive for tryptase (Fig. 3C and G for HMC-1 and LAD2, respectively) identifying the compartment as mast cell granules.

In order to test whether the function of granules is not disturbed by directing exogenously expressed TNF-EGFP fusion protein into this compartment, TNF release from stimulated mast cells was assessed. HMC-1 and LAD2 cells were transfected with p1-234hTNF-EGFP and cultured for 24 hours; a subset of cells was then incubated with 20 μM CHX for 60 minutes. For TNF release cells were incubated for 60 minutes in culture medium alone, in culture medium containing 100 ng/ml PMA and 1 μM ionomycin, or in culture medium containing both stimuli and 20 μM CHX . Non-transfected HMC-1 cells and cells expressing 1-234hTNF-EGFP, following treatment with PMA/Ionomycin, released 176 and 841 pg of TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 22 and 402 pg TNF /106 cells. Basal release from non-stimulated cells was 20 and 120 pg of TNF /106 cells for non-transfected and transfected cells, respectively (Fig. 3D). The amounts of TNF released by HMC-1 cells within 30 minutes from stimulation, while not in all cases significantly different between transfection groups and treatments, fit the trend that is set by the statistically significant differences measured 60 minutes of stimulation thus supporting the presence of preformed cytokine in the cells (data not shown). Non-transfected LAD2 cells and cells expressing 1-234hTNF-EGFP, following treatment with PMA/Ionomycin, released 27 and 747 pg of TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 3 and 495 pg TNF /106 cells. Basal release from non-stimulated cells was 2 and 27 pg of TNF /106 cells for non-transfected and transfected cells, respectively (Fig. 3H).

These results demonstrated that 1-234hTNF fusion protein is indeed sorted to, and released from mast cell granules and raised the question of the trafficking route utilized for delivery of 1-234hTNF-EGFP to this compartment.

1-234hTNF-EGFP is exposed on the cell membrane on its way to granules

In absence of carbohydrate residues TNF in HMC-1 cells might not be efficiently retrieved from the secretory pathway and thus become exposed on the cell membrane and eventually re-internalized for granule delivery. To test this hypothesis HMC-1 cells were transfected with p1-234hTNF-EGFP and 12 hours posttransfection surface proteins were biotinylated. Subsequently, cells were disrupted (control) or cultured for another 8 hours and disrupted. Cellular organelles were separated by centrifugation on a Percoll gradient and β-hexosaminidase (granular enzyme) activity in these fractions was determined to localize the granule-containing fraction (Fig. 4A).

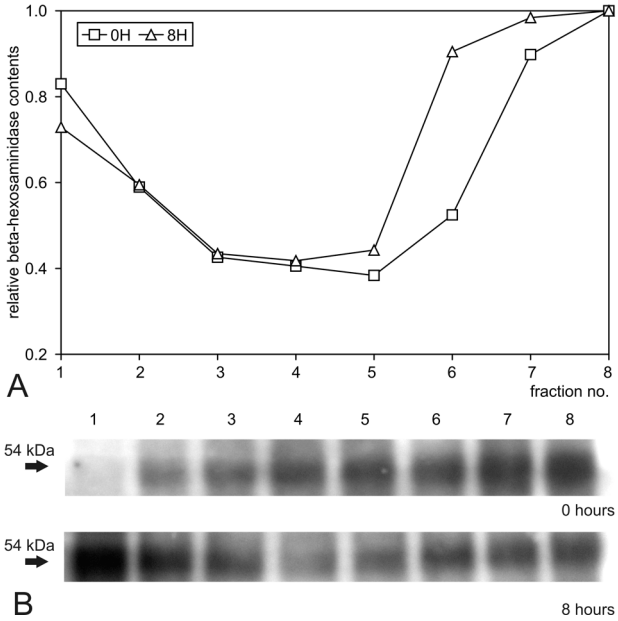


Figure 4 1-234hTNF-EGFP is exposed on the outer membrane on the way to the granules. Cell surface of HMC-1 cells transfected with p1-234hTNF-EGFP was biotinylated 12 hours post-transfection. Cells were disrupted immediately (0H) or following 8 hours chase (8H) and subject to subcellular fractionation. (A) beta-hexosaminidase activity in fractions from heaviest (1) to lightest (8); granule-associated activity in fractions 1-2. (B) anti-GFP Western blot analysis of biotinylated proteins immunoprecipitated from fractions by streptavidin beads.

Additionally, biotinylated proteins were immuno precipitated from fractions using streptavidin-sepharose and analyzed by Western blot using anti-EGFP antibodies. This allowed for detection of EGFP-containing proteins that were exposed on the outer membrane. In fractions obtained from cells disrupted directly after biotinylation, the majority of biotinylated, EGFP-containing protein was detected in light fractions (6-8) that represent cytosol and cell membrane. In fractions obtained from cells that were cultured for 8 hours before disruption, the majority of this protein was found in fractions 1 and 2, containing heavier, β-hexosaminidase-containing organelles, including mast cell granules (Fig. 4B). This indicates that during the 8 hours chase period 1-234hTNF-EGFP protein was translocated from the cell membrane into mast cell granules, though the mechanism of this translocation is not known.

N-terminal domain is dispensable for targeting of TNF to mast cell granules

We addressed the question of processes and amino acid motifs responsible for endocytosis and further trafficking of 1-234hTNF-EGFP to mast cell granules. Deletion mutants 21-234hTNF-EYFP and 46-234hTNF-EYFP were employed to localize the region responsible for endoplasmatic reticulum entry and granular targeting of TNF. Expression of 1-, 21- and 46-234hTNF-EYFP was examined by transfection of HMC-1 cells with respective plasmids and Western blot analysis of lysates with the use of both anti-GFP and anti-TNF antibodies. Both antibodies detected proteins of apparent molecular masses of 54, 52 and 49 kDa in lysates of cells transfected with p1-, 21- and 46-234hTNF-EYFP, respectively (Fig. 5A-B). In order to analyze intracellular localization of truncated mutant proteins HMC-1 cells were cotransfected with p1-234hTNF-ECFP, employed as a granular marker, and p21-234hTNF-EYFP or p46-234hTNF-EYFP or p1-234hTNF-EYFP. 24 hours posttransfection cells were imaged under confocal microscope. Both 21-234hTNF-EYFP and 1-234hTNF-EYFP exhibited granular fluorescence pattern and colocalized with 1-234hTNF-ECFP in mast cell granules (Fig. 5 C and D). In contrast cells expressing 46-234hTNF-EYFP exhibited

diffuse cytoplasmic and nuclear fluorescence with weakly fluorescent vesicles in yellow channel and granular fluorescence in cyan channel. Vesicular structures visible in yellow channel colocalized well with 1-234hTNF-ECFP which identified them as mast cell granules (Fig. 5E).

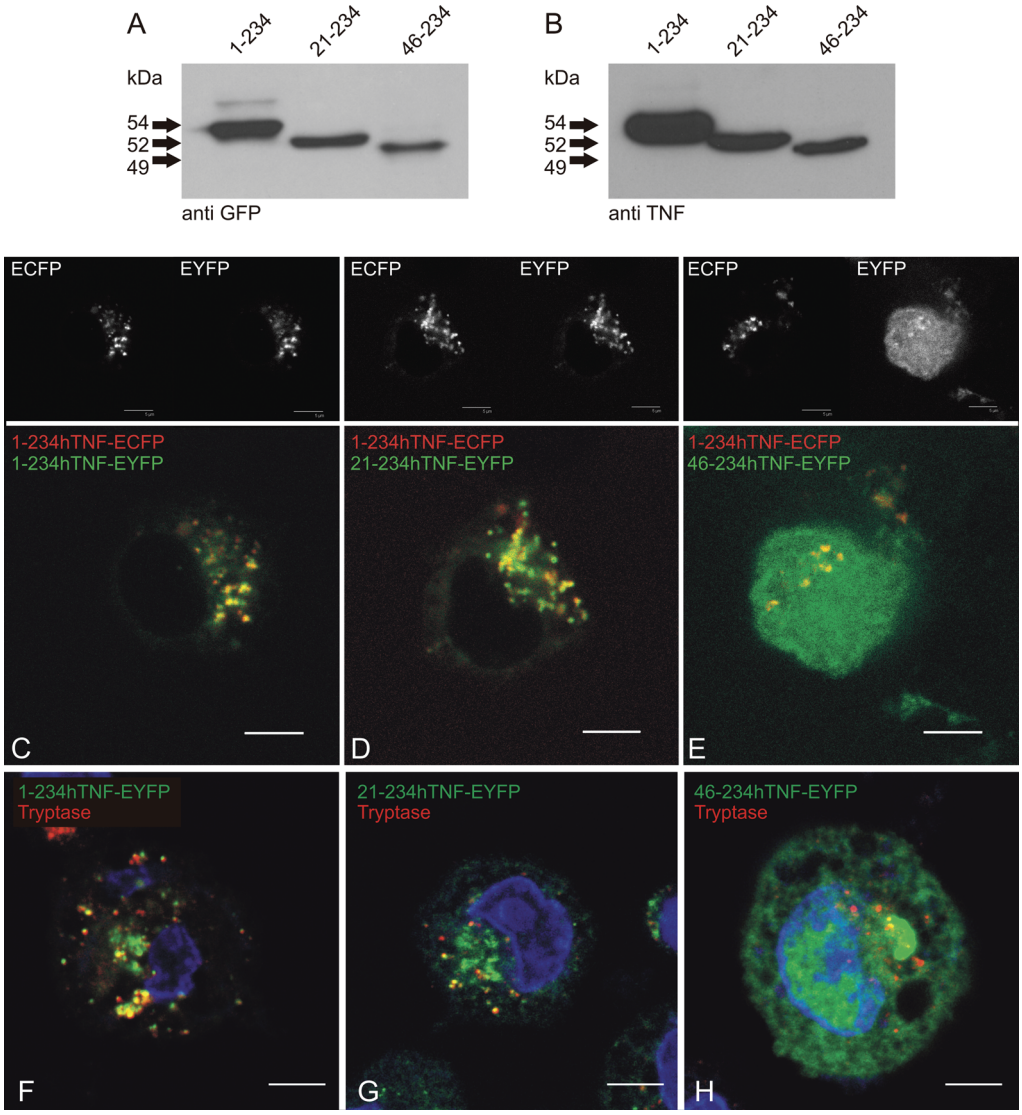


Figure 5 N-terminal domain is dispensable for granular targeting of TNF. (A-B) anti-GFP and anti-TNF Western blot analysis of lysates of HMC-1 cells transfected with p1-, 21- or 46-234hTNF-EYFP. (C-E) confocal microscopy analysis of HMC-1 cells cotransfected with p1-234hTNF-ECFP and p1-, 21- or 46-234hTNF-EYFP and imaged 24 hours post-transfection. In overlay images ECFP is represented by false color red and EYFP by false color green. Colocalization is represented by yellow color. Representative confocal sections are shown. Bars represent 5 µm. (F-H) confocal microscopy analysis of LAD2 cells transfected with p1-, 21- or 46-234hTNF-EYFP and 24 hours posttransfection stained for tryptase. In overlay images EYFP is represented by false color green, tryptase stain by false color red and nuclei are visualized by TO-PRO3 stain (blue channel). Colocalization of EYFP and tryptase is represented by yellow color. Representative confocal sections are shown. Bars represent 5 µm.

In order to ascertain these results LAD2 cells were transfected with p1-, p21- or p46-234hTNF-EYFP and 24 hours post-transfection stained for tryptase. In cells transfected with p1-234hTNF-EYFP or p21-234hTNF-EYFP vesicular pattern of fluorescence was observed in green channel (EYFP) which colocalized well with granules visualized by tryptase staining (red channel) (Fig. 5F and G). Conversely, cells transfected with p46-234hTNF-EYFP exhibited diffuse cytoplasmic and nuclear pattern in the green channel with no specific colocalization observed between 46-234hTNF-EYFP and tryptase (Fig. 5H).

These data indicate that N-terminal 20 amino acids of TNF are dispensable for delivery of this protein to mast cell granules and that 21-45 region of TNF is necessary for its efficient endoplasmatic reticulum entry and subsequent trafficking.

1-234- and 21-234- but not 46-234hTNF-EGFP are actively released from cells

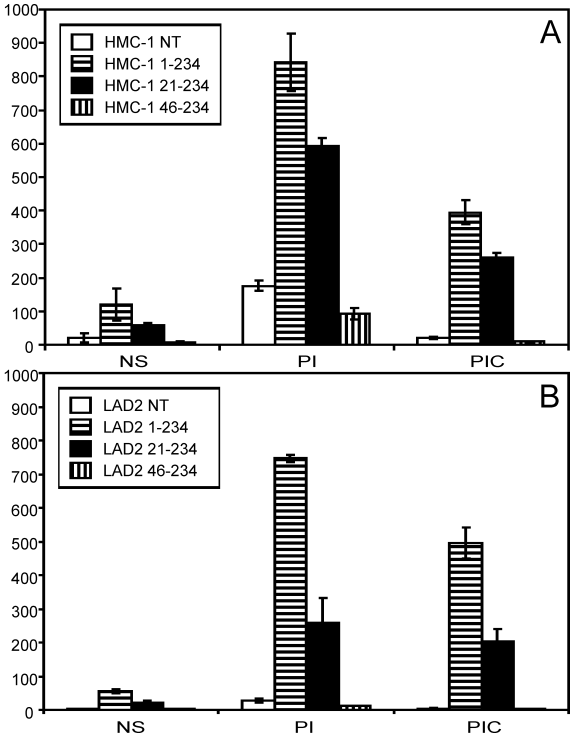


Figure 6 1- and 21-234 but not 46-234 TNF-GFP are actively released from cells. (A-B) ELISA analysis of TNF release from HMC-1 and LAD2 cells. Non-transfected cells (NT), cells transfected with p1-, 21- or 46-234hTNF-EGFP (1-234, 21-234, 46-234 respectively). Stimuli: non-stimulated (NS), PMA/ionomycin (PI), PMA/ionomycin/cycloheximide (PIC). Supernatants for TNF concentration measurements were collected from HMC-1 and LAD2 cultures 60 minutes post-stimulation (A and B, respectively). For detailed description see text. The differences in TNF release resulting from transfection or treatments, measured at 1 hour time-point were found statistically significant.

Mast cells' ability to release N-terminal deletion mutants of TNF-EGFP upon stimulation was assayed. HMC-1 and LAD2 cells were transfected with p46-234hTNF-EGFP, p21-234hTNF-EGFP or p1-234hTNF-EGFP and cultured for 24 hours; a subset of cells was then incubated with 20 μM CHX for 60 minutes. For TNF release cells were incubated in culture medium alone, in culture medium containing 100 ng/ml PMA and 1 μM ionomycin or in culture medium containing both stimuli and 20 μM CHX . After 60 minutes of stimulation and/or inhibition, culture supernatants were collected and TNF concentration was assayed. Results obtained for HMC-1 cells are shown in Fig. 6A. Cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP or non-transfected cells following treatment with PMA/Ionomycin released 92, 593, 854 and 162 pg TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 9, 262, 410 and 17 pg TNF /106 cells for respective transfectants. Basal

release from non-stimulated cells was 6, 59, 123 and 16 pg TNF /106 cells for cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP and non-transfected cells, respectively. The amounts of TNF released by HMC-1 cells within 30 minutes from stimulation, while not in all cases significantly different between transfection groups and treatments, fit the trend that is set by the statistically significant differences measured 60 minutes of stimulation (data not shown). TNF concentrations measured in LAD2 culture supernatants are shown in Fig. 6B. Cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP or non-transfected cells following treatment with PMA/Ionomycin released 11, 258, 747 and 27 pg TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 3, 203, 495 and 3 pg TNF /106 cells for respective transfectants. Basal release from non-stimulated cells was 3, 22, 55 and 2 pg TNF /106 cells for cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP and non-transfected cells, respectively.

These data show that both 21-234hTNF-EGFP and 1-234hTNF-EGFP are delivered to mast cell granules and released upon stimulation although in the case of 21-234hTNF-EGFP this process is less efficient as compared to 1-234hTNF-EGFP.

Discussion

Among many cytokine-releasing cells mast cells are the only ones known to store preformed TNF in their cytoplasmic granules and release it upon activation. While intracellular trafficking, processing and release of TNF were investigated in macrophages (26), the noticeable difference is that in mast cells there apparently operate mechanisms responsible for diverting TNF from the constitutive secretory pathway to mast cell granules.

We have shown recently, that in rodent mast cells TNF trafficking to cytoplasmic granules is dependent on N-linked glycosylation (10). This mechanism however does not seem to be evolutionarily conserved, since it is predominantly found in Rodentia while in Artiodactyla N86 residue is replaced by alanine which excludes any glycosylation. In contrast in Primates the N-linked glycosylation motif is replaced by potential O-linked glycosylation site (Fig. 1A). This posttranslational modification may also be involved in protein trafficking (25, 27) but its presence is more context-dependent than N-linked glycosylation. Neural network-based computer prediction (28) indicated that in human TNF T83 residue might be glycosylated (Fig. 1B). Our results obtained by two different approaches suggest opposite (Fig. 1 C-D). It has to be noted, however, that posttranslational modifications may be cell lineage-specific as O-glycosylation of TNF in human B-cell lymphoblastoid cell line BALL-1 has been reported (29). This might reflect involvement of differential, cell line-specific trafficking mechanisms for delivery of the protein cargo to the target compartment such as mast cell granules, or release to extracellular space.

EGFP fusion proteins have been successfully employed to follow intracellular trafficking of many proteins, including mast cell granular proteins CD63, VAMP-7, syntaxin-3 and phospholipase D (30-33). In this work we chose a similar strategy

and transiently transfected human mast cell line HMC-1 or LAD2 with several TNF-EGFP fusion proteins to visualize intracellular trafficking of TNF in these cells. HMC-1 cells are often utilized as a model system for investigation of mast cell biology, including cytokine physiology (34-36). It has to be mentioned, however, that these cells lack several phenotypic features that are characteristic for normal human mast cells, including high-affinity IgE receptors (34), and thus conclusions concerning human mast cells have to be drawn with caution. LAD2 is a recently developed cell line that closely resembles primary culture of CD34+-derived human mast cells (23). Cell surface marker expression, enzyme contents and functional FcεRI and FcγRI receptors make it a good model system for mast cells investigation. Low growth rate, possibly allowing LAD2 cells to exhibit a more mature phenotype, precludes experiments requiring large amounts of cells such as subcellular fractionation presented in Fig. 4.

The EGFP fused to full-length TNF, when expressed in both HMC-1 and LAD2 cells, accumulated predominantly in cytoplasmic granules, as visualized by colocalization with tryptase, a mast cell granular marker (Fig. 3C and G, respectively). This was not the case for fusion proteins consisting of either TNF propeptide or mature chain fused to EGFP (1-76hTNF-EGFP or 77-234hTNF-EGFP). The former produced vesicular pattern, but organelles it accumulated in apparently are not mast cell granules, as shown by lack of significant colocalization with tryptase (Fig. 3A and E, respectively). This suggests that the protein enters endoplasmatic reticulum, but due to lack of additional signals located in mature chain it is missorted. Fusion protein consisting of mature chain fused directly to EGFP exhibits diffuse fluorescence pattern (Fig. 3B and F, respectively) which indicates that in absence of propeptide, ER entry is impaired and hypothesized granular targeting motifs cannot act. Observed differences in amounts of TNF released by non-transfected and p1-234hTNF-EGFP-transfected cells, even in the presence of CHX, suggest that the fusion protein is not only sorted to, but also efficiently released from mast cell granules.

Because human TNF expressed in HMC-1 cells apparently is not glycosylated it cannot be retrieved from the secretory pathway by carbohydrate-dependent mechanism and might reach the outer membrane. We tested this hypothesis and showed that TNF is transiently exposed on the outer membrane, reendocytosed and ultimately delivered to mast cell granules (Fig. 4). A protein that reaches cell membrane can be reendocytosed through interactions of its cytoplasmic tail with a cytoplasmic protein (reviewed in (37)), by an extracellular receptor (38) or passively with the membrane. Endocytosis of uncleaved TNF has been demonstrated in macrophages (39) and we addressed the question of molecular features involved in reendocytosis of TNF leading to its storage in mast cell granules.

Using EYFP fusion proteins we have shown that truncation of the 20 N-terminal amino acids, constituting majority of the cytoplasmic tail of TNF, does not interfere with its transport to mast cell granules (Fig. 5D and G). Contrarily, deletion of 45 N-terminal amino acids dramatically decreases TNF ER entry although the fraction of 46-234hTNF-EGFP that is translocated into ER (only in HMC-1 cells) seems to

be sorted to mast cell granules (Fig. 5E and H). This localizes an ER-entry motif to 21-45 region of TNF which is in agreement with earlier data obtained in vitro (40). Sorting of 21-234hTNF-EYFP to mast cell granules suggests that TNF granular targeting is mediated by a mechanism that is operating on the outer side of the cell membrane, independently of the cytoplasmic tail of the cytokine. This is further supported by the observation, that 1-76hTNF-EGFP is not sorted to granules, despite its complete cytoplasmic tail. It has to be noted, however, that palmitoylation of human TNF on C30 has been reported (41) and a regulatory role for the lipid residue has been postulated with regard to endocytosis efficiency (42, 43). This reversible posttranslational modification could provide additional level of regulation of TNF endocytosis and granule delivery rate.

Functional characterization of the target compartment reached by 1-, 21- and 46-234hTNF-EYFP as compared to endogenous TNF revealed, that upon stimulation in the presence of CHX mast cells expressing 1- or 21-234hTNF-EYFP release significantly more TNF than cells that are non-transfected or transfected with 46-234hTNF-EYFP (Fig. 6). These data indicate that in absence of majority of its cytoplasmic tail TNF is still efficiently delivered to, stored in and released from mast cell granules.

In this report we present data indicating that glycosylation is not necessary for efficient sorting and release of TNF from human mast cell granules. We also identify a new trafficking route of this cytokine in human mast cells which involves transient exposure to extracellular space followed by endocytosis. The exact nature of this endocytotic mechanism is not known, but the data gathered suggest a mechanism involving recognition of mature chain by a hypothetical receptor mediating delivery to granules. Based on the data presented here it might be hypothesized that regulatory mechanism diverting TNF from constitutive secretory pathway to mast cell granules might be TNF cleavage-dependent. Resting cells express low levels of TNF converting enzyme (TACE) and uncleaved TNF is endocytosed and stored in mast cell granules. Following activation TACE is induced and released into extracellular space (44, 45). Consequently, TNF is processed as it follows the secretory pathway and also during exposure on cell membrane which results in increased release into environment.

Further studies will be required to test the above hypothesis. Identification of the motif recognized in the TNF molecule by the granule-targeting endocytotic mechanism will be particularly important, because interference with the mechanism of TNF trafficking is of potential therapeutic relevance for mast cell-derived TNF-related diseases (46, 47) and reviewed in (48)).

Materials and methods

Plasmid construction

The DNA fragment coding for TNF was obtained by RT-PCR. Briefly, 5 μg of total human RNA (isolated from HMC-1 cell line) was subject to RT reaction, using MMLV reverse transcriptase (Gibco) and random hexamer oligonucleotides in a final volume of 20 μl. cDNA was amplified using AccuTaq polymerase (Sigma) and GGAAAGGACACCATGAGCACTG and GTCCTCCTCACAGGGCAATGAT oligonucleotides

(all oligonucleotide sequences are given in 5' to 3' direction). Product of PCR reaction was cloned into pT-Adv plasmid (Clontech) resulting in plasmid pTA-hTNF. pTA-hTNF was sequenced to verify the integrity of the TNF reading frame. To obtain plasmids encoding for fusion proteins consisting of 1-234, 1-76, 77-234 fragments of TNF and EGFP respective fragments of TNF ORF were PCR-amplified from pTA-hTNF using Pvu polymerase (Fermentas MBI), digested with indicated restriction enzymes and in-frame cloned into pEGFP-N1, pECFP-N1 (enhanced cyan fluorescent protein) and pEYFP-N1 (enhanced yellow fluorescent protein) plasmids (Clontech) digested with XhoI and AgeI. The following forward/reverse oligonucleotide pairs were used: TATACTCGAGATGAGCACTGAAAGCATGATCCGGG/TGACCGGTAATTCGG CTGGTCCTCCTACCAG**(1-234,XhoI/AgeI)**,TATACTCGAGATGAGCACTGAAAGCATG ATCCGGG/ATGACCGGTGGGATTCCAGGACATAATCTGACTG**(1-76,XhoI/AgeI)** and GTCACTCGAGATGGTCAGATCATCTTCTCGAACCCCG/TGACCGGTAATTCGGCTGGTCCTC CTACCAG**(77-234, XhoI/AgeI**). Resulting plasmids were named p1-234, p1-76 and p77-234hTNF-ECFP (EGFP/EYFP), respectively, and sequenced to confirm the integrity of the fusion proteins' ORFs. To prepare p21-234hTNF-EYFP and p46-234hTNF-EYFP corresponding fragments of fusion reading frame were amplified from p1-234hTNF-EYFP, digested with indicated restriction enzymes and ligated into XhoI/NotI digested pEYFP-N1 (4 kbp fragment). The following forward/reverse oligonucleotide pairs were used: CTGCTCGAGGAGGCGCTCCCCAAGATGACA/AGTCGCGGCCGCTTTACTTGTACAG CTCGTCC**(21-234,XhoI/NotI)** and CTGCTCGAGGCAGGCGCCACCATGCTCTTC/AG TCGCGGCCGCTTTACTTGTACAGCTCGTCC**(46-234,XhoI/NotI)**. Resulting plasmids were sequenced to confirm the integrity of the fusion proteins ORFs.

Cell culture, transfection and ELISA

The human mast cell line HMC-1 (22) was a generous gift from Dr. J. Butterfield, (Mayo Clinic, Rochester, MN). Cells were maintained in Iscove's medium supplemented with 10% heat-inactivated FBS and 2 mM glutamine. At the density of ca. 1.5*106 cells/ml cells were diluted to 0.4*106 cells/ml. Cells were grown in 5% CO2 at 37°C in fully humidified atmosphere. All media components were purchased from Sigma. LAD2 cells (23) were a generous gift from Dr. A. S. Kirshenbaum, NIH, Bethesda, USA. Cells were cultured in serum-free media (StemPro-34, Life Technologies, Grand Island, NY) supplemented with 2mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin (complete SFM) and 100 ng/ml recombinant human stem cell factor. For transfection 25-50 µg DNA was added to 2-10*106 (HMC-1) or 5*105 (LAD2) cells in 400µl culture medium and electroporated in Gene Pulser II (Biorad) at 250V (HMC-1) or 300V (LAD2), 950µF. Immediately following transfection cells were diluted to 5*105 cells/ml in fresh medium. For TNF release/inhibitor studies cells were incubated, where applicable, with 20 µM cycloheximide (CHX) applied 60 minutes before stimulation. For stimulation 1 µM ionomycin and 100 ng/ml PMA were added for the indicated time. Cell suspension density was adjusted to 1*106 cells/ml. Viability of the cells subject to concentrations of CHX used in experiments was assessed by AnnexinV-PE/7-AAD staining and established as >90%. Non-inhibited cells exhibited 95% viability. TNF ELISA was performed using a commercial kit (Sanquin) according to manufacturer's instructions.

SDS-PAGE and Western blotting

For SDS-PAGE cells were collected and lysed in 0.2% Triton X-100 supplemented with protease inhibitor cocktail (Roche Applied Science) and 1 mM PMSF. For deglycosylation analysis cell lysate containing 10 µg total protein was treated with neuraminidase (New England Biolabs) according to manufacturer's instructions. For fusion protein expression analysis 10 µg of total lysate was loaded per lane of the gel. Samples were resolved on 8 or 10% polyacrylamide gels under denaturing conditions. Following electrophoresis proteins were transferred to Hybond N membrane (Amersham) in wet blotter (Biorad) at 400 mA for 60 min. Western blotting was conducted according to standard procedures using mouse monoclonal anti-GFP (Roche Applied Science) and HRP-conjugated goat anti-mouse (Dako) antibodies or goat anti-TNF and HRP-conjugated donkey anti-goat antibodies (both from Santa Cruz Biotechnology). Chemiluminescent detection was performed using an ECL kit (Amersham) according to manufacturer's protocol.

Subcellular fractionation

HMC-1 cells were transfected with p1-234hTNF-EGFP and 12 hours post-transfection cell surface proteins were biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology) according to manufacturer's instructions. Immediately after labeling or after indicated chase period cells were fractionated as described with minor modifications (24). Briefly, 5-10*107cells were suspended in 2 ml of 0.34 M sucrose, 5 mM HEPES (pH 7.3), 0.5 mM EDTA, 1 mM PMSF (supplemented with cocktail of protease inhibitors) and disrupted by 20 passages through a 21-gauge needle. Unbroken cells and nuclei were removed by centrifugation for 10 minutes at 700g. 6 mL of 20% Percoll in 15 mM HEPES pH 7.4/0.25 M sucrose was layered on top of a 1-mL cushion of saturated sucrose. The 700g supernatant of the cell homogenate was layered on top of the Percoll and centrifuged in SS-34 rotor at 16.4 krpm (32.000 g) for 45 minutes. Fractions (1 ml each) were collected from the bottom with a peristaltic pump. Beta-hexosaminidase activity assay in collected fractions was performed by mixing of 50 µl of 2 mM 4-Nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) in citrate buffer pH 4.5 and 50 µl of each fraction diluted with the same buffer and incubating in 37°C for 60 minutes. Following incubation 150 µl of 0.1 M carbonate buffer pH 10 was added and absorbance measured at 405 nm. Controls showing no influence of Percoll on beta-hexosaminidase assay results were performed. For biotinylated proteins distribution analysis fraction samples were diluted with phosphate buffered saline (PBS) containing 0.1 % Triton X-100 and protease inhibitors and incubated overnight at 4°C with streptavidin-sepharose beads. The beads were washed 5 times with 0.5% Triton X-100 in PBS, boiled for 5 minutes in SDS-PAGE sample buffer and analyzed by Western Blot using anti-GFP antibodies (Roche Applied Science).

Microscopy

For live imaging HMC-1 cells were placed in LabTek chambered coverslips (Nunc). For tryptase staining cells were washed and resuspended in cold PBS and incubated on poly-L-lysine slides. Following adhesion cells were washed with PBS, fixed in 4%

formaldehyde/0.1 % glutaraldehyde/PBS. All further steps were performed in 0.05% saponin/2.5 % BSA/PBS. Briefly, cells were incubated for 1 hour at RT with 1:100 mouse monoclonal anti-human tryptase antibodies (Dako). Following thorough washing cells were incubated for 1 hour at RT with 1:200 TRITC-conjugated goat anti-mouse F(ab)2 (Sigma) and washed. After final wash cells were mounted in 5 mg/ml DABCO (Sigma)/Fluoromount G (Southern Biotech). Confocal microscopy was performed on Leica TCS SP2 AOBS microscope. Image processing was performed in Leica Confocal Software package. All monochrome images are presented in unprocessed form. For visualization purposes color restoration in multicolor images was accompanied by linear adjustments of brightness/contrast.

Sequence and statistical analysis

Predictions of O-linked glycosylation sites were performed using software hosted on Center of Biological Sequence Analysis, Technical University of Denmark DTU (http://www.cbs.dtu.dk/services/). TNF protein sequence alignment was performed with Vector NTI software. Two-way ANOVA followed by Holm-Sidak t-test were used to determine statistical significance of observed differences

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Chapter 4

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

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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.

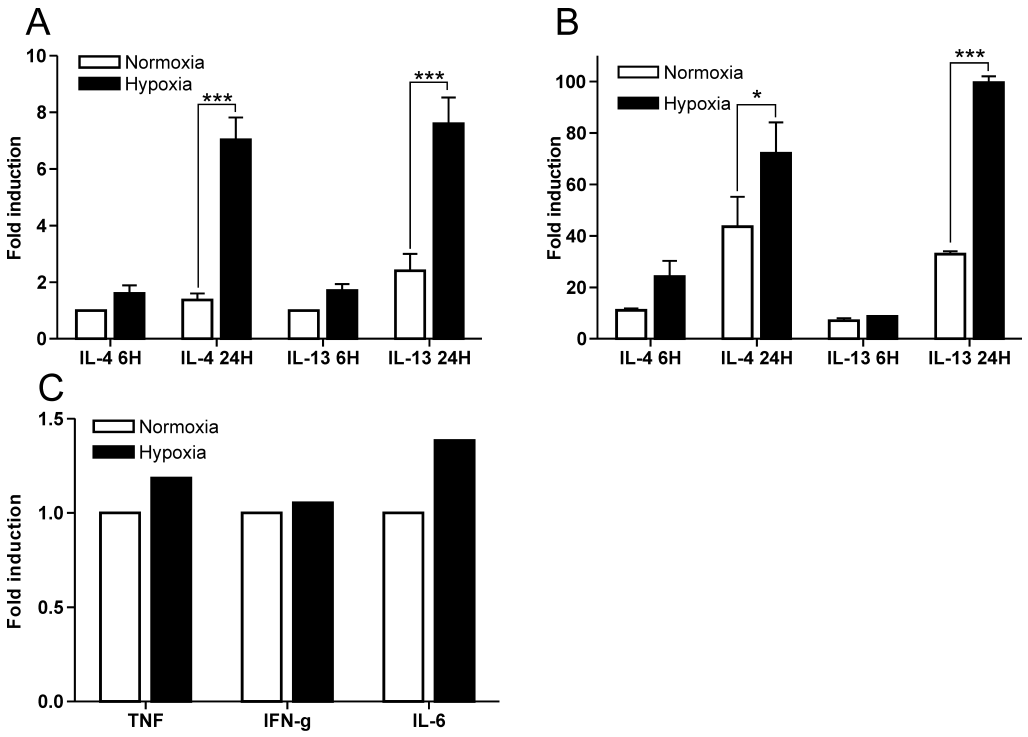

Figure 1 Mast cells release IL-4 and IL-13 in hypoxic conditions. HMC-1 mast cells were incubated in normoxic (21% O2) or hypoxic (1% O2) conditions for 6 or 24 hours in the absence (A) or presence (B) of 100 ng/ml PMA and 1 μM ionomycin. Concentration of IL-4 and IL-13 in the culture medium was assayed 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).

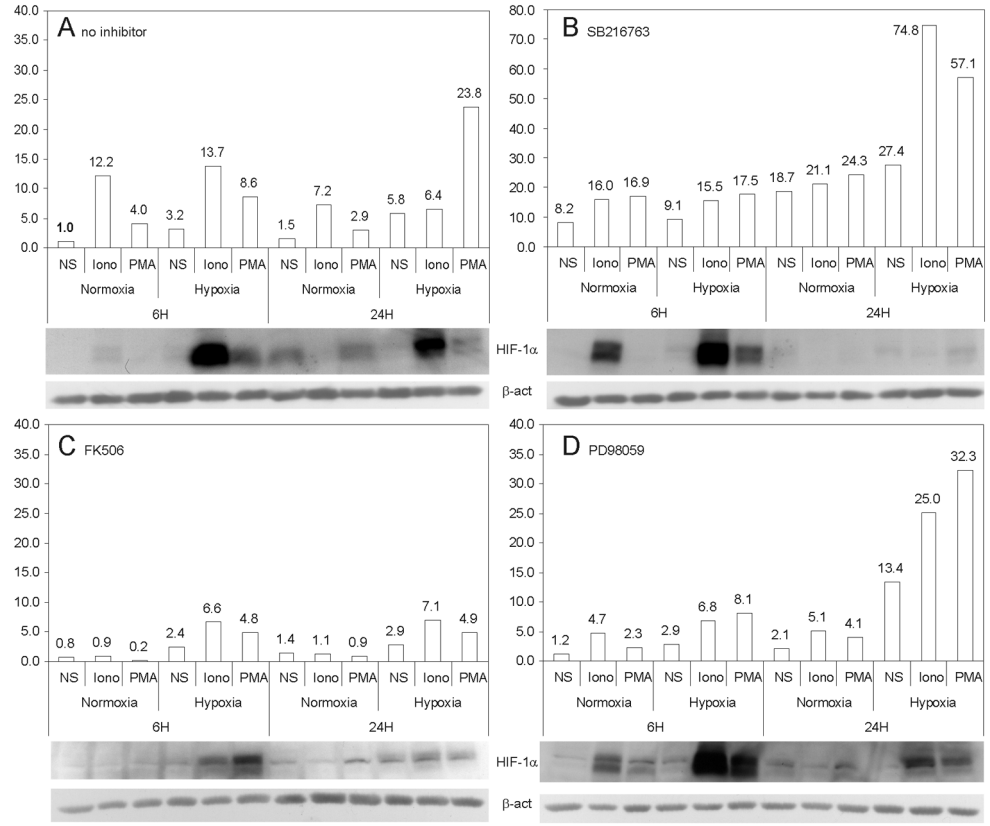

Figure 2 IL-4 release in hypoxia is dependent on calcineurin and GSK-3 but not on HIF-1α. HMC-1 mast cells preincubated for 1 hour in culture medium (A), 10 μM SB216763 (B), 5 μM FK506 (C) or 50 μM PD98059 (D) were incubated for 6 or 24 hours in normoxic or hypoxic conditions with or without addition 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.

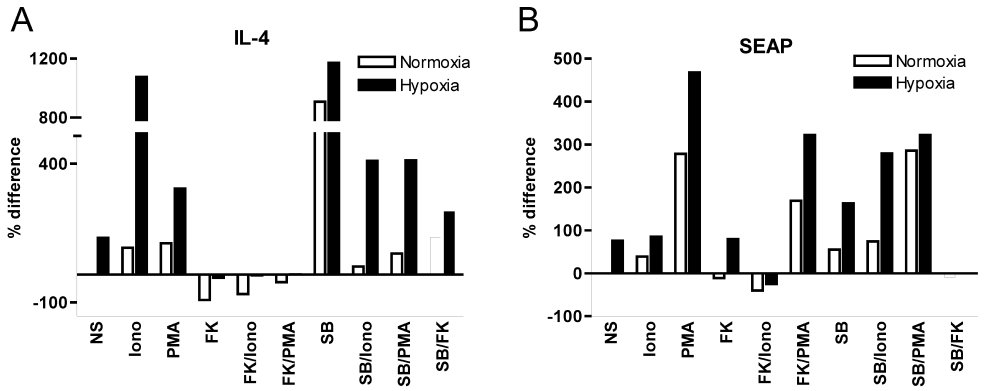

Figure 3 Hypoxia-induced IL-4 stimulation is partly dependent on NFAT. HMC-1 mast cells transfected with a plasmid encoding for SEAP under transcriptional control of NFAT responsive element and 24 hours post-transfection preincubated for 1 hour with 5 μM FK506 or 10 μM SB216763. Following preincubation 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.

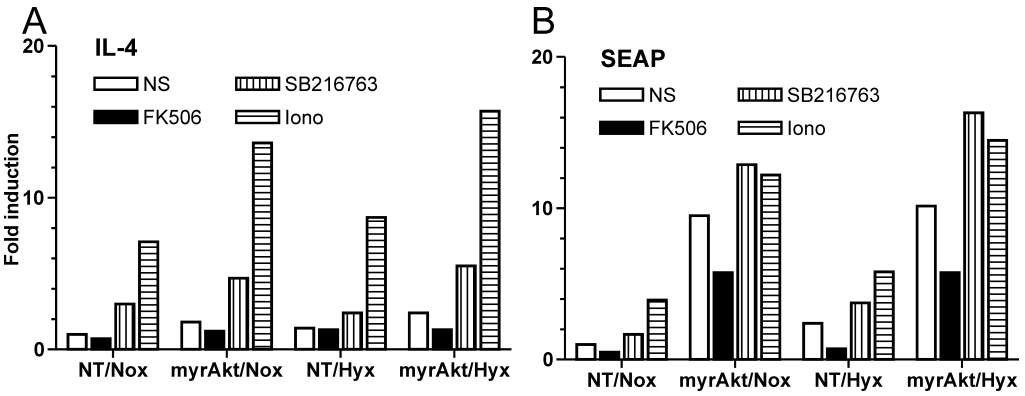

Figure 4 IL-4 expression is regulated by Akt. HMC-1 mast cells were transfected with plasmids encoding for constitutively active Akt (myrAkt) and SEAP under transcriptional control of NFAT responsive element and 24 hours post-transfection preincubated for 1 hour with 5 μM FK506 or 10 μM SB216763. Following 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.

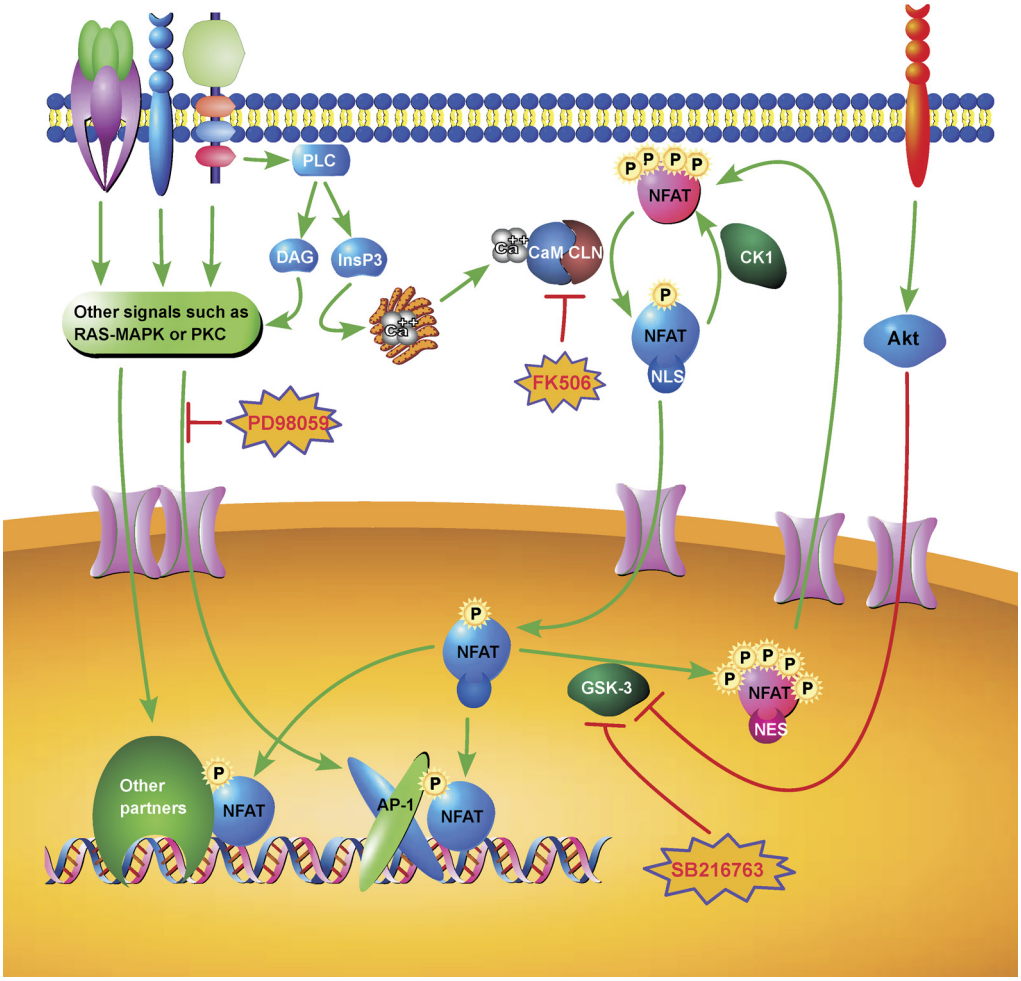

Figure 5 Scheme depicting the proposed model of IL-4 expression regulation in mast cells under hypoxic conditions

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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Chapter 5

Mercuric ions activate calcineurin and upregulate NFAT-dependent IL-4 promoter activity in mast cells

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Abstract

Mercuric ions induce abnormal immune responses in vivo. IL-4 is necessary for certain elements of Hg^{2+}-induced immunomodulation and its expression is upregulated in mast cells and lymphocytes following exposure to $HgCl_2$. In search for molecular mechanism of mercury-mediated IL-4 expression we investigated the effect of mercuric ions on IL-4 promoter activity in mouse mast cells. $HgCl_2$ upregulated IL-4 promoter activity in mast cells in a process, which required NFAT binding site of minimal IL-4 promoter and was sensitive to calcineurin (CaN) inhibitor FK506. Furthermore, Hg^{2+}-upregulated transcription driven by artificial NFAT-dependent promoter containing three NFAT sites and this transcription was sensitive to inhibitors of CaN and to dominant negative mutant of CaN. Finally, we observed that low concentrations of Hg^{2+} increased activity of purified rCaN in vitro. These observations are consistent with the hypothesis that Hg^{2+} ions increase activity of CaN that in turn upregulates NFAT, which binds to specific DNA motif present in IL-4 promoter resulting in IL-4 expression. Thus, Hg^{2+} ions activate CaN/NFAT signaling pathway in mast cells and this molecular mechanism could be important for mediating immunotoxic activities of mercuric compounds observed in vivo.

Introduction

Mercuric compounds are known to cause various adverse effects on immune system. Pathological changes observed in experimental animals exposed to Hg^{2+} ions include autoimmune glomerulonephritis (1), polyclonal arthritis (2), and induction of polyclonal IgE (3). Cellular and molecular mechanisms that are possibly involved in these pathological immune responses include Hg^{2+}-mediated changes in the expression of certain cytokines (4). One of the cytokines upregulated in vivo following administration of $HgCl_2$ is interleukin-4 (5, 6) necessary for induction of IgE and IgG1 (5) and upregulation of MHC II (7) in $HgCl_2$ treated animals. Hg^{2+}-mediated upregulation of IL-4 expression was reproduced in vitro in isolated lymphocytes and mast cells (8, 9).

Several molecular mechanisms explaining Hg^{2+}-mediated IL-4 expression in lymphocytes and mast cells have been proposed (10-12). Among molecules suspected of mediating the effect of Hg^{2+} on IL-4 expression are PKC, P type calcium channels, calcineurin (CaN), and C_Jun N-terminal kinase (10, 12). The possible engagement of CaN in Hg^{2+}-mediated cell responses is supported by observations that CaN inhibitor CsA inhibits Hg^{2+}-mediated IL-4 expression in lymphocytes in vitro and prevents development of Hg^{2+}- induced pathological immune response in vivo (13).

Antigen-mediated IL-4 expression in immune cells is predominantly controlled at the level of gene transcription by regulatory elements located in a proximal promoter (14-16). CaN a protein phosphatase capable of dephosphorylation nuclear transcription factor NFAT resulting in its translocation from cytoplasm to nucleus (17, 18) plays essential role in this regulatory mechanism (19-23). Therefore, we decided to investigate the role of CaN/NFAT signaling pathway in Hg^{2+} -mediated expression of IL-4 in mast cells. Data presented in this report support the critical role of CaN in Hg^{2+}-mediated IL-4 expression and show that Hg^{2+} directly interact with this phosphatase increasing its activity.

Results

It has been shown previously that Hg^{2+} ions mediate IL-4 secretion in mast cells and that this process requires de novo transcription of cytokine mRNA (8). To test if Hg^{2+} ions are able to upregulate IL-4 promoter activity, C57 cells or BMMC were transfected with DNA reporter construct -87 IL-4 pCAT, in which CAT expression is under control of minimal proximal promoter of IL-4. Following transfection cells were sensitized with DNP-specific IgE, and incubated with $HgCl_2$ or antigen (DNP-HSA). CAT concentrations in cell lysates were determined by ELISA and normalized for total protein concentrations.

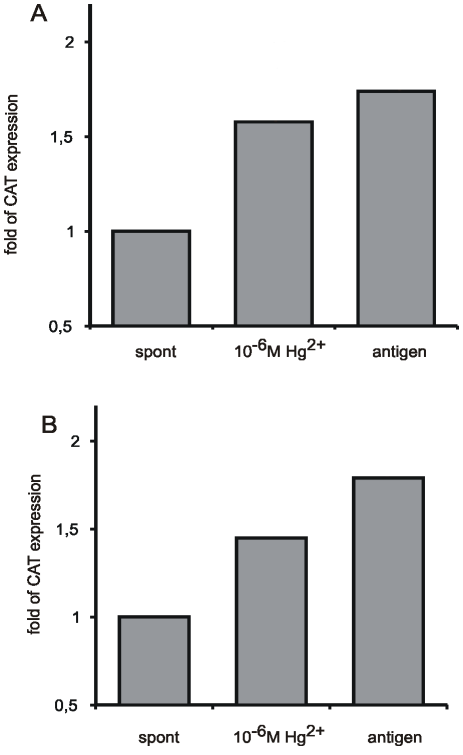

Figure 1 Mercuric ions increase IL-4 promoter activity in mast cells. BMMC (A) and C57.1 (B) mast cells were transfected with -87p IL-4 CAT, sensitized with IgE and incubated for 4 h with medium only (spont.), 10^{-6}M $HgCl_2$ (Hg^{2+}), or 50ng/ml DNP-HSA (antigen). Mast cells were collected by centrifugation, lysed and CAT concentration was determined by ELISA. CAT content was normalized for total protein concentrations and is expressed as fold increase compared to control. Bars represent the mean of 4 (A) and 2 (B) independent experiments each of them performed in duplicate.

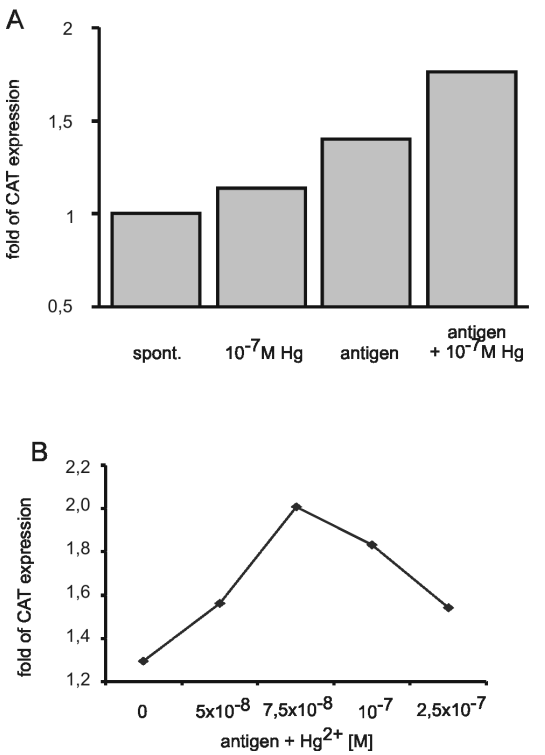

Figure 2 Mercuric ions enhance antigen-mediated IL-4 promoter activity in mast cells. A. C57.1 mast cells were transfected with -87 IL-4 pCAT sensitized with IgE and incubated for 4 h with medium only (spont), 10^{-7}M $HgCl_2$ (Hg^{2+}), 50ng/ml DNP-HSA (antigen), or combination of both 10^{-7}M $HgCl_2$ and 50ng/ml DNP-HSA (antigen + Hg^{2+}). B. C57.1 mast cells were transfected with -87 IL-4 pCAT sensitized with IgE and incubated for 4 h with a single dose of antigen (10 ng/ml DNP-HSA) and increasing concentrations of $HgCl_2$. In each experimental design (A and B) after incubation cells were collected by centrifugation, lysed and concentration of CAT in cell lysates was determined with ELISA. CAT content was normalized for total protein concentrations and is expressed as fold increase compared to control. Each point represents the mean of 2 independent experiments, each of them performed in duplicate.

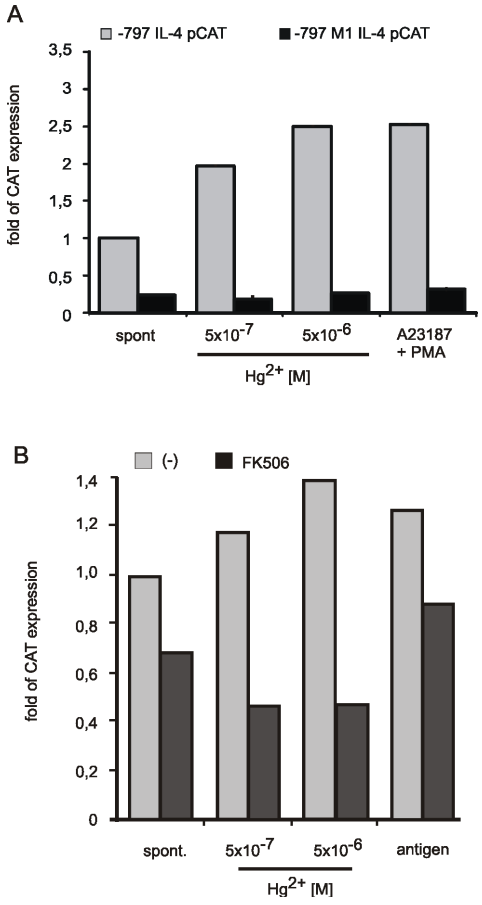

Figure 3 The effect of mercuric ions on IL-4 promoter activity depends on NFAT-binding site and CaN activity. A. C57.1 mast cells were transfected with -797 IL-4 pCAT or -797 M1 IL-4 pCAT , sensitized with IgE and incubated for 4 h with medium only (spont), 10^{-6}M $HgCl_2$ (Hg^{2+}) or combination of 10^{-6}M calcium ionophore and 50 ng/ml phorbol ester (A23187/PMA). B. C 57.1 mast cells were transfected with -87 IL-4 pCAT construct then sensitized with IgE and incubated for 4 h with medium only, $5x10^{-6}$M $HgCl_2$ (Hg^{2+}), or 50 ng/ml DNP-HSA (antigen) in the absence or presence of 1 μM FK506. In each experimental design (A and B) after incubation cells were collected by centrifugation, lysed and concentration of CAT was determined with ELISA. CAT content was normalized for total protein concentrations and is expressed as pg CAT per mg of total protein (A) or as fold increase compared to control (B). Bars represent data from one of two experiments performed in triplicate with similar results.

As seen in Fig. 1 A and B 10^{-9} M $HgCl_2$ significantly increased CAT expression in both BMMC and C57 mast cells. Subsequently we tested the effect of combination of Hg^{2+} ions and antigen on minimal IL-4 promoter activity in mast cells. Low concentrations of $HgCl_2$ (10^{-7} M) increased antigen-mediated activity of minimal IL-4 promoter (-87 to 5 bp) (Fig. 2 A) in mast cells. This effect of Hg^{2+} ions on antigen-mediated IL-4 promoter activity was dose-dependent with maximum enhancement observed for $7,5x10^{-8}$ M (Fig. 2 B). Thus, $HgCl_2$ alone or in combination with antigen/IgE upregulated IL-4 promoter activity in mast cells.

Next, we investigated whether Hg^{2+} -mediated IL-4 promoter activity requires intact NFAT binding site present in position -78 to -70 bp. BMMC were transfected with plasmids -797 IL-4 pCAT (wild type) and -797 M1 IL-4 pCAT, in which mutation in position -71 to –76 bp prevents NFAT binding to promoter. As seen in Fig. 3 A both ionophore 23187 / PMA and $HgCl_2$-induced reporter gene expression only in mast cells transfected with wild type but not with mutated IL-4 promoter DNA construct. Similar data were also obtained for antigen-stimulated BMMC (data not shown). These data suggest that NFAT binding site present in P1 NFAT/AP-1 composite site is required for Hg^{2+} ions-mediated IL-4 promoter activity. Availability of NFAT capable to bind to DNA depends on activity of CaN that dephosphorylates NFAT protein. Therefore we have investigated the effect of CaN inhibitor, FK506, on Hg^{2+} ions-mediated IL-4 promoter activity. Mast cells were transfected with -87 IL-4 pCAT promoter construct, and stimulated with $HgCl_2$ ($5x10^{-6}$ M) or antigen (50 ng/ml DNP HSA) in the absence or presence of 1 μM FK506. As seen in Fig. 3B, FK506 prevented both Hg^{2+} ions and antigen-stimulated increase in reporter gene expression. Thus, $HgCl_2$ upregulated IL-4 promoter activity in mast cells in a process that requires

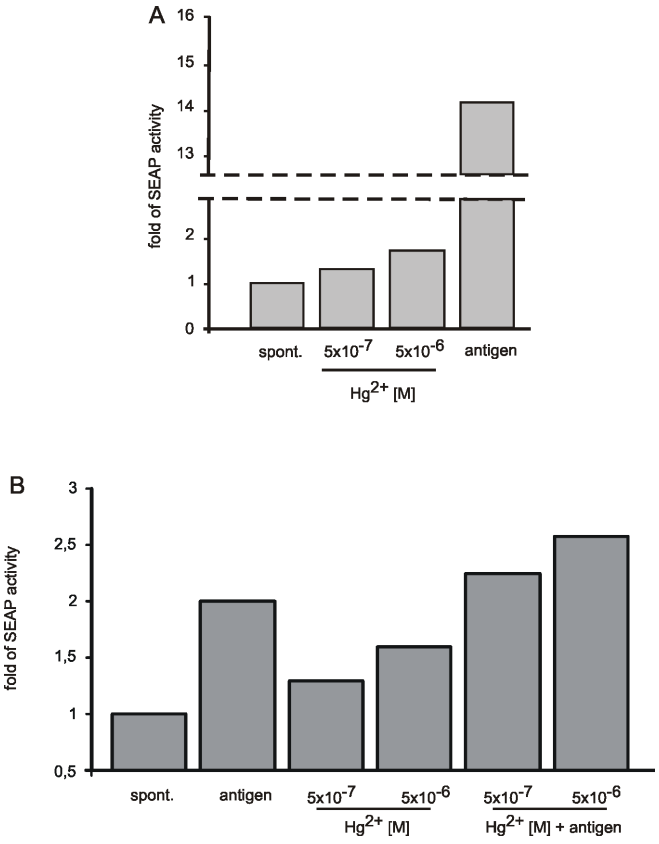

Figure 4 Mercuric ions increase NFAT-dependent SEAP expression in mast cells. (A) BMMC were transfected with pNFAT-SEAP, sensitized with IgE and stimulated with increasing concentrations of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen). (B) C57.1 mast cells were transfected with pNFAT-SEAP, sensitized with IgE and stimulated with increasing concentrations of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen) or combination of both as indicated. Each point represents the mean of 2 independent experiments, each of them performed in duplicate.

NFAT binding motif in IL-4 promoter and CaN activity. We have next decided to verify if Hg^{2+} ions induce CaN-dependent NFAT driven transcription in mast cells employing DNA construct pNFAT-SEAP, in which SEAP expression is controlled with an artificial NFAT-dependent promoter. Mast cells were transfected with pNFAT-SEAP promoter construct and stimulated with $HgCl_2$ ($5x10^{-6}$ M) or antigen (50 ng/ml DNP HSA) in the absence or presence of CaN inhibitors (1 μM FK506 or 1 μg/ml CsA). As seen in Fig. 4 A, incubation of BMMC cells in the presence of $HgCl_2$ resulted in an increased level of NFAT-dependent expression of reporter gene and this effect of $HgCl_2$ was dose-dependent. Similar results were obtained for C57.1 mast cells stimulated with $HgCl_2$ alone or in combination with antigen (Fig. 4 B). Therefore we next investigated the effect of CaN inhibitors on Hg-stimulated SEAP expression in mast cells. CaN inhibitors CsA and FK506 effectively blocked expression of SEAP in mast cells stimulated with $HgCl_2$, antigen and combination of both stimuli (Fig. 5A). To further verify if Hg^{2+}-induced transcription requires CaN activity, dominant negative variant of CaN A subunit was expressed in mast cells cotransfected with pNFAT-SEAP. Mast cells were cotransfected with pNFAT-SEAP and pDN-CLN-GFP or pEGFP-N1 (control) and stimulated with $HgCl_2$ antigen, and combination of both stimuli. As seen in Fig. 5 B expression of dominant negative variant of CaN resulted in abrogation of NFAT-dependent expression of SEAP in mast cells stimulated with $HgCl_2$, antigen, and combination of both stimuli. Thus, both pharmacological inhibition of CaN and expression of dominant negative mutant of this enzyme inhibited Hg^{2+} ions induced NFAT-dependent expression of reporter gene in mast cells.

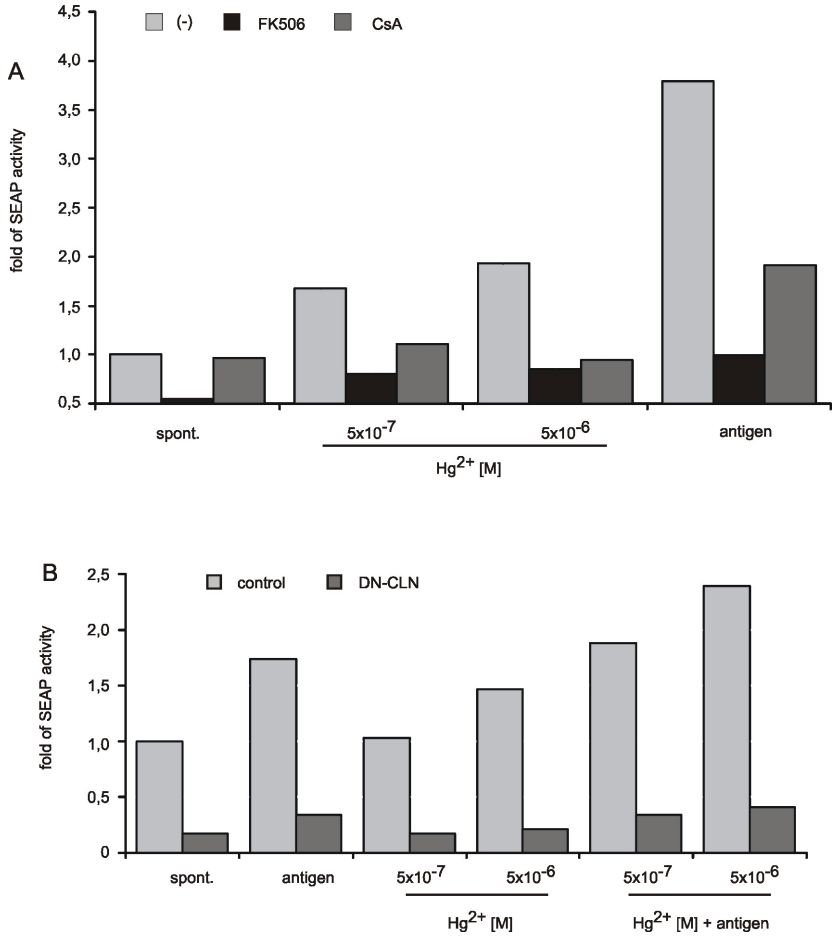

Figure 5 NFAT-driven SEAP transcription induced by mercuric ions in mast cells requires CaN activity (A) C57.1 mast cells were transfected with pNFAT-SEAP, sensitized with IgE and stimulated with increasing concentrations of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen) in the absence or presence of 1 μM FK506 or 1 μg/ml CsA. Supernatants were collected by centrifugation and SEAP activity concentration was determined with chemiluminescence-based assay. (B) C57.1 mast cells were cotransfected with pNFAT-SEAP and pDN-CLN-GFP (DN-CLN) or pEGFP-N2 (Control), sensitized with IgE and stimulated with indicated concentration of $HgCl_2$ or with 10ng/ml DNP-HSA (antigen) or combination of both stimuli. Supernatants were collected by centrifugation and SEAP activity concentration was determined with chemiluminescence-based assay. Each point in A and B represents the mean of 2 independent experiments, each of them performed in triplicate.

It is known that Ni^{2+} and Mn^{2+} are able to increase CaN activity in vitro (37). Therefore we have next tested direct effect of Hg^{2+} on enzymatic activity of CaN in vitro. As seen in Fig. 6 addition of 10^{-8} M Hg^{2+} to the assay buffer has significantly increased activity of human rCaN. In contrast, higher concentrations of Hg^{2+} (10^{-5}M) completely inhibited this enzymatic activity. These effects of Hg^{2+} on the amount of released phosphate were observed at the presence of $5x10^{-4}$M Ca^{2+} and were absent in control reactions where either calmodulin or CaN was absent. We have next repeated this experiment using bovine CaN purified from brain rather than human recombinant enzyme and observed similar effect of Hg^{2+} on enzymatic activity (data not shown). Thus, low concentrations of Hg^{2+} increased enzymatic activity of CaN in vitro.

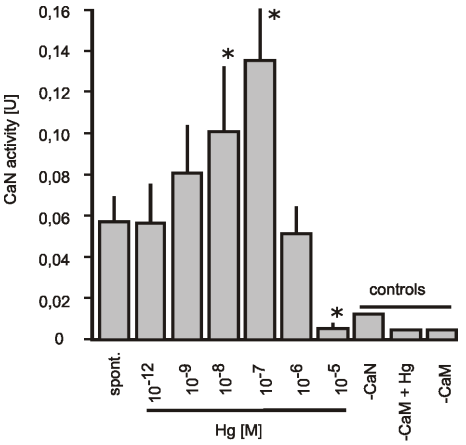

Figure 6 The effect of mercuric ions on CaN activity in vitro. Activity of human rCaN was measured in reaction performed for 10 min at 37 C° in the presence of 2.5 μM calmodulin, 500 μM CaCl2, and in the absence (spont.) or presence of indicated concentration of $HgCl_2$. The amount of PO43- was determined with malachite green., 1 Unit is defined as the enzymatic activity that releases 1 nmol PO43- per 1 min. Controls represent reaction performed without CaN (-CaN), without calmodulin (-CaM), and without calmodulin in the presence of 10-8M $HgCl_2$ (-CaM+Hg). Data represents results of 7 independent experiments, each of them performed in triplicates. Each bar represent means ± SEM. * - Statistically significant difference at P < 0.05.

Discussion

IL-4 expression is regulated at the level of transcription (14-16), and the sequence -87bp to +1 in IL-4 promoter, containing NFAT/AP1 composite binding site, is sufficient to induce transcription in antigen activated mast cells (26). We observed in two different types of murine mast cells that this DNA sequence mediated upregulation of transcription following incubation with Hg^{2+} ions (Fig.1 A and B). This observation is consistent with Hg^{2+}-mediated activation of IL-4 promoter sequences –726 and +26 observed in RBL-2H3 rat mast cell line (11). The effect of Hg^{2+} on -87bp to +1 IL-4 promoter activity was observed at concentration that mediates upregulation of IL-4 expression in mast cells (9) (8). Hg^{2+} ions have also mediated enhancement of antigen-driven IL-4 promoter activity in mast cells stimulated with optimal dose of antigen (Fig. 2 A and B) that might explain previously observed effect of Hg^{2+} ions on antigen-mediated IL-4 secretion (8). These observations are consistent with the hypothesis that Hg^{2+} increase IL-4 promoter activity resulting in de novo transcription and translation of IL-4 in mast cells.

Binding NFAT to IL-4 promoter is necessary for IL-4 expression in mast cells (26) and in T cells (29). NFAT/AP1 composite binding site present within the region –88 bp to –60 bp of IL-4 promoter has been shown to be critical for inducible IL-4 promoter activity in mast cells (26) and in T lymphocytes (14) activated with antigen and PMA/ ionomycine. Promoter construct with mutation within this NFAT binding sequence did not mediate transcription in mast cells activated with antigen or Hg^{2+} ions (Fig. 3 A) suggesting similar mechanisms of promoter activation. NFAT is regulated by CaN that controls shuttling of NFAT to nucleus (17, 18). We observed that CaN inhibitor FK506 suppressed Hg^{2+} ions-mediated and antigen-mediated IL-4 promoter activity in mast cells (Fig. 3 B). FK506 was earlier reported to inhibit IL-4 expression in activated mast cells (23, 30). This inhibitor has been also reported to inhibit activity of IL-2 promoter that, similarly to IL-4 promoter, contains NFAT/AP1 composite binding site critical for its activity (31). These facts and our results suggest that upregulation of IL-4 expression in mast cells exposed to Hg^{2+} is mediated by CaN/ NFAT signal transduction pathway. In agreement with this hypothesis exposure of

BMMC and C57.1 mast cells to Hg^{2+} ions alone or in combination with antigen resulted in induction of NFAT-dependent gene expression from pNFAT-SEAP reporter plasmid (Fig. 4A and B) that was effectively blocked with CaN inhibitors FK506 and CsA (Fig. 5A). Hypothesis that Hg^{2+} activate NFAT in mast cells and this process depends on CaN activity is further supported by the effect of co-expression of dominant negative variant of CaN that abrogated Hg^{2+}-mediated NFAT-dependent expression of reported gene (Fig.5B).

Other metal ions, namely nickel and vanadium, have previously been reported to activate CaN/NFAT signaling pathway in fibroblasts, possibly by generation of reactive oxygen species (32, 33). The exposure of mast cells to Hg^{2+} ions also results in generation of reactive oxygen species (34) and mercuric ions-mediated activation of NFAT driven transcription could involve reactive oxygen species. However, Hg^{2+} enter the cell and accumulate in different cellular compartments (35), where they could directly interact with CaN. In support of such hypothesis we observed that Hg^{2+} (Fig. 6) significantly increased CaN activity in vitro at concentrations lower than those needed to activate NFAT-dependent transcription in vivo (Fig.1 A and B). As far as we know, it is the first observation that Hg^{2+} ions are able to interfere with CaN activity. The difference in Hg^{2+} concentrations activating NFAT-driven transcription in vivo and CaN activity in vitro could be explained by the fact that only a fraction of internalized Hg^{2+} is present in cytoplasmic compartment (36) where it interacts not only with CaN but also with other proteins. Other divalent metal ions (Ni^{2+} and Mn^{2+}) are known to increase CaN activity in vitro but the mechanism of their action is not well understood (37). Activity of CaN depends on Fe^{2+} and Zn^{2+} present in the bimetal catalytic center (38, 39) and it is conceivable that other metal ions, including Hg^{2+}, could also interact with this domain of CaN molecule. However, it is not clear if purified CaN retains natural cofactors Fe^{2+} and Zn^{2+} in metal catalytic center (37). or is partially depleted of these ions (39). Thus it could not be ruled out that Hg^{2+} substitute for depleted metal ions. We have, however, observed similar effects (235 % compared to 221 % increase at 10^{-7} M Hg^{2+}, Fig 6 and data not shown) of Hg^{2+} with CaN preparations of different origin (recombinant human and purified bovine) and, more importantly, of different specific activities (1490 nmol PO43-/min/mg and 27.6 nmol PO43-/min/mg) that is consistent with hypothesis that Hg^{2+} are able to increase activity of CaN under optimal conditions.

In conclusion, our data support hypothesis that Hg^{2+} ions directly increase activity of CaN that in turn upregulates NFAT that binds to specific DNA motif present in IL-4 promoter resulting in IL-4 expression in mast cells. This molecular mechanism, possibly in cooperation with other signaling pathways (10-12), may be involved in initiation of Th2 immune response observed in humans (40) and experimental animals exposed to mercuric compounds (3, 5-7) partly explaining immunotoxic activities of mercuric compounds.

Acknowledgements

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Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), FCS, HEPES, L-glutamine, 2-ME, penicillin/streptomycin, BSA, dinitrophenyl-conjugated human serum albumin (DNP-HSA), PMA, leupeptin, aprotinin, TLCK, TPCK, and PMSF (Sigma Chemical Company, St. Louis, MO, USA), A23187 (Calbiochem, La Jolla, CA, USA), FK506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan, pNFAT-SEAP and pEGFP-N1 (Clontech), human rCaN (specific activity 1492 nmol/min/mg), bovine CaN purified from brain (specific activity 27.6 nmol/min/mg), and BIOMOL GREEN Calcineurin Assay Kit (Biomol Research Laboratories, Inc. Plymouth Meeting, PA, USA), analytical grade $HgCl_2$ (POCH, Gliwice, Poland), were purchased from indicated manufacturers. Reporter gene constructs -87 IL-4 pCAT, -797 IL-4 pCAT, and -797 M1 IL-4 pCAT were obtained from Dr M. Brown, Emory University School of Medicine Atlanta, Georgia, USA. Expression vector pDN-CLN-GFP coding dominant negative variant of was obtained from Dr B. Kaminska, Nencki Institute of Experimental Biology, Warsaw, Poland. This plasmid codes for a mutant of subunit A of CaN with deleted catalytic domain fused to EGFP and its expression results in inhibition of CaN activity and NFAT-dependent transcription (Kaminska personal communication). Murine monoclonal anti-dinitrophenyl (DNP) – specific IgE was obtained from the culture of hybridoma Hi-DNP-ε-26.82 (24).

Culture and transfection of mast cells

Murine mast cells C57.1 (25) were cultured in DMEM supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 25mM HEPES, and 100 μg/ml penicillin/streptomycin (complete DMEM). Murine Bone Marrow Derived Mast Cells (BMMC) were obtained in 4-6 week culture of cells from bone marrow of female BALB/c mice (6 to 9 weeks old) in RPMI 1640 supplemented with 20% WEHI-3 conditioned medium as a source of IL-3, 10% FCS, 4 mM L-glutamine, 25mM HEPES, and 100 μg/ml penicillin/streptomycin (complete RPMI). Cells were cultured at 370C in a CO2 incubator. For transfection, mast cells were collected by centrifugation and 107 cells were suspended in 0.4 ml complete DMEM. Cell suspension was mixed with 50 μg plasmid DNA in a total volume of 0,4 ml and electroporated at 1000 μF and 0,25 kV using Bio-Rad Gene Pulser II. Following electroporation cells were transferred to culture flask, cell density adjusted to $2.5x10^5$ per ml and cultured for 16-18 h in a CO_2 incubator.

Sensitization and stimulation of mast cells

Transfected mast cells were collected by centrifugation, and suspended in complete DMEM or complete RPMI supplemented with 20% anti-DNP IgE containing supernatant to final density 10^6 cells/ml. Cells were incubated for 1,5 hour followed

by three washings with medium. Sensitized mast cells were suspended in media at density of 10^6 cells/ml and incubated in medium alone, in the presence of indicated concentrations of $HgCl_2$, antigen, or combination of both. In some experiments CsA or FK506 dissolved appropriately in DMSO or EtOH, or those solvents alone, were added at indicated concentrations to mast cell suspension 30 min before stimulation with $HgCl_2$ or antigen. Following 4 hours incubation mast cells and supernatants were collected by centrifugation and frozen for further analysis.

Gene reporter activity assays

Reporter gene constructs -87 IL-4 pCAT, and -797 IL-4 pCAT contain respectively sequences -87 to +5, and -797 to +5 of murine il-4 gene inserted upstream of open reading frame of chloramphenicol transferase (CAT) (14, 26) -797 M1 IL-4 pCAT differs from -797 IL-4 pCAT by mutation of sequence AATTTT in position -76 to -71 into sequence CTGCAG (26). Plasmid pNFAT-SEAP contains three tandem repeats of NFAT consensus sequence fused to a TATA like promoter region from Herpes simplex virus thymidine kinase promoter. To measure the expression of chloramphenicol acetylotranspherase (CAT) reporter gene mast cells were lysed in the presence of proteases inhibitors (aprotinin, leupeptin, TPCK, TLCK, PMSF) and CAT content was determined using ELISA (Roche) and normalized to total protein content measured with BCA (Pierce) assay. To measure the expression of secreted alkaline phosphatase (SEAP) reporter gene, enzymatic activity of SEAP released into supernatant was determined by chemiluminescence based assay (Great EscAPe SEAP Kit, Clontech) and luminometry.

Calcineurin activity assay

Activity of CaN was measured using BIOMOL GREEN Calcineurin Assay Kit, according to manufacturer's protocol. Briefly, recombinant human CaN and bovine calmodulin dissolved in assay buffer containing 100mM Tris, pH 7.5, 200mM NaCl, 12 mM MgCl2, 1mM DTT, 0.05% NP-40, and 1mMCaCl2 were mixed in selected wells of microtiter plate. Increasing concentrations of $HgCl_2$ dissolved in dH_2O or dH_2O were added to selected wells and the plate was incubated for 10 min. Next, 0.15 mM RII phosphopeptide (27) as a CaN substrate was added and plate was incubated for additional 10 min. Determination of released phosphate concentration was performed using malachite green (28). In some experiments human rCaN was replaced with bovine CaN purified from brain.

Statistical analysis

One way repeated measures ANOVA followed by Bonferroni t-test were used to determine statistical significance of observed differences.

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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.

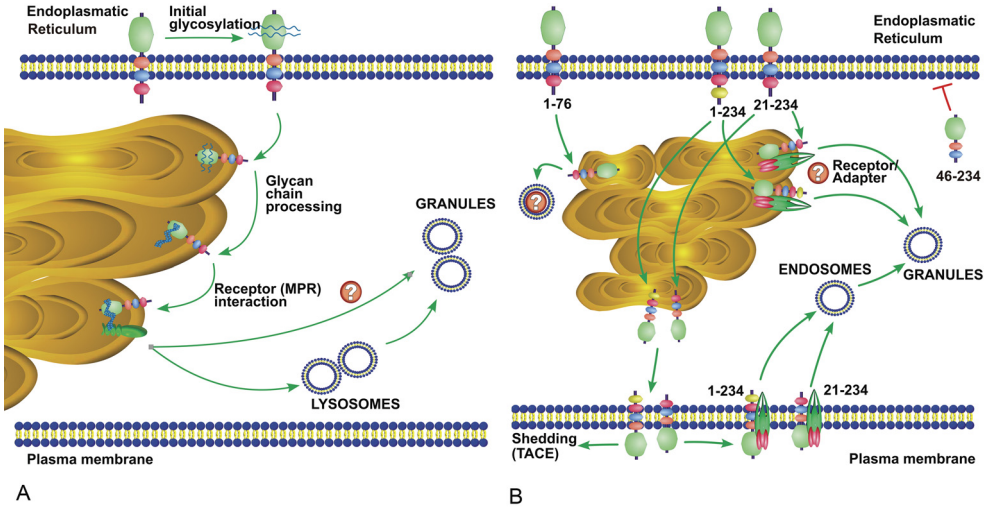


Figure 1 Proposed models of TNF trafficking to mast cell granules in rodents (A) and human (B)

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).

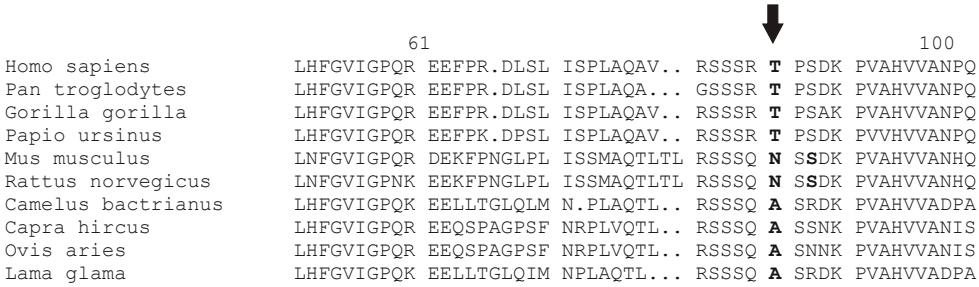


Figure 2 Multiple alignment of TNF sequences representing Primates, Rodentia and Artiodactyla, showing low conservation of potentially glycosylated residues (indicated by an arrow)

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:

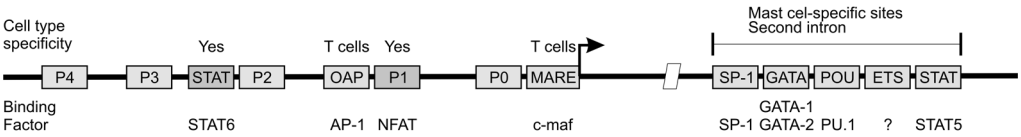
1. STAT binding site that is not required for IL-4 expression in mast cells (94), but is an obligatory element in Th2 cells development (95, 96)
2. c-maf binding site (MARE) that is specifically required by Th2 cells, but not for mast cells for IL-4 expression (97)
3. P1 NFAT binding site (see below).

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.

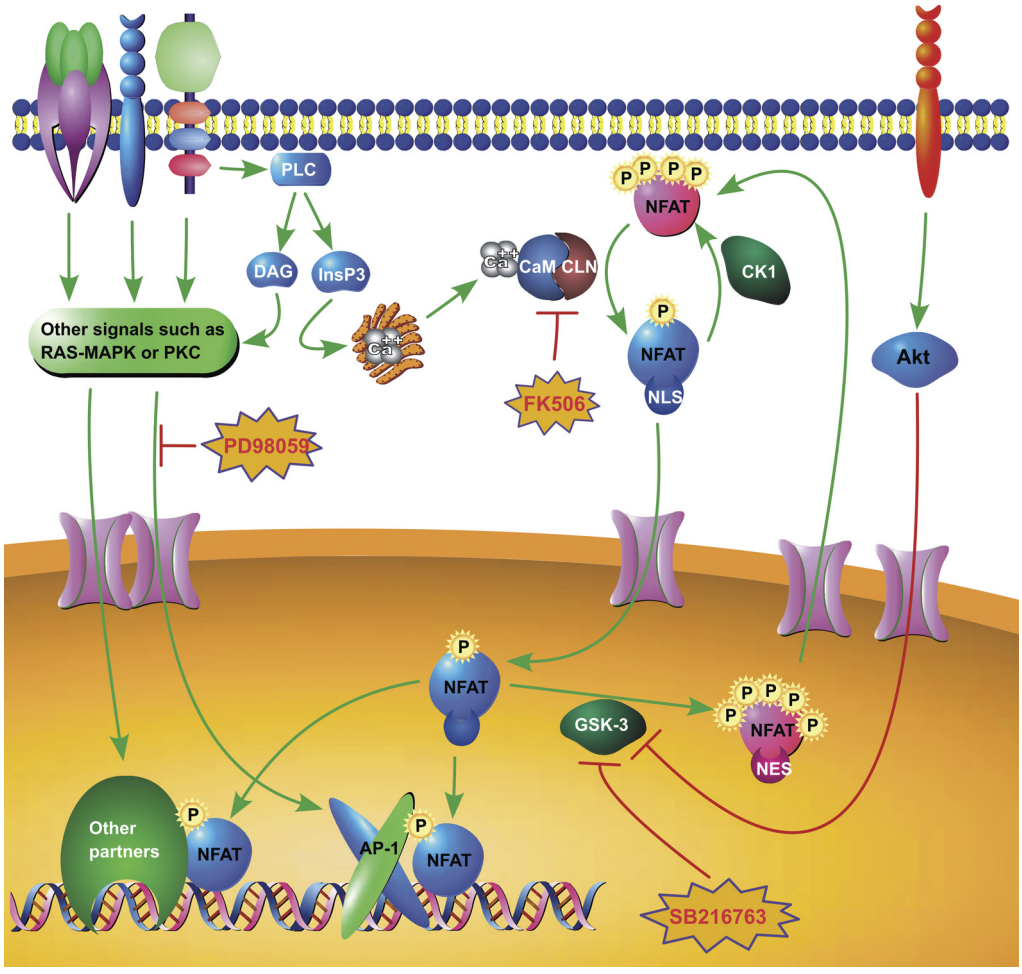


Figure 4 A proposed model of IL-4 transcriptional activity regulation under hypoxia and Hg++ exposure

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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Summary

Nederlandse Samenvatting

Summary

The inflammatory process

The entire host reaction to pathophysiological stimuli, including bacterial infections, parasite infestation or exposure to irritant environmental substances and conditions, is named inflammation. Many distinct inflammatory pathways exist, each of them involving a different set of cells and mediators. Individual events in course of the inflammatory process are controlled by cytokines and other small regulatory molecules which in this context are named inflammatory mediators. A given mediator may exert its effect directly or indirectly by regulating the activity of other mediators. This complicated interplay of the network mediators gives rise to an integrated response. Although the mechanisms constituting inflammation evolved to eliminate injurious substances or limit their spreading through the organism, they may be the cause of excessive damage in inflamed tissue when injury is severe or when they become misregulated. In the course of sepsis, it is not the inducing pathogens, but the excessive immune inflammatory responses that are responsible for severe health problems. Allergic reactions are examples of excessive immune inflammatory response against innocuous substances such as dust, pollen, food or drugs. The same mechanisms of immunological inflammation are partly responsible for pathogenic consequences of autoimmune diseases.

Mast cells and their granules

Mast cells are tissue-dwelling cells that are predominantly located at the interfaces of the organism and the exterior, such as skin, gut mucosal membranes and lung. They are evolutionarily old cells that play multiple roles in many modes of immune response, including innate and antibody-dependent reactions. Ultrastructural analysis of mast cell granules reveals several subtypes such as scroll-containing, crystal-containing, particle-containing and homogeneously electron-dense content-containing granules although the functional importance of this heterogeneity is not clear. The granules were initially considered a storage organelle for the products of a cell; these products could be rapidly released upon appropriate stimulation. Many highly bioactive mediators are found in mast cell granules; apart from the long-known ones such as chymase, tryptase, histamine or heparin an increasing number of cytokines are being discovered: bFGF, SCF, VEGF, IL-4 and TNF, to name just a few.

It is often observed that mast cells secrete bioactive compounds, including cytokines, without full degranulation. This mode of granule cargo release was shown to be mediated by vesicular transport and apparently involves additional regulatory checkpoints. The mechanisms responsible for selective release of granule cargo without the full degranulation remain largely unclear.

TNF intracellular trafficking in mast cells

In order to gain insight into the process of intracellular TNF-alpha trafficking in mast cells we have employed a strategy involving expression of constructs coding for TNF-alpha-EGFP fusion protein transiently expressed in RBL-2H3 rat mast cells (chapter 2). The EGFP fusion protein containing the entire sequence of the transmembrane TNF-alpha accumulated predominantly in cytoplasmic secretory granules. The unequivocal identification of the compartment in which TNF-alpha fusion protein accumulated is supported by colocalization with three independent markers for secretory granules. The kinetics of appearance of the full TNF-EGFP fusion protein in subsequent compartments of transfected mast cells supports the hypothesis that it is sorted into cytoplasmic granules via a ER/Golgi secretory pathway. Additionally, ectopically expressed TNF-EGFP fusion protein is not only sorted to mast cell granules, but also efficiently released. Inhibitor studies confirmed that TNF is sorted with the use of ER/Golgi pathway and that it is retrieved from the secretory pathway at the stage of late Golgi. This retrieval seems to be mediated by an MPR (mannose-6-phosphate receptor) system. Inhibition of N-linked glycosylation by tunicamycin or mutation (N86S) results in abrogation of granular sorting indicating that this process is carbohydrate-dependent and supporting the hypothesis involving MPRs.

In human mast cells, however, carbohydrate-dependent trafficking does not seem to be in operation since the N86 residue is not conserved and deglycosylation studies seem to indicate that TNF is not glycosylated in human mast cells (chapter 3). Analysis of the trafficking patterns of deletion mutants of TNF-EGFP revealed that residues 21-46 are critical for ER entry of TNF and its subsequent sorting. In contrast, majority of the cytoplasmic tail of TNF is expendable for its granular sorting. Surface biotinylation experiments showed that TNF is transiently exposed on the outer membrane and re-endocytosed on its way to the granules and that this process is dependent on the lumenal/extracellular part of the TNF molecule, suggesting an interaction with a receptor or an accessory protein. These important differences in modes of TNF trafficking between rodent and human systems stress potential weaknesses of rodent models when TNF biology in mast cells is considered.

Regulation of IL-4 expression in mast cells by environmental stress

Mast cells play an important regulatory role in the course of inflammatory process. Until recently they were considered the cells that, when activated, will augment the development of inflammatory state. Recent reports, however, indicate that mast cells may also contribute to immune tolerance and the resolution of an inflammatory state. This prompted our research concerning the regulation of IL-4, a potentially anti-inflammatory cytokine expression in mast cells.

In many pathological situations mast cells are exposed to hypoxic condition. 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. However, there is a separate, degranulation independent, phase of cytokine production that involves *de novo* synthesis and release of these mediators. In chapter 4 we

show that in hypoxic conditions IL-4 (and IL-13) expression and release is induced, as opposed to IL-6, TNF and IFN-gamma. This induction, under various experimental conditions, does not correlate with stabilization of HIF-1 alpha, a major transducer of hypoxic signalling. Instead, we show that regulation of IL-4 promoter activity in hypoxia is mediated by Akt/GSK-3 and calcineurin pathways that converge at the level of NFAT, a known regulator of IL-4 and IL-13 promoter activity in mast cells.

Treatment with mercuric ions has long been used as a model system for systemic autoimmune inflammation including arthritis, vasculitis and glomerulonephritis. Such treatment causes polyclonal B cells activation and enhanced IgE and IL-4 production. The involvement of mast cells and mast cell-derived IL-4 in full elicitation of these responses has been suggested. The molecular mechanism underlying mercury-induced IL-4 expression is not known and therefore in chapter 5 we aimed at establishing signal transduction pathways responsible for this induction. We have shown that in murine mast cells Hg activates IL-4 production and this effect is dose-dependent, additive to IgE/antigen stimulation and critically depends on P1 NFAT binding site in IL-4 promoter. Inhibitor and dominant-negative mutant studies demonstrated involvement of calcineurin in the regulation of mercury-induced IL-4 expression; this hypothesis was further reinforced by the *in vitro* assay of calcineurin activity in the presence of mercuric ions.

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.

Nederlandse Samenvatting

Het ontstekingsproces

Het totaal van reacties op pathofysiologische stimuli, zoals bacteriële en parasitaire infecties, of blootstelling aan irriterende omgevingsfactoren wordt ontsteking of inflammatie genoemd. Het ontstekingsproces wordt gecontroleerd door cytokinen en andere regulerende kleine moleculen, ook wel ontstekingsmediatoren genoemd. Het complexe samenspel tussen ontstekingsmediatoren en cytokinen intergreert tot de ontstekingsreactie. Er bestaan veel verschillende combinaties van cytokinen, mediatoren en celtypen die allemaal tot ontsteking kunnen leiden.

Alhoewel de ontstekingsreactie bedoeld is om de schadelijke effecten van een externe component te elimineren, of de verspreiding in het lichaam te beperken, kan ontsteking leiden tot excessieve schade wanneer de effecten van de externe component groot is, of wanneer het ontstekingsproces wordt ontregeld. In het geval van sepsis is het niet zo zeer het inducerende pathogeen, maar de geïnduceerde ontstekingsreactie die verantwoordelijk is voor de ernstige gezondheidsproblemen. Allergische reacties zijn voorbeelden van een overdreven immuungemedieerde ontstekingsreactie tegen een onschuldig antigeen, zoals pollen, voedsel of geneesmiddelen. Vergelijkbare mechanismen van immuungemedieerde ontstekingen zijn voor een deel verantwoordelijk voor de pathologische reacties bij auto-immuunziekten.

Mestcellen

Mestcellen zijn weefselgebonden cellen die zich voornamelijk bevinden op de plaatsen waar contact is met het externe milieu, zoals huid, darm en de long. Mestcellen zijn evolutionair geconserveerde cellen die belangrijke rollen spelen in verschillende immuunreacties, zowel aangeboren als antistofafhankelijke immuunresponsen.

Op basis van de ultrastructuur van de korrels, ook wel granula genoemd, in mestcellen kunnen deze cellen worden onderverdeeld in verschillende subtypen, zoals kristal-bevattende, rol-bevattende en homogeen electrodense granula. De functionele implicaties van deze verschillende typen granula in de mestcellen is niet duidelijk. Oorspronkelijk zijn de granula beschreven als opslagorganel voor de producten van de cel; na stimulatie kunnen de producten snel worden uitgescheiden. Mestcelgranula bevatten vele zeer bioactieve mediators. Naast de bekende mediatoren zoals histamine, heparine en tryptase wordt een toenemend aantal cytokines beschreven die zijn opgeslagen in de granula van mestcellen, zoals basisch fibroblast groei factor (bFGF), stemcel factor (SCF), vasculair endotheel groei factor (VEGF), interleukine-4 (IL-4) en tumor-necrose factor (TNF).

Mestcellen kunnen bioactieve stoffen uitscheiden zonder volledige degranulatie. Deze vorm van vrijmaken van een deel van de granula verloopt via vesiculair transport en lijkt extra regulatoire checkpoints te bevatten. De signalen die aanzetten tot dit selectieve vesiculaire transport zijn nog niet opgehelderd.

Transport van TNF in mestcellen

Om inzicht te krijgen in de mechanismen bij het transport van TNF in mestcellen is gebruik gemaakt van TNF-EGFP (enhanced green fluorescent protein) fusie-eiwitten die tot expressie werden gebracht in de RBL-2H3 ratte mestcel (Hoofdstuk 2). Wanneer de volledige sequentie van transmembraan TNF werd gekoppeld aan EGFP, trad ophoping in de cytoplasmatische secretoire granula op. De identiteit van de secretoire granula werd vastgesteld middels 3 verschillende, onafhankelijke markers. Na stimulatie van mestcellen werd het TNF-EGFP fusie-eiwit ook efficiënt uitgescheiden. De kinetiek van het verschijnen van de TNF- EGFP in de granula suggereert dat dit via een endoplasmatisch reticulum (ER)/Golgi secretoir pad plaatsvindt. Middels het gebruik van selectieve remmers werd dit verder bewezen. Tevens werd zo vastgesteld dat TNF transport naar de granula optreedt vanuit de stadium van late Golgi. Het transport naar de granula wordt gemedieerd via een MPR (mannose-6-phosphate receptor) systeem. Remming van N-linked glycosylering met tunicamycine of mutering van de selectieve site op TNF (N86S) voorkomt transport naar de granula. Dit impliceert dat de granula sortering carbohydraat afhankelijk is en dat MPR's betrokken zijn.

Opmerkelijk is dat in mestcellen van humane origine de carbohydraat-afhankelijk beweging van TNF niet relevant is, omdat het N86 residu niet geconserveerd is en TNF niet is geglycosyleerd (Hoofdstuk 3). Analyse van deletiemutanten van TNF toonden dat residuen 21-46 belangrijk zijn voor binnenkomen in het ER en de daaropvolgende sortering. Opmerkelijk is dat het grootste deel van het cytoplasmatische gedeelte van TNF hierbij niet relevant is. Oppervlakte biotinilering toonde dat TNF transient tot expressie komt op de buitenmembraan en "re-endocyteert" naar de granula en dat dit proces afhankelijk is van het luminale/extracellulaire gedeelte van het TNF molecuul. Dit laatste zou wijzen op een rol van een interactie tussen een receptor en een accessoir eiwit.

De verschillen in het transport van TNF tussen rat en humane systemen onderschrijven de potentiële zwakke punten van ratmodellen wanneer TNF biologie in mestcellen wordt onderzocht.

Regulatie van IL-4 expressie in mestcellen door omgevingsstress.

Mestcellen spelen een belangrijke rol in het verloop van ontsteking. Mestcellen worden beschouwd als cellen die na activatie een sterk pro-inflammatoir effect hebben. Zeer recent is gebleken dat mestcellen ook een belangrijk rol spelen bij immuuntolerantie en resolutie van ontsteking. Wij hebben onderzoek gedaan aan de regulatie van IL-4 in mestcellen, een belangrijk anti-inflammatoire cytokine.

In diverse pathologische situaties worden mestcellen blootgesteld aan hypoxische condities. Mestcellen dragen bij aan het ontstaan van ontsteking in deze weefsels, onder andere via het vrijmaken van TNF dat wordt vrijgemaakt naar reperfusie. Er is echter een apart, degranulatie-onafhankelijk fase van cytokine productie, waarbij *de novo* synthese en vrijmaking van mediatoren optreedt. In Hoofdstuk 4 hebben wij aangetoond dat tijdens hypoxie de mestcellen IL-4 (en IL-13) synthetiseren

en vrijmaken, terwijl pro-inflammatoire cytokinen, zoals IL-6, TNF en interferon-γ in mestcellen niet toenemen. Opvallend is dat er geen relatie bestaat tussen de hypoxie geïnduceerde productie van IL-4 en de stabilisatie van HIF-1-α, een belangrijke transducer van hypoxie signalering. De regulatie van IL-4 promotor wordt gemedieerd via Akt/GSK-3 en calcineurine routes welke uiteindelijk op het niveau van NFAT samenkomen. NFAT is een belangrijke regulator van IL-4 en IL-13 promotor activiteit in mestcellen. .

Behandeling met kwikionen wordt reeds lange tijd gebruikt in modelsystemen voor systemische autoimmuunziekten, zoals artritis, vasculitis en glomerulonefritis. Kwikion behandeling leidt onder andere tot polyclonale B cell activatie en verhoogde IgE en IL-4 productie. Mestcellen en de IL-4 uit mestcellen lijken een rol te spelen in het ontstaan van deze aandoeningen na kwikionen. Wij hebben onderzocht in Hoofdstuk 5 wat het moleculair mechanisme is voor de kwikgeïnduceerde IL-4 productie door mestcellen. In muize mestcellen hebben wij aangetoond dat de kwikgeïnduceerde IL-4 productie door mestcellen dosis afhankelijk is en additief aan een IgE-antigeen gemedieerde stimulatie. Bij deze IL-4 productie is de P1 NFAT binding plek op de IL-4 promotor relevant. De calcineurine route was relevant in de IL-4 productie, zoals werd aangetoond door middel van experimenten met remmers en analyse van dominant-negatieve mutanten. Tevens bleek dat calcineurine direct door kwikionen wordt geactiveerd.

Algemene conclusies

Zoals hierboven beschreven worden mestcellen gereguleerd door unieke signaaltransductie routes. Mestcellen zijn weefselgebonden cellen die zich vooral bevinden op de grens tussen organisme en de omgeving. Deze eigenschap in combinatie met het feit dat mestcellen grote hoeveelheden potente bioactieve componenten in hun granula hebben opgeslagen, maakt deze cellen perfecte kandidaten voor de initiëring van immuun-, of niet immuun-gemedieerde reacties op microbiële en parasitaire infecties en expositie aan UV, hypoxia en verontreinigingen in het milieu (zoals kwikionen). Mestcellen initiëren niet alleen de immuunrespons, maar kunnen deze respons ook nog moduleren. Gezien het feit dat mestcellen nu steeds meer worden gezien als cellen met een pro- én anti-inflammatoire functie, moeten mestcellen worden gerespecteerd als belangrijke onderdelen in de immunoregulatie en als therapeutisch doel in de behandeling van ontstekingsaandoeningen en immuundeficienties.

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Finally the time has come to look back and reflect on the past few years – the quest for PhD. For all this years – at the university and later on – my girlfriend Ania has always provided me with unconditional support. Always full of understanding for the life of a scientist: working overtime and weekends, need for mobility and obviously the salary of a scientist. She has never complained and always expressed her belief in me. Now is the time and place to express my appreciation: thank you, this couldn't be done without you!

How have I reached this point? From time to time an evaluative look back certainly is worthwhile. I think that all this starts before you know what science is at all. It is the parents that are responsible for seeding this appreciation for knowledge. In my case it started with a Little Chemist kit and ended up with blowing up the toilet at school. At all times (well, maybe except for the toilet case) my parents were full of understanding, supported me and showed interest in my progress. For this I can't thank them enough. I hope they are proud of what I achieved and that they see their efforts rewarded. I also hope there's more to come.

In the distant times before I entered the university there were two persons important for my further proceedings. Zdzislaw Zaleski was my chemistry teacher in secondary school. He was able to awaken ambition and competition spirit in some students and induce them to invest their free time and extra work in preparations for the National Chemistry Olympiad. He also spent his free time in the lab preparing his team for the contest. Thanks to him I had the opportunity to represent my school in the "Top 100" final thrice and won myself an entry to the university. Meanwhile my interests drifted somewhat towards biology but never far enough from chemistry to make me a botanist. I found molecular biology a field where these two disciplines meet forming the foundations of life. When the time came to decide what to study I had the flirt with an idea of getting rich. I applied for both the leading business school and science department. Succeeding in both I faced the dilemma, a choice that seemingly was as clear as it gets but still not an easy one. As I see it now, it was my elder brother whose advice finally made my scientific spirit win. I am grateful for that every time I see an army of uniformed people leaving one of those skyscrapers, which is not often since being a scientist certainly is not a nine-to-five job.

It was at the Warsaw University that I've had my first contact with real science. I was a student of Inter-Faculty Individual Studies in Mathematics and Natural Sciences where one could shape their own study programme picking and mixing from seven departments as seemingly distant as biology, mathematics, geography or psychology. Thus it was important for an inexperienced student to have a tutor helping the potential of the student and his interests meet. My tutor was Dr. Aleksandra Dmochowska from the Department of Genetics who helped me through the pitfalls of choosing the lectures and becoming a molecular biologist with an extended chemistry background. It was also her who encouraged me to join the group of Professor Piotr Weglenski for the research part of my master thesis. I worked under the supervision

of Dr Agnieszka Dzikowska and she was the person that really introduced me to labwork. She has always emphasized the need for accuracy, scientific soundness and carefulness in experimental work. How many times did she stop my premature jumping to conclusions with this one question concerning proper controls… This, and the project itself that involved seeding and reseeding of thousands of colonies of Aspergillus nidulans taught me patience, being critical towards what you're doing and that scientific success is 1 percent of luck and 99 percent of hard work. She also was an invaluable help in preparation of my thesis, the first piece of scientific text I had to write.

Several friendships of these made during my university time survived the trial of time. I'd like to thank Marcin von Grotthuss, Justyna McIntyre and Wojtek Dragan for being good friends, discussing science at parties and other stuff that I'm not really supposed to mention here. Special thanks to Marcin, my paranymph, for winding up the scientific competition spirit that keeps pushing us forward in the quest for our first Nature papers.

Now, with a diploma in my pocket I had to decide what to do. I realized that the most career-wise option would be emigration. I also realized that having received a PhD I certainly wouldn't come back to Poland for a postdoc. I decided it was too early to leave everything for a decade or more and applied for a PhD student position at the International Institute of Molecular and Cell Biology in Warsaw, within a joint initiative of IIMCB and Utrecht University. The project was coordinated by Professors Willem Gispen and Michal Witt who were always very helpful when any problems related to international cooperation emerged. I was accepted for the position in the Laboratory of Molecular Immunology headed by Dr Jaroslaw Dastych, a co-promoter of this thesis and my supervisor in Warsaw. Himself being interested in mast cells he designed a project concerning intracellular trafficking of TNF into mast cell granules. At this point I became a cell biologist. All I had were a fluorescent microscope with a 40x dry objective and a digital camera of the type you take for holidays. I couldn't see anything and when I did I still couldn't photograph it. Now, with an expertise on confocal microscopy I see it was like van Leeuwenhoek trying to see mitochondria. Nevertheless, with the help of my fellow PhD students Dominika Trzaska, Violetta Adamczewska and Ula Wyrzykowska from Laboratory of Molecular Immunology I managed to obtain my first publishable data. We shared the difficulties of colonizing the brand-new institute with Aleksandra Szybinska, Sanne Mikkelsen, Marta Bucko, Grzegorz Kudla, Leszek Lipinski, Lech Trzeciak and many others that, though not mentioned due to space constraints, are certainly not forgotten!

I have to mention that the publication of this paper, my first first-author one, with all its 6 figures in colour was financed by IIMCB, courtesy of Professor Jacek Kuznicki, the director of the institute.

Our Dutch counterparts were Professor Carla Bruijnzeel-Koomen and Dr Edward Knol, a promoter and co-promoter of this thesis, respectively. During my stay in Utrecht they were very helpful in terms of both scientific guidance and everyday life.

The problematics and tools necessary for my project were new to the Department of Allergology and Dermatology but Edward has made every effort to provide me with what I needed or contact me with the people who could help. At this point I have to thank Professors Paul van Diest, Alain Kummer, Paul Coffer and Leo Koenderman for sharing their resources and providing creative environment. When, as usually in a new lab, I was totally lost Miranda Buitenhuis, Adrie van Ieperen-van Dijk and Marloes Laaper-Ertmann helped me to find my way. Onno ten Berge, Inge Haeck, DirkJan Hijnen, Jorg van Loosdregt, Marja Oldhoff and Evert Nijhuis are the guys from my Dermatology room who made the life merry and rich in stroopwaffels. Bert Ruiter and Peter Lee always ready for the joke, fortunately not a practical one. I also have to thank Jantine Korpel and Miranda Jacobs-Verhoef, the secretaries that guided me through the maze of Dutch formalities. Obviously everyone in the department contributed to the great atmosphere for which I thank to the people not mentioned but certainly not forgotten.

In search of equipment and reagents I explored neighbouring departments making friends and receiving help from Marnix Wieffer from Cell Biology Department and the gang from Pathology Department: Pieter deKoning, Eelke Gort, Lennert Ploeger, Niels Bovenschen and Dick van Wichen. Special thanks to Arjan Groot, with whom I spent countless hours in a quiet and deserted lab, biking home well after midnight. Since we happened to live in the same house we've also had our bit of fun together. Thanks, Arie!

I have left The Netherlands richer in a pack of new friends and a box of results that later on turned out to be publishable. A note for any young microscopist: find a sponsor! Publishing a paper full of colour figures is pretty expensive; this time it was Carla and Edward that covered the expense instead of telling me to convert the figures to greyscale.

Having finished the Dutch period I returned to IIMCB and took up a position in the Department of Molecular Biology headed by Professors Maciej and Alicja Zylicz. Scientifically I started a completely new chapter but all along I received from them full moral and financial support I ever needed. The time needed to actually write the thesis has been stolen from my labwork. This thesis wouldn't have come into being without my new bosses and I'm extremely grateful for that. Professor Jacek Kuznicki, the director of IIMCB also showed a vivid interest in my proceedings and finally financed printing of this thesis.

Realizing that the list of people, who in this or other way contributed to my being here, is far from exhaustive, I'm closing this chapter and moving on to new challenges I'm certain that there is something in the words of the song that is occasionally heard around the Anfield Road: "You'll Never Walk Alone".

Curriculum vitae

Education

Warsaw University, Warsaw, Poland, M. Sc. in molecular biology, 2000
Thesis: "Cloning of Aspergillus nidulans SuH gene", passed "*cum laude*"
Promoter: Prof. Piotr Weglenski, supervisor: Agnieszka Dzikowska, PhD

Employment

From 2006	Research Assistant at the International Institute of Molecular and Cell Biology, Warsaw, Poland, Department of Molecular Biology, headed by prof. Maciej Zylicz Involvement of molecular chaperones in p53 aggregation under stress conditions
2004-2005	Research Assistant at the University Medical Center, Utrecht, The Netherlands Department of Allergology/Dermatology, prof. Carla Bruijnzeel-Koomen Regulation of cytokine expression in mast cells.
2000-2004	Research Assistant at the International Institute of Molecular and Cell Biology, Warsaw, Poland, Laboratory of Molecular Immunology, headed by Jaroslaw Dastych, PhD TNF trafficking in rodent and human mast cells

Grants and Awards

2005	EMBO Short-term fellowship
2005	FEBS Collaborative Experimental Scholarship for Central & Eastern Europe
2004	Best Poster Award in New Scientist category, Microscience2004, London
2000-2004	PhD Scholarship in cooperation with IIMCB, Poland funded by Utrecht University, The Netherlands
2001	Polish State Committee for Scientific Research grant: "The role of TNF-α propeptide in storage of this cytokine in mast cell granules"
2000	Erasmus/Socrates Stipend, research conducted at Manchester University, UK Cellular stress and GADD153 expression in primary murine hepatocytes

Research experience and courses

My current research activities comprise mostly molecular biology of the cell. Major techniques I specialize in are fluorescence microscopy and confocal microscopy, supported by protein expression analysis. I also have experience with mammalian cell culture, transfection and cloning.

Participation in the following research projects

European Union 6th Framework Programe Project "A Prospective Analysis of the Mechanisms of Nuclear Hormone Receptors and their Potential as Tools or the Assessment of Developmental Toxicity"

European Union 5th Framework Programe Project "A new Technology for Fluorescent 'Cell Chip' Immunotoxicity Testing" ("Tools and methods useful in characterising the immunotoxic activity of xenobiotic substances" patent co-holder)

State Committee for Scientific Research grant: "Characterisation of Promoter Sequences, Transcription Factors and Signal Transduction Pathways involved in the Activation of IL-4 Gene Expression by Heavy Metal Ions"

State Committee for Scientific Research grant: "The role of TNF-α propeptide in storage of this cytokine in mast cell granules"

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