Hematopoietic secretory granules as vehicles for the local delivery of cytokines and soluble cytokine receptors at sites of inflammation

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ABSTRACT. Cytokines play an important role in the regulation of homeostasis and inflammation. A de-regulated cytokine function can subsequently promote chronic inflammation. This is supported by clinical evidence showing the beneficial effect of inhibiting TNF-α through injection of antibodies and soluble receptor in disorders such as rheumatoid arthritis and Crohn's disease. Systemic anti-TNF-α therapy however is associated with infectious complications. We therefore suggest a concept for the local deposition of therapeutically active agents into areas of inflammation or malignancy, based on the use of hematopoietic storage and secretory granules as delivery vehicles. Hematopoietic cells are induced to express the therapeutically active protein and to store it in the secretory lysosomes. The cells migrate into a tumour or site of inflammation, where the cells become activated and release the contents of their secretory lysosomes resulting in the local delivery of the therapeutically active protein. In support of this concept, gene transfer and granule loading can be achieved using the soluble TNF-α receptor (sTNFR1) after cDNA expression in hematopoietic cell lines. Endoplasmic reticulum (ER)-export can be facilitated by the addition of a transmembrane domain, and constitutive secretion can be prevented by incorporating a cytosol-sorting signal resulting in secretory lysosome targeting. The sTNFR1 is released from the transmembrane domain by proteolytic cleavage and finally, regulated sTNFR1-secretion can be triggered by a calcium signal. *In vivo* investigations are currently determining the feasibility of local protein delivery at sites of inflammation.

Keywords: inflammation, tumour necrosis factor, TNF-α, soluble TNF-α receptor, sTNFR, secretory lysosome, NK-cell, granulocyte, mast cell, secretory lysosome targeting, local delivery

BACKGROUND

Introduction

Inflammation is a normal, beneficial local response to injury. However, when the inflammatory reaction can no longer be regulated, systemic complications can arise and conditions can become critical e.g. in septic syndrome [1]. Furthermore, persistent inflammation occupies a central position in the pathophysiology of many diseases. It can promote a chronic inflammatory disorder or even neoplastic transformation. New anti-inflammatory treatment modalities include anti-tumour necrosis factor alpha (TNF-α) therapy, leukotriene receptor blockers, cyclooxygenase inhibitors, and many more. In particular, inhibition of cytokines such as TNF-α and IL-1 is beneficial in the treatment of rheumatoid arthritis and other chronic inflammatory disorders. However, these treatments are systemic and therefore bring with them an increased risk of severe infection. In this review, the principle is suggested that secretory granules of hematopoietic cells can be used as vehicles for the local delivery of therapeutic agents to tumours or at sites inflammation. These granules contain anti-microbial agents and other agents of importance in innate immunity. The aim of the principle is to load the granules with the protein of interest, achieve regulated secretion of the granules at a specific site of deposition, and thereby target the protein at an inflammatory focus. So far, after expressing cDNA in hematopoietic cells, our *in vitro* studies have achieved granule targeting and regulated secretion of anti-inflammatory proteins, e.g. soluble TNF-α receptor (sTNFR) [2]. We are currently investigating the principle of targeted protein delivery *in vivo*, at sites of local inflammation.

Cytokines in inflammation – starts and stops

Tissue injury or infection triggers pro-inflammatory cytokine production and/or release from mast cells and macrophages that stand on guard to promote the inflammatory reaction *(Figure 1)*. Released cytokines activate the endothelium, which primarily attracts neutrophils to

Cells on guard in tissue	Guard cell action	Effector cells recruited	Effector cell actions
Mast cells	Leukotrienes	Neutrophils	Respiratory burst
Macrophages	Histamine		Degranulation
APCs	Chemokines	Lymphocytes	Cytokines
	Cytokines		Cytotoxicity
	(TNF, IL-1 etc)		
	Antigens		

Figure 1
Simplified scheme of the early stage inflammatory process triggered by injury and infection. APC: antigen-presenting cell.

the injury focus. Other hematopoietic cells are recruited at later stages. A lack of leukocyte recruitment could be deleterious by contributing to the spread of an infection (dysfunctional start signal). Moreover, an over-stimulated leukocyte mobilization may contribute to prolonged inflammation (hyperfunctional start signal or dysfunctional stop signal). Neutrophil recruitment is co-ordinated by selectin activation to facilitate rolling along the vascular endothelium, leukocyte integrin activation to facilitate neutrophil sticking on the endothelium surface, and finally, chemokine gradients that direct neutrophil transmigration to the injury site (the battlefield). Furthermore, the pro-inflammatory cytokines activate additional cytokines that amplify the inflammatory response and generate downstream secondary responses. In contrast, stop signals switch the inflammation process towards healing and tissue repair. To achieve repair, TNF-α, IFN-γ, TGF-β and other cytokines change from being pro-inflammatory to becoming anti-inflammatory [3]. Removal of apoptotic neutrophils by macrophage ingestion is important in preventing local tissue damage. An additional continuous, active suppression of inflammation is also necessary. This is suggested by the finding that the disruption of many genes predisposes both mice and humans to inflammatory disease. The gene products necessary for suppressing an inflammation exacerbation frequently have a role in (A) clearance of immune complexes, (B) activation, proliferation and apoptosis of inflammatory cells, and (C) inhibition of oxidative injury [3]. The knowledge of specific functions of these gene products is likely to generate new ideas both for understanding the underlying pathophysiology in different inflammatory disorders and for the development of new therapeutic modalities.

Acute deleterious inflammation – chemokine dysfunction

Bacterial infections can give rise to life-threatening complications such as sepsis and septic shock through host soluble TNF-receptor response modulation by the invading organisms [4, 5]. Thus, virulent strains of group A streptococcus can cause severe, life-threatening, invasive disease including necrotising soft-tissue infections, sepsis and toxic-shock syndrome [6]. The interaction of these bacteria with host cells has been shown to cause an increased pathogenicity and an up-regulation of genetic elements relevant for virulence [7, 8]. Furthermore, evidence has been presented to suggest that deficient leukocyte recruitment to the injury site might contribute to the spread of an infection [9]. Here, the authors suggested that the bacteria multiplied and spread rapidly because of a lack of IL-8, resulting in retardation of neutrophil recruitment. The neutrophil reduction in necrotic tissues was partly caused by a group A streptococcus protease that degraded IL-8. Furthermore, administration in mice of the bacterial peptide SilCR, whose gene is mutated in the virulent group A streptococcus strains, abrogated chemokine proteolysis and restored neutrophil migration [9]. The principle of local delivery by local deposition of chemokine or protease inhibitors discussed in this review could well be feasible in these types of serious conditions.

Chronic inflammatory disorders – anti-cytokine therapy

Inflammation is self-limiting unless dysfunction creates a persistent pathological process such as in chronic inflammation or neoplastic transformation. Among the pro-inflammatory cytokines, TNF-α [10-13] has a particularly strong beneficial and protective effect in homeostasis. Nevertheless, TNF-α can also give rise to deleterious systemic effects when over-produced. Furthermore, TNF-α can induce the expression of other pro-inflammatory cytokines, such as IL-1 as well as chemokines [14-15]. TNF-α may therefore induce chronic inflammation both directly and indirectly. A state in which TNF is continually over-expressed is therefore likely to become destructive and result in tissue damage, such as in chronic inflammatory arthritis and Crohn's inflammatory bowel disease [16]. Consequently, cytokine inhibitors such as soluble TNF-α receptors (first identified as TNF-α-binding proteins in biological fluids) [17-19], as well as antibodies against TNF-α [20], have become promising therapeutic possibilities in these diseases [21].

Despite possible harmful pro-inflammatory activities, TNF-α may inhibit the development of autoimmunity [22] suggesting a possible beneficial immunosuppressive TNF-α effect *(Figure 2)*. In support of this, TNF-α prevented autoimmune diabetes in transgenic non-obese diabetic (NOD) mice [23]. Furthermore, blocking TNF-α in multiple sclerosis patients resulted in disease exacerbation indicating a need for TNF-α in suppressing autoimmunity [24]. However, the pro-inflammatory and immunosuppressive properties of TNF-α can, in fact, be uncoupled from each other. Thus, signalling through the p55 TNF-α receptor (TNFR1) was not required for TNF-α-mediated immunosuppression and protection against experimental autoimmune encephalitis [25]. In contrast, the same receptor was necessary for the occurrence of the detrimental effects of TNF-α during inflammation. Obviously, the beneficial and deleterious effects of TNF-α are dependent on its concentration, and the tissue and disease in context.

Proinflammatory TNF-α (p55 receptor)	Immunosuppressive TNF-α (p75 receptor)
Inflammation	
Crohn's disease	
Rheumatoid arthritis	
Sepsis	Sepsis
Multiple sclerosis	Multiple sclerosis
Diabetes	Diabetes (NOD)

Figure 2
Pro-inflammatory and immunosuppressive TNF-α actions.

Systemic administration of anti-TNF-α antibody or soluble TNF-receptor fusion protein can control signs and symptoms in patients with rheumatoid arthritis and reduce inflammation and joint destruction [21]. However, even if the anti-TNF-α therapy was beneficial, it did not cure the disease. Anti-TNF-α-treatment down-regulated the cytokine cascade in these patients. The concentration of IL-6 was normalized and a reduction in IL-8, MCP-1 and VEGF was observed. Furthermore, leukocyte trafficking into the inflamed joint was reduced [26]. Significant clinical responses were achieved with both an IgG1 murine-human chimeric antibody (infliximab) [27], and a fusion protein formed between recombinant soluble human TNF-α receptor and human IgG1 (etanercept) [28-30]. Approximately two-thirds of the rheumatoid arthritis patients responded to the therapy within two to four weeks, with one-third being non-responders. The variable response to anti-TNF-α therapy might be explained by TNF-α promoter gene polymorphism [31] or disease phenotype heterogeneity. A variation in host genetic cytokine profiles might also explain the non-response to anti-TNF therapy, e.g. in some patients TNF-α was not the main causative mediator of the disease. Furthermore, alternate genes with as yet unidentified products may predict the response to treatment in rheumatoid arthritis [32].

Anti-TNF-α therapy with the monoclonal antibody (infliximab) is also beneficial in Crohn's disease [33]. In contrast, soluble TNF-α-receptor has not been proven to be effective against this disease [34, 35]. As in rheumatoid arthritis, one-third of patients were non-responders to the monoclonal antibody. The pathogenic source of TNF-α in Crohn's disease is assumed to be $CD4^+$ T cells [31]. The anti-TNF-α antibody promotes apoptosis of these effector T cells by binding the membrane bound TNF-α that seems to be responsible for the bowel inflammation. The lack of clinical effect by soluble TNF-α receptor (etanercept) supports this proposal. TNF-α receptor polymorphisms have also been associated with the variable therapy response in Crohn's disease [36].

Manipulating the effects of a cytokine that has an important role in normal biological function would have considerable adverse effects upon a patient. This is clearly observed in anti-TNF-α therapy where side effects such as infections, autoimmune reactions, non-Hodgkin's lymphomas and neurological complications such as CNS demyelination and multiple sclerosis are seen. However, by far the most serious infectious complication aassociated with anti-TNF-α therapy is reactivation of tuberculosis [22]. This is more often observed in rheumatoid arthritis patients than in Crohn's disease patients. If the principle of local delivery discussed in this review is feasible, it is possible that these side effects could be reduced.

Other inflammatory regulators, such as the IL-1 receptor antagonist [37], decoy receptors e.g. IL-1 receptor type III, and the anti-inflammatory cytokine IL-10 [38] may also counteract inflammatory responses in rheumatoid arthritis and Crohn's disease. A small molecule approach to anti-TNF-α therapy has also been suggested based on thalidomide, p38 MAP kinase inhibitors and TACE inhibitors [39].

Neoplastic disease – inflammatory response

Ever since the middle of the 18th century, when Virchow postulated that cancer originated from sites of chronic inflammation, a relationship between inflammation and cancer has been observed. The finding that non-steroidal anti-inflammatory drugs (NSAIDs) have a preventive effect in gastrointestinal cancer also supports a role for inflammation in malignant transformation [40]. Many tumour cells produce cytokines and chemokines that stimulate the production of inflammatory cells and their migration to the tumour site infiltrate [41]. As a consequence, most tumours become surrounded by an array of inflammatory cells such as macrophages, neutrophils, dendritic cells, eosinophils and mast cells. This inflammatory infiltrate has a dual function. It is essential for the initiation of an adaptive immune response and can produce a direct anti-tumour response. Moreover, it can also promote tumour growth and be responsible for tumour dissemination. Thus, as part of the normal host response, tumour-infiltrating inflammatory cells may promote early neoplastic progression.

A major component of the inflammatory infiltrate in neoplastic disease is the tumour-associated macrophage (TAM) [42]. This cell is derived from peripheral monocytes, which are recruited to the tumour site primarily by the monocyte chemotactic protein. This chemokine is produced after stimulation by pro-inflammatory cytokines, such as TNF-α [43]. Although TAMs have the capacity to kill neoplastic cells after activation, they also release angiogenetic growth factors, cytokines and proteases, which in contrast promote neoplastic progression. Furthermore, it is important to know that TAMs produce IL-10 [44] and oxygen radicals [45]. These can block the anti-tumour response of cytotoxic T-cells and NK-cells, cells that are endowed with protective activity against tumours and that are frequently found within tumours. According to a well establish theory in tumour immunology, the functions of intra- or peritumoural lymphocytes are impaired, in contrast to T or NK-cells in the neighbouring tissue or in peripheral blood. This is referred to as tumour-induced immunosuppression and could be explained, at least in part, by TAM activity.

In support of this, clinical observations show high TAM density within malignant tumours to be inversely related to disease severity and prognosis. Thus, the degree of TAM infiltration correlates to tumour invasiveness in colorectal carcinomas [46]. Furthermore, an increase in tumour TAMs predicts both reduced relapse-free and overall survival in patients with invasive breast cancer [47]. In addition, a high number of TAMs correlates to local progression of malignant melanoma [48, 49] and gastric carcinoma [50].

It is obvious from the preceding discussion that pro-inflammatory cytokines, TNF-α in particular, may play a key role upstream in early malignancy *(Figure 3)* by setting the stage for downstream mediators that enhance tumour development and spreading [51]. Neutralizing TNF-α may therefore have a role in cancer prevention and therapy at an early stage of tumour progression. Furthermore, a change from an inflammatory response into a lymphocyte-based tumour response would benefit the host by suppressing the tumour growth. A local delivery concept could therefore be valid approach for tumour therapy, with local anti-TNF-α therapy and local pro-lymphocyte response therapy.

Our local delivery concept could use neutrophils as well as T- and NK-cells as vehicles for the transport of substances

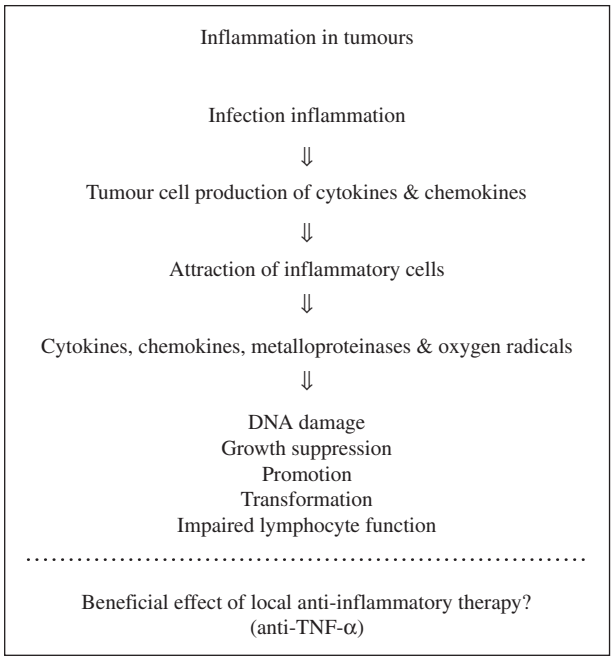

Figure 3
The role of pro-inflammatory cytokine (TNF-α) in tumours.

into tumours. Anti-TNF-α therapy facilitated by granulocytes might reduce the activity of the inflammatory cells surrounding tumours, and NK-cells that patrol tissues and destroy tumour cells by release of lytic granules could be of particular interest as potential vehicles for the local delivery of therapeutic molecules in tumours. Many studies have already been conducted to improve the NK-cell tumour cell toxicity (see review [52]). Furthermore, local delivery of chemokines by NK-cells themselves may amplify the accumulation of NK-cells at the tumour site and sustain an anti-tumour effect.

Biogenesis of hematopoietic granules – the vehicles

Neutrophils, mast cells, NK-cells, cytotoxic T-lymphocytes (CTLs) and other hematopoietic cells have a critical role in host defence. Neutrophils eliminate microorganisms by phagocytosis and phagosomal killing [53]. Mast cells have a primary role in allergic reactions but also play a critical role in autoimmune diseases [54]. NK-cells and CTLs destroy virus-infected and malignant cells by the action of cytolytic granule proteins, including perforin and serine proteases, released by regulated secretion [55, 56]. Hematopoietic cells in general accumulate bioactive agents in subsets of granules synthesized in precursor cells. These stored bioactive agents can be released from mature cells [57, 58]. Neutrophils have many subsets of storage granules. Lysosome-like azurophil granules are manufactured first and equipped with anti-microbial proteins and cationic serine proteases as well as lysosome hydrolases during the promyelocyte stage of maturation [57, 58]. Hematopoietic cells commonly have lysosome-related organelles that store cytolytic proteins of a cell-specific character together with lysosome hydrolases [55, 56]. These organelles are also called secretory lysosomes because of their combined ability for storage, regulated secretion and lysosome activity [56]. Their structural morphology comprises an external limiting membrane, internal membrane-bound vesicles, dense cores of tightly packed proteins, and a matrix compartment [56]. The internal vesicles are formed in an unusual manner by inward budding towards the compartment lumen, in a similar manner to viruses. They may serve a lytic function while the dense core and matrix compartments serve a storage function [56].

In addition to secretory lysosomes, hematopoietic cells have other storage granules. Among these are specific (secondary) neutrophil granules manufactured after the azurophil granules during the myelocyte stage of maturation. These granules are equipped with anti-microbial proteins, matrix metalloproteinases and other constituents [57]. Such organelles may also be used as possible vehicles for targeting the inflamed site with exogenous proteins. The degranulation dynamics differ between the various granule subsets. Specific granules are mobilized more readily than azurophil granules [57], and some specific granule release may even occur as a response to neutrophil activation during transmigration into tissue. Targeting exogenous proteins to these readily mobilized storage granules may therefore make it possible to affect extravasation and trafficking of blood cells. Easily mobilized storage organelles are also present in other hematopoietic cells such as cytotoxic T-cells and NK-cells [59].

Granule targeting and regulated secretion – loading and unloading the vehicles

An active sorting process postulates that proteins destined for granule targeting bind to a sorting receptor in the trans-Golgi network (TGN) for selective delivery into the granule biogenesis pathway. The binding to a receptor requires a sorting signal. A protein lacking a sorting signal would be excluded from sorting and directed to the constitutive secretory pathway by default. In contrast, a passive sorting process postulates that the delivery into the granule biogenesis pathway is non-selective and independent of receptor binding. These potential alternatives should be viewed in light of the existence of different kinds of granules, all with a unique composition, present in the same cell, e.g. the neutrophil. Thus, different granules may require different protein targeting mechanisms.

Retrieval of newly synthesized proteins from constitutive secretion is indispensable for granule targeting. Secretory lysosome targeting of certain proteins requires sorting determinants that are recognized by the transport machinery thus allowing entry into the cargo route. Our principle of granule targeting is based on the use of such sorting determinants incorporated into exogenous proteins not normally expressed in hematopoietic cells. To become secretory lysosome residents, many trans-membrane proteins require a cytosol-sorting signal with a tyrosine (Y) motif. This motif may conform to the lysosome sorting sequence YXXØ, used in coat recruitment, membrane invagination and transport vesicle formation [56, 60]. The X positions can accommodate any amino acid, while the Ø position comprises of residues with bulky hydrophobic side chains. When serving as a secretory lysosome-targeting signal, the YXXØ sequence is located at the carboxy-terminal close to the trans-membrane domain [60]. On the other hand, the secretory lysosome targeting determinants of granule matrix proteins are largely unknown [58]. An exception is the mannose-6-phosphate receptor (MPR) that targets hydrolases to lysosomes in many cells. The MPR system is also responsible for

granzyme targeting to secretory lysosomes in NK cells and CTLs [61]. Neutrophils, however, seem to be independent of this system [58]. The MPR system brings cargo from the TGN to secretory lysosomes by the endosome route. Hematopoietic cells may also use a non-endosome route to secretory lysosomes. Efficient targeting of the subcompartments of these organelles may require separate transport routes to avoid mixing of incompatible proteins.

Secretory lysosome degranulation at the inflamed site is calcium-dependent [56]. Furthermore, synaptotagmins on secretory lysosomes act as calcium-sensors to regulate exocytosis [62]. A calcium-signal can trigger the extracellular release of granule matrix and dense core proteins, after fusion of the secretory lysosome-limiting membrane with the plasma membrane. The internal vesicles of secretory lysosomes, called exosomes, can be released intact [63]. Membrane fusion processes are mediated by vesicle (v)- and target (t)-SNARES [64, 65] and by small isoforms of GTPases of the Rab protein family [66].

Granule delivery at the inflamed and neoplastic site – local action

If a "foreign" protein were targeted to specific granules and moreover, was stable during storage, it would be delivered to an inflamed site by degranulation. We have observed that certain exogenous, non-hematopoietic proteins, that are expressed by cDNA transfection, can be targeted to granules in hematopoietic cells [67]. Thus, the targeting mechanism(s) may not be unique for endogenous granule proteins, but may also be applicable for exogenous proteins. Further, the co-existence of lysosome enzymes and hematopoietic serine proteases with several antibiotic proteins in secretory lysosomes, indicates that co-storage is possible without degradation. Consequently, a strategy that takes advantage of a sorting signal for granule targeting should be generally applicable for protein targeting to secretory lysosomes of hematopoietic precursor cells. If local release of the protein of interest, e.g. an anti-cytokine agent, could be achieved at an inflamed site, the approach would have great therapeutic potential in dampening an inflammatory reaction. Furthermore, a principle for local delivery and local action of cytokines and soluble cytokine receptors would have the advantage of inducing low systemic effects.

EXPERIMENTAL SUPPORT FOR THE PRINCIPLE

Cell models for granule targeting and regulated secretion

Hematopoietic cell lines with granule biogenesis have been used as models in targeting and secretion studies. Characterization of sorting, processing and secretion has been performed after cDNA expression of the gene of interest in the murine myeloblast-like leukaemia 32D cell line [68] and the rat basophilic leukaemia (RBL) [69] cell line. These cells have been used frequently in protein targeting and processing studies [58]. Furthermore, experiments have been performed in the NK-cell lines YT-Indy [70] and NK-92 [71]. The dense core content of CTL and NK-cell secretory lysosomes comprises of perforin and granzymes packed in complexes with ser-gly-rich proteoglycans [72]. While the dense core content can be secreted as intact proteoglycan complexes, the internal vesicles can actually be secreted intact to the cell exterior as exosomes [73]. During such conditions, the matrix content is assumed to be released in a soluble state.

ER protein export – quality control

Secretory and membrane proteins acquire their native conformation in the endoplasmic reticulum (ER) before exiting to the sorting station, the Golgi. Improperly folded proteins are identified by a conformation-based quality control and diverted for degradation in the proteasome [74]. The cDNA expression for granule targeting of the sTNFR1 gene product was not successful because ER retention and degradation of the expressed protein could not be prevented *(Figure 4A)*. Furthermore, the ER-exported portion of sTNFR1 was constitutively secreted [75]. Similar observations have been made for other truncated proteins, e.g. propeptide-deleted myeloperoxidase [76]. Moreover, chimeric proteins were also subject to ER retention and degradation [75]. By anchoring sTNFR1 by a transmembrane domain (tm), this problem was diminished. However, although the exit of sTNFR1-tm to the Golgi was greatly enhanced, there was no granule targeting *(Figure 4A)* [3].

Preventing constitutive secretion – retention signal requirement

Not all proteins that are transported to the Golgi compartment of hematopoietic cells will be granule targeted but some will be constitutively secreted. The finding that some proteins escape retrieval and become constitutively secreted suggests the necessity for cell- and/or protein-specific mechanisms for granule targeting. The finding that chimeras and multimers thereof are prevented from targeting suggests that protein conformation is important [77]. When truncated proteins are expressed in hematopoietic cells, secretion instead of sorting is observed. Therefore, even if an sTNFR1-tm construct was cleared by the ER quality control and released via the secretory route to the Golgi sorting station, it could still be lost from the cell by constitutive secretion [75]. Results from subcellular separations have indicated an accumulation of sTNFR1-tm in fractions corresponding to the ER, Golgi and plasma membrane, but significant secretory lysosome sorting of this protein was not in evidence. Instead, sTNFR1-tm seemed to be translocated to the plasma membrane, where sTNFR1 was shed by proteolytic cleavage. A retention signal for granule targeting was lacking *(Figure 4A)*.

Achieving secretory lysosome targeting – sorting signal requirement

In order to accomplish secretory lysosome targeting, a sorting signal is required. The cytosol sorting sequence SIRSGYEVM, designated construct Y and conforming to the secretory lysosome sorting sequence YXXØ, was incorporated to rescue the sTNFR1-tm-Y construct from constitutive secretion and direct it to the secretory lysosomes [78]. The Y sequence corresponds to the tyrosine-based sorting motif of CD63, a secretory lysosome membrane protein [79, 80]. We assumed this motif should enable targeting of sTNFR1-tm-Y in a similar manner to that of CD63 itself. In fact, targeting of sTNFR1-tm-Y to secretory lysosomes through this sorting motif was more

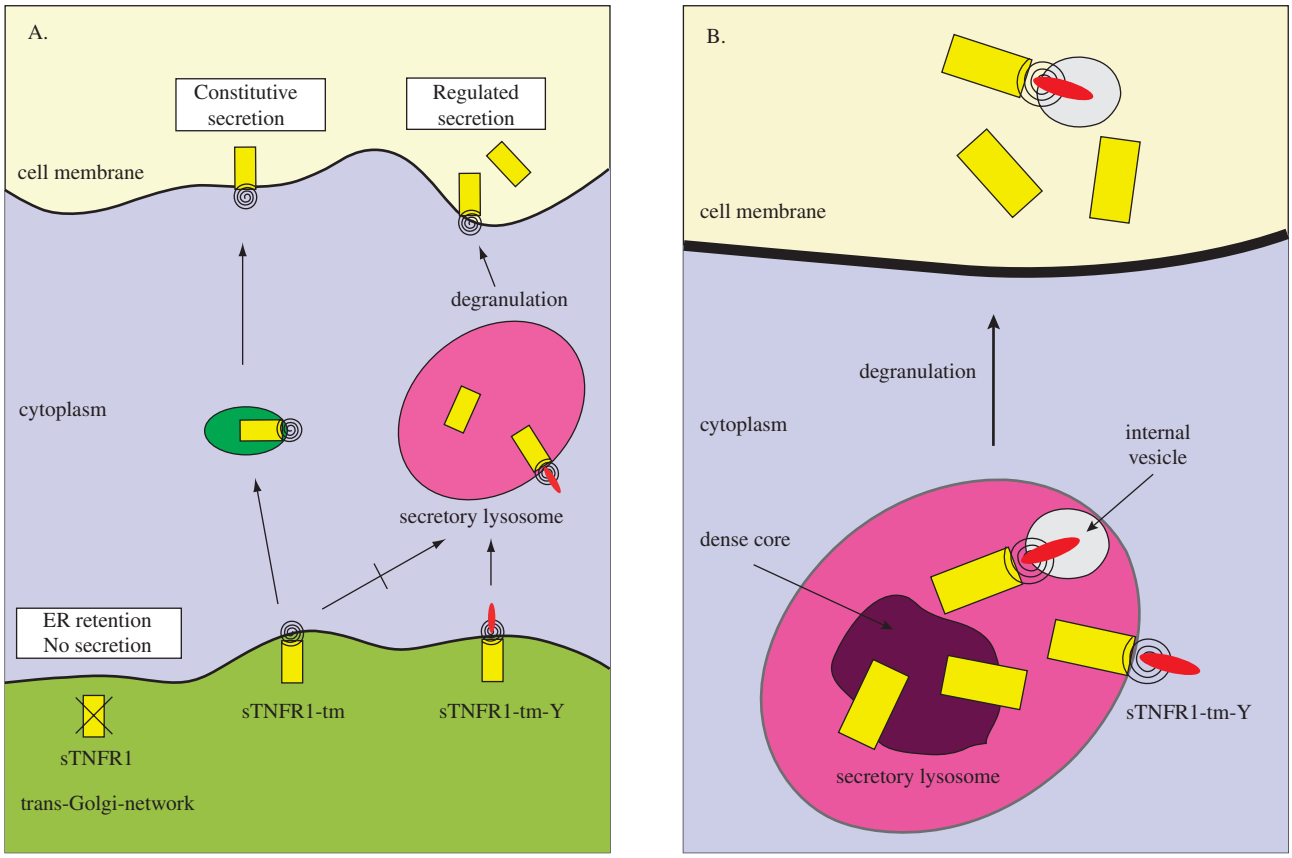

Figure 4

Secretory lysosome targeting in hematopoietic cells as a principle for targeting of selected proteins to areas of inflammation and tumours. **(A)** Schematic figure of the fate of the expression of different constructs. Expression of the **sTNFR1**-rendered retention in the endoplasmatic reticulum and degradation. Addition of a transmembraneous region, **sTNFR1-tm**, allowed transport to the trans-Golgi network but the product was constitutively secreted and no secretory lysosome targeting was seen. Free sTNFR1 was cleaved from the cell membrane by proteolytic cleavage. A tyrosine-based sorting motif (Y), corresponding to the sorting motif in CD63, achieved secretory lysosome sorting of **sTNFR1-tm-Y. (B)** Schematic figure of the secretory lysosome after expression of sTNFR1-tm-Y. sTNFR1-tm-Y was predominantly observed in the interior of the secretory lysosome but also seen on the limiting membrane. The localisation was probably due to transfer to internal vesicles and/or hydrolytic cleavage of sTNFR1-tm-Y generating sTNFR1 localised to the dense core. Upon degranulation both sTNFR1 and sTNFR1-tm-Y localised to internal vesicles destined for release.

efficient than the endogenous secretory lysosome targeting of granzyme B through the MPR-system *(Figure 4A)*.

The secretory lysosome localization of sTNFR1-tm-Y in RBL cells was confirmed by subcellular fractionation, immunofluorescence microscopy, and immunoelectron-microscopy [78]. Processing was observed for the newly synthesized sTNFR1-tm-Y resulting in generation of a lower molecular weight form. This processed form is likely to consist of sTNFR1 released by limited proteolysis of sTNFR1-tm-Y in the secretory lysosome. Immuno-electron-microscopy results verified the co-localization of sTNFR1-tm-Y and its processed form with endogenous secretory lysosome constituents. Thus, co-localization was demonstrated both with rat mast cell protease–II (RMCP-II) [69] and lysosome-associated membrane protein-1 (LAMP-1) [81]. However, the sTNFR1-tm-Y was mostly observed in the interior of the secretory lysosome, while LAMP-1 was seen along its outer limiting membrane *(Figure 4B)*.

These results are consistent with transfer of sTNFR1-tm-Y into the internal vesicles of secretory lysosomes [73]. Such transfer is reported to require a mono-ubiquitin tag and subsequent binding by the escorting complex ESCRT-1 [82]. CD63 transfer to internal vesicles does, however, not require ubiquitination [83]. The cytosol-sorting motif (Y) at the carboxy-terminal of TNFR1-tm-Y would be localized to the inside during formation of internal vesicles by inward budding. Furthermore, the major amino-terminal part of the construct would be on the outside of the internal vesicle membrane. Delivery to internal vesicles was assumed to be combined with hydrolytic cleavage of sTNFR1-tm-Y to generate sTNFR1 for release into the lumen of the secretory lysosome. Consequently, sTNFR1 would be deposited in the secretory lysosome matrix and/or dense core *(Figure 4B)*.

We also hypothesize that a therapeutic intervention could take advantage of lytic granule targeting in NK-cells, whose primary role is in the defence against virus-infected and tumour cells. If anti-tumour proteins were targeted to the lytic granules of NK-cells, they would eventually be delivered at the tumour site and be therapeutically beneficial after degranulation. Accordingly, it may be feasible to use NK-cell precursors to achieve lytic granule targeting of a gene product. The NK-cell could then migrate in the tissue and find a tumour, whereupon the gene product would be delivered. The genes for several non-NK-cell proteins have been expressed in NK-cell lines [2]. A normal liver secretory protein, α_1-antitrypsin, can be constitutively secreted with very low retention. Conversely, another liver secretory protein, α_1-microglobulin, can be

largely retained in the cells. However, a major amount of newly synthesized endogenous granzyme B is also constitutively secreted, indicating that this cell line has a rather low sorting capacity for soluble proteins.

Expression of sTNFR1-tm-Y in an NK-cell line resulted, however, in efficient intracellular retention. Thus, the secretory lysosome-sorting signal (Y) was also able to promote efficient intracellular retention in NK-cells. The results demonstrated export to the Golgi of newly synthesized sTNFR1-tm-Y, targeting to dense organelles and concomitant proteolytic processing, with production of a lower molecular weight form corresponding to sTNFR1. The targeting of sTNFR1-tm-Y was more efficient than that of the endogenous secretory lysosome constituent granzyme B. Results from immunofluorescence microscopy verified the co-localization of sTNFR1 with the endogenous CD63 in the NK-cell line transfected with s-TNFR1-tm-Y. The distribution pattern of sTNFR1 and the endogenous CD63 was similar. In addition, immunoelectron-microscopy confirmed co-localization of sTNFR1-tm-Y with both granzyme B and CD63. Granule profiles often show small internal vesicles or dense core structures. The CD63 labelling was observed on the outer membrane and on the parallel tubular arrays, and the granzyme B labelling was observed on the dense core and the tubular arrays. Consequently, sTNFR1-tm-Y and its processed form were present in the same granules as granzyme B and CD63, consistent with targeting of sTNFR1-tm-Y to secretory lysosomes.

Achieving regulated secretion

The secretory lysosome is two organelles in one, possibly formed from the merger of a lysosome and a regulated secretory granule. Fusion would be made possible by the formation of compartments whose contents are kept apart by separate delivery routes that prevent direct interactions. Such interaction between constituents from different compartments can be essential for protein activation and may finally occur during secretion when compartment contents are mixed. Regulated exocytosis is critical for the function of hematopoietic cells [56]. In contrast to constitutive secretion, regulated secretion relies on extracellular signals. CTLs secrete when targets are recognized. NK-cells secrete into the intercellular cleft that is created after recognition and binding to a tumour cell in a calcium-dependent manner. Mast cells respond with secretion to IgE cross-linking. Regulated secretion of sTNFR1 and endogenous secretory lysosome constituents can be achieved in sTNFR1-tm-Y transfected NK-92 cells (a human NK-cell line) with a calcium-ionophore (Hansson *et al.* unpublished).

Concluding remarks

Pro-inflammatory and anti-inflammatory cytokines regulate homeostasis. Dysregulated cytokine function, in particular of TNF-α, can give rise to chronic inflammatory disorders and even cell transformation. Inhibiting TNF-α by injection of antibodies or soluble receptors has been proved successful in some of these disorders, although there are associated systemic side effects, such as infectious complications.

Our results suggest a potential for using the storage organelles of hematopoietic cells as vehicles for targeting sites of inflammation with therapeutically active agents that might prevent such systemic effects. The *principle* is summarized in *Figure 5*. The present research is predominantly based upon results from the expression of a soluble TNF-α receptor form in hematopoietic cell lines, and the targeting to secretory lysosomes for storage and regulated secretion. A transmembrane form of the protein was required to facilitate ER-export, and a cytosol-sorting signal for secretory lysosomes was required to overcome constitutive secretion and achieve granule targeting. Our results to date are based on studies of secretory lysosome targeting in transformed myeloblastic, basophilic and NK-cells. However, the results reported are equally applicable to non-transformed hematopoietic precursor cells, since most hematopoietic cells have a lysosome compartment with secretory properties.

The secretory lysosome environment may be potentially degradative because of the presence of catalytically active hydrolytic enzymes. Stability and resistance to proteolysis in this environment are crucial. The half-life of heterologously expressed, non-hematopoietic proteins may be limited, as they are not suited to this environment. However, co-localization is possible between endogenous degradative enzymes and antibiotic proteins or other granule proteins. Furthermore, although the tm-Y fragment was removed by proteolytic cleavage leaving behind the

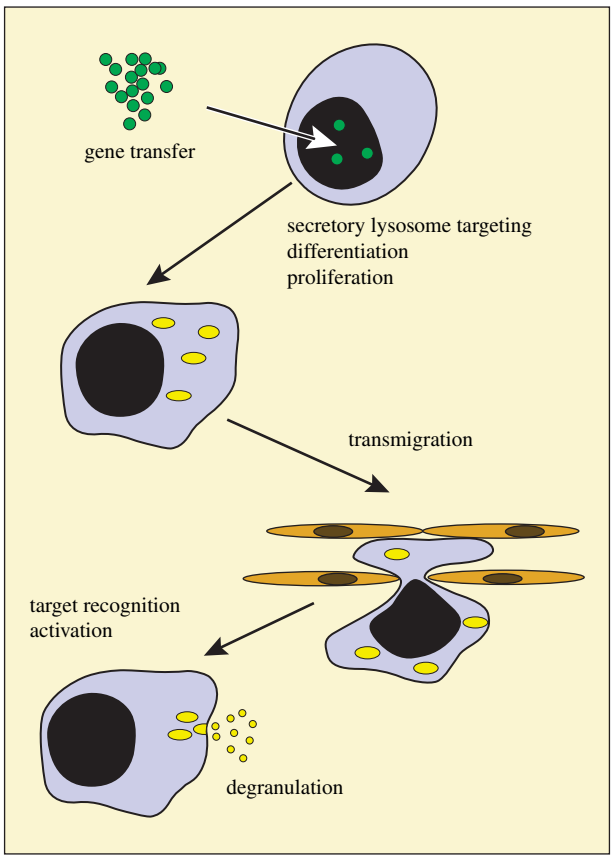

Figure 5
Secretory lysosomes as a vehicle for the delivery of exogenous therapeutic molecules at sites of inflammation or into tumours. The figure shows a schematic diagram of the proposed principle. Gene transfer and granule loading of encoded protein is achieved in hematopoietic progenitor cells, differentiation is induced with cytokines, and mature cells are directed to inflamed or tumour sites by chemotactic gradients. During degranulation, the targeted protein is delivered at the inflamed focus or into the tumour.

membrane-free sTNFR1, the sTNFR1-tm-Y seemed to be rather stable in this environment.

The secretory lysosome function differs between hematopoietic cells. The NK-cell and CTL secretory lysosome secretes to the cell exterior upon extracellular stimulation, whilst the neutrophil secretory lysosome (azurophil granule) secretes primarily to the phagosome during phagocytosis. The azurophil granules would be suitable vehicles for delivering proteins into the phagosome to promote antimicrobial defence. On the other hand, NK-cell and CTL secretory lysosomes would be suitable vehicles for extracellular delivery to promote anti-tumour defence. Finally, our results to date are based on studies in transformed hematopoietic cell lines. It remains to be seen whether secretory lysosome targeting in transformed cell lines differs from that in normal hematopoietic cells, whose granules are the final vehicles for delivering agents at an inflamed site. Targeting and regulated secretion may be more efficient in normal than in transformed hematopoietic cells.

Experiments in animals with inflammatory and malignant disease are now feasible and will be able to corroborate our principle. For this purpose, gene transfer in normal hematopoietic cells will be carried out in combination with hematopoietic cell transfer in the animal models. Continued exploration of this concept is also likely to shed light on the mechanism for hematopoietic granule formation, regulation of secretion and granule deposition at the inflamed site.

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