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Rosén, Hanna

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PO Box 117
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Mechanisms for Targeting of Proteins to Secretory Lysosomes of Haematopoietic Cells

Doctoral Thesis

by

Hanna Rosén

Division of Hematology and Transfusion Medicine

Lund University, Sweden

LUND UNIVERSITY

Faculty of Medicine

This thesis will be defended on May 24, 2006, at 13:00
in Segerfalksalen, Wallenberg Neurocentrum, BMC, Sölvegatan17, Lund

Faculty opponent:
Docent Birgitta Rasmusson
Department of Molecular and Clinical Medicine
Faculty of Health Sciences
Linköping University, Sweden

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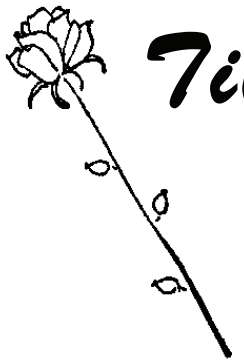

Till min familj

"The truth is rarely pure,
and never simple."

Oscar Wilde

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Original papers

This thesis is based on the following publications and manuscripts, which will be referred to in the text by their Roman numerals:

I. Hanna Rosén, Ying Gao, Ellinor Johnsson and Inge Olsson.
Artificially controlled aggregation of proteins and targeting in hematopoietic cells.
Journal of Leukocyte Biology 74(5):800-809, 2003.

II. Hanna Rosén, Linda Källquist and Inge Olsson.
Neutrophil pro-elastase sorting involving the plasma membrane is conformation dependent.
Manuscript.

III. Hanna Rosén, Jero Calafat, Lars Holmberg and Inge Olsson.
Sorting of Von Willebrand factor to lysosome-related granules of haematopoietic cells.
Biochemical and Biophysical Research Communications 315(3):671-678, 2004.

IV. Ying Gao, Hanna Rosén, Ellinor Johnsson, Jero Calafat, Hans Tapper and Inge Olsson.
Sorting of soluble TNF-receptor for granule storage in hematopoietic cells as a principle for targeting of selected proteins to inflamed sites.
Blood, 102(2):682-688, 2003.

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Paper **IV** was originally published in *Blood.* Gao Y, Rosén H, Johnsson E, Calafat J, Tapper H and Olsson I. Sorting of soluble TNF-receptor for granule storage in hematopoietic cells as a principle for targeting of selected proteins to inflamed sites. Blood. 2003;102:682-8. © the American Society of Hematology.

Selected abbreviations

AP-2 and 3	adaptor proteins 2 and 3
CD63 / LAMP-3	cluster of differentiation 63 / lysosome-associated membrane protein-3
cDNA	complementary deoxyribonucleic acid
COP I and II	coatomer proteins I and II
CTL	cytotoxic T-lymphocyte
egfp	enhanced green fluorescent protein
ELA	elastase
Endo-H	endoglycosidase H = Endo-β-N-acetylglucos-aminidase H
ERAD	endoplasmatic reticulum associated degradation
ERGIC	endoplasmatic reticulum-Golgi intermediate compartment
ER	endoplasmic reticulum
FCS	furin cleavage site
FKBP	FK506 binding protein
hGH	human growth hormone
Ig	immunoglobulin
IL	interleukin
IP	immunoprecipitation
kDa	kilodalton
LAMP-1 and 2	lysosome-associated membrane proteins 1 and 2
lgp 120	lysosomal glycoprotein 120
LPS	lipopolysaccharide
MHC class I and II	major histocompatibility complex class I and II
MPO	myeloperoxidase

MPR	mannose 6-phosphate receptor
NK-cell	natural killer cell
N-glycanase	*N*-glycosidase F = Endo-β-N-glucosaminidase F
N-linked	asparagine-linked
PCR	polymerase chain reaction
PKC	protein kinase C
PMA	phorbol myristate acetate
RBL	rat basophilic leukemia/ mast cell tumour line
RMCP-II	rat mast cell protease II
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sTNFR	soluble tumour necrosis factor receptor
TGN	*trans*-Golgi network
TLRs	toll like receptors
32D	murine myeloid 32D cell line
VWF	von Willebrand factor

Introduction

The cells of the innate immune system are essential for the host's first line defence against infection. The immune cells derive from the bone marrow, where they are developed from haematopoietic stem cells. The mature cells circulate in the blood until recruitment by chemotactic agents to a site of infection. Here, they transmigrate into the tissues and become activated by unspecific receptor binding to pathogen-associated molecular patterns. Their mission is then to kill the microorganisms, which may be done through several mechanisms.

In the case of the neutrophil, which is one of the major constituents of the innate immune system, and the white blood cell in focus in this work, one way to exert anti-microbial activity includes the production and release of antibiotic proteins and peptides. These serve as mediators in the inflammatory reactions.

Newly synthesized anti-microbial proteins and peptides are transported through the secretory pathway of the cell, where they are modified in a number of ways to finally obtain a native conformation required for its functionality. To avoid intracellular accumulation of proteins with non-native conformations, a protein quality control targets unfolded or misfolded proteins for intracellular degradation. Completely folded antimicrobial proteins are targeted for storage in secretory lysosomes and other granules of haematopoietic cells and released in the inflammatory process by regulated secretion.

However, the neutrophil granule contents are very potent agents, which may cause damage to the surrounding tissues upon extracellular release in an inflammatory response. Thus, even though the inflammatory response is crucial in an individual's defence against infections, its actions may

also confer negative effects. Rapid resolution of the acute inflammation upon completion of its task is of significant importance, since persistent inflammation may lead to severe tissue damage and organ failure.

Basic knowledge about biosynthesis, targeting and secretion of the neutrophil granule proteins is important for the understanding of the role of these immune cells in the emerge of inflammatory diseases.
Therefore, the aim of this thesis is to increase the knowledge about mechanisms underlying targeting of proteins to secretory lysosomes of haematopoietic cells.

Background

Haematopoiesis

The biogenesis of blood cells

The cells of the blood originate from pluripotent haematopoietic stem cells residing in the bone marrow of adult individuals. Stem cells have a capability of self-renewal, which allows them to maintain haematopoiesis throughout the lifetime of the host. During haematopoietic differentiation, the stem cells divide and generate more differentiated daughter cells, whose genetic program become fixed towards a single lineage and the pluripotency is then gradually lost during the differentiation process. The classical model for haematopoietic differentiation involves the restricted ability to generate myeloid versus lymphoid progenitor cells and lead to the generation of cells called common myeloid progenitors and common lymphoid progenitors. The myeloid progenitors do after further differentiation give rise to mature myeloid cells as granulocytes, monocytes, erythrocytes and megakaryocytes. The lymphoid progenitors differentiate into mature lymphocytes, including B- and T-cells [1]. However, recent studies in mice have provoked a debate about the common precursor cells and alternative or complementary models of the haematopoietic differentiation have evolved [2, 3]. The process of haematopoietic differentiation is subject to intrinsic regulation by transcription factors and extrinsic regulation by locally produced cytokines [1].

Innate immunity

The innate immune system [4], in contrast to the adaptive immune system involving specific T- and B-lymphocyte effector mechanisms [5], confers rapid non-specific responses to infection and does not differ between different individuals. This first line of defence against microorganism invasion involves the epithelial cells of the skin and mucosal tissue as a mechanical barrier, as well as effector cells such as cytotoxic T-cells, NK-cells, macrophages and neutrophils [6]. The proteins of the complement system [7], is also an important component contributing to phagocyte recruitment, microbial opsonization and bacteriolysis [6].

Innate immune recognition

The recognition of pathogens by the cells of the innate immune system is not based on binding to specific antigens, but to structurally conserved pathogen-associated molecular patterns. The receptors that bind to these structures belong to a family of transmembrane proteins showing high homology to the *Drosophila* receptor Toll, and are therefore termed Toll like receptors (TLRs) [6, 8]. There are at least ten members of this family (TLRs 1-10) in humans and mice and they are differently expressed among the immune cells, but have also been observed on vascular endothelial cells, cardiac myocytes, intestinal endothelial cells and adipocytes. The TLRs on the immune effector cells have been reported to respond to different stimuli and together they recognize a wide range of microbial ligands, including cell-wall components of yeast and mycobacteria, CpG dinucleotide motifs in bacterial DNA, the glycolipid LPS in the outer membrane of Gram-negative bacteria, bacterial lipoproteins and cell-wall peptidoglycans, lipoteichoic acids of Gram-positive bacteria and viral RNA. [4, 6, 8]. The TLR signalling pathways

leading to activation of different transcription factors, may be dependent or independent of an adaptor molecule [4, 8]. In addition to the TLRs, antigen presenting cells (APCs), such as macrophages and dendritic cells, also selectively express the type 3 complement receptor (CR3), scavenger receptors, a β-glucan receptor (Dectin-1), the mannose receptor and other C type lectins, that are capable of pattern recognition of exogenous as well as endogenous ligands. [9].

Effector molecules and mediators

An important component of the innate immune system consists of the antimicrobial peptides. These are relatively small (<10 kDa) endogenous cationic and amphipathic peptides. They are constitutively expressed (or in a few cases induced), and have been broadly classified into several groups, based on properties such as secondary and tertiary structure, length and absence or presence of disulfide bridges. These peptides make a very important contribution to the natural defences of most living organisms in that they posses virucidal, bactericidal, fungicidal as well as tumouricidal properties, and thereby exhibit a broad-spectrum activity against a wide range of pathogens. The mechanisms used by antimicrobial peptides have been shown to include disruption of the cell membrane, activation of autolysis of bacteria, inhibition of protein synthesis, activation of essential protein degradation, ion channel formation and interference with transport and energy metabolism [10]. Lysozyme and cathelicidin released by neutrophils and epithelial cells [11], as well as the β-defensins [12] of epithelial cells, are examples of important antimicrobial peptides. Some β-defensins as well as cathelicidin have been shown to, in addition to their antimicrobial action, also function as chemoattractants for certain immune cells like

monocytes, neutrophils, T-lymphocytes, memory T-cells and immature dendritic cells [6].

Another group of molecules, acting as mediators in the inflammatory response, is the cytokines [13]. These soluble polypeptide or glycoprotein mediators are active as a part of a complex network involved in the regulation of the immune and inflammatory responses. Important collaborators for the cytokines are the chemokines [13-15], a specialized group of small polypeptides that are able to induce directed cell migration in a variety of cells. Both cytokines and chemokines act by binding and signalling through specific receptors present on most cell types of the immune system, as well as on a large number of other host cell types [6, 13].

Cytotoxic T-cells and NK-cells

Cytotoxic T-cells (CTLs) and Natural Killer cells (NK-cells) are cytolytic cells of the lymphoid lineage that play an important role in the host's acute phase defence against pathogens, before the development of adaptive immune responses. The T-cell receptors of CTLs recognize viral peptides exposed on MHC class I molecules on virus-infected cells, whereas the NK-cells are activated in an antigen-independent manner by reduced levels or absence of MHC class I on virus-infected or malignant tumour cells [16].

Both CTLs and NK-cells kill their targets by means of granule- and FAS-mediated pathways, which induce apoptosis in the target cell [17, 18]. Both cytolytic cell types have granules containing various proteins that are known to be involved in the triggering of the target cell death program. Included here are the pore-forming protein perforin and the granzyme family of serine proteases. CTLs also produce and secrete cytokines such as tumour-necrosis factor (TNF) and interferon-γ (IFN-γ),

having cytotoxic action on target cells when delivered in the vicinity [16]. IFN-γ, which is released also by activated NK-cells, does also have a limiting effect on tumour angiogenesis and promotes the development of specific immune responses [19].

Macrophages and neutrophils

Macrophages and neutrophils are cells of the myeloid lineage that play a major part as critical phagocytotic effector cells in the innate immune system. Interactions between specific receptors on these cells with ligands on microorganisms lead to internalization of the pathogen into a phagosome. The phagosome will then mature by a series of fusion and fission events with components of the endocytic pathway, culminating in the formation of the mature phagolysosome. Here, the pathogen is killed by oxidative burst reactions, proteases and lysosomal hydrolases, and pathogen-derived peptides are generated for presentation to T-cells on MHC class II molecules on the cell surface [20-22]. The macrophages do also play an important role in the clearance of apoptotic cells in the resolution of an inflammatory response [23].

The neutrophils circulate in the bloodstream and interact transiently with endothelial cell molecules, called selectins, in a rolling-and-release tumbleweed-like motion. At a site of infection, the chemoattractant gradient originating in the peripheral tissue activates neutrophil responses, resulting in tight adhesion to endothelium through proteins of the β_2-integrin family [24], as well as to the extracellular matrix [25]. To arrive at the extravascular site of infection, the neutrophil then transmigrate between [26], or directly through [27], the endothelial cells in a process called diapedesis. The invading pathogenic microorganisms are then phagocytosed and killed [25].

The neutrophil kills pathogens in mainly two ways; oxidative killing by the NADPH oxidase system, generating O_2^-, which is converted to the bactericidal oxidant hypochlorous acid (HOCl) by myeloperoxidase (MPO) [28], and nonoxidative killing by granule proteins [25].

Neutrophil granules

Neutrophil granules are developed sequentially during the maturation process of the neutrophil. The different types of granules differ in size, density, protein content and tendency for extracellular secretion, reflecting the stage of myeloid differentiation at which they are formed **(Figure 1)**. The earliest granules appear at the promyelocyte stage and are called primary or azurophilic granules and are lysosome-like. They store serine proteases, myeloperoxidase (MPO) [29], antibiotic peptides and lysosomal enzymes in functionally active forms. Later, specific and gelatinase granules appear, which store metalloproteases and antibiotic peptides as inactive forms. The inactive proteins are activated only after the degranulation when the content from the different granules are mixed in the extracellular space or in the phagocytic vacuole. At maturity, highly mobilizable secretory vesicles are also manufactured. Stimulation of the neutrophil will lead to extracellular granule secretion in the following order: secretory, gelatinase, specific, and azurophilic. Specific and azurophilic granules are most likely to fuse with the phagocytic vacuole [30, 31].

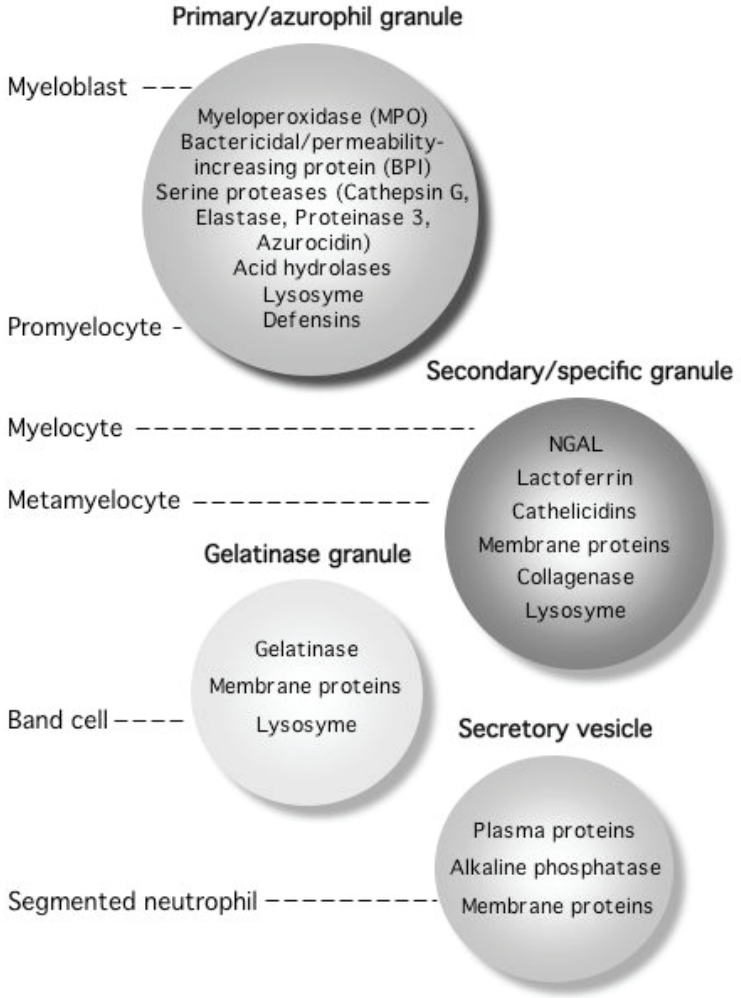

Figure 1. ***Characteristic neutrophil granule contents at the different stages of granulopoietic differentiation.***

Processing and sorting of neutrophil granule proteins

The fact that each granule protein is packaged in its appropriate granule leads to sequential mobilization of the different granule proteins as well as separation of proteins that cannot coexist. The joint accumulation of an

array of proteins in the same granule subset requires joint expression. This implies that successive expression of genes encoding granule proteins, governed by stage- and tissue-specific transcription factors, ensures that granule proteins synthesized at the same time during the neutrophil maturation process will localize to the same granules. This timing leads to joint storage of gene products in granules [30, 32].

However, the structural requirements for a neutrophil granule protein to be sorted for storage instead of secreted through the non-regulated pathway, is not fully understood.

Studies of processing of the serine proteases elastase, cathepsin G and proteinase 3, suggest a similar processing of these proteins, including removal of an amino-terminal and a carboxy-terminal propeptide. The cleavage of the propeptides was implied to take place in pre-granule and granule structures [33]. Therefore, studies involving deletions and mutations of the propeptides were performed. However, the removal of the propeptides of these proteins did not interfere with the targeting for storage in azurophil granules [34, 35]. This was strengthened by results from investigations of the non-catalytic serine protease azurocidin [36]. The role of glycosylation in targeting of cathepsin G was also investigated by mutations in the cDNA in order to prevent the addition of oligosaccharides. No effect on the targeting could be observed, excluding the mannose 6-phosphate receptor from being involved in the targeting to granules [37]. Taken together, the results from these studies suggest that neither glycosylation nor final processing is a prerequisite for sorting for storage of neutrophil serine proteases in granules [35].

Moreover, metalloproteases are targeted and stored as proforms in specific granules, and are subjected to propeptide cleavage and activation first after degranulation. Also, in addition to the unprocessed forms of the serine proteases, mature forms have been observed to be targeted to the

azurophil granules, suggesting that both an immature and a mature conformation is compatible with targeting for storage in granules [34, 36].

Structural features may however be required for targeting of some proteins. Upon deletion of the amino-terminal propeptide of myeloperoxidase (MPO), the normal processing was blocked and targeting was inhibited [38]. Therefore, it can be concluded that different properties may be required for individual proteins and that the neutrophil granule proteins may not be targeted in an identical manner [33].

The azurophil granules are lysosome-like in that they, in addition to the anti-bacterial proteins and peptides, store acid hydrolases and contain the membrane protein CD63 [39]. They are therefore considered to correspond to the dual-function organelle termed the secretory lysosome, which is found in many cells of the immune system [40]. Secretory lysosomes and mechanisms for protein targeting to this organelle is covered on **pages 27-30** in this thesis.

Mast cells

The mast cell, which belongs to the myeloid lineage and is associated with blood vessels throughout the body, has been well studied as an effector cell in IgE-dependent allergic reactions [41, 42]. However, the mast cell also plays a critical role in innate immune responses, where it contributes to the early host defence by several mechanisms. The activation of a mast cell, via crosslinking of cell surface IgE-receptors, induced by pathogens, their products, or other mediators associated with tissue damage, leads to rapid release of potential preformed or newly generated mediators effecting the vasculature. The release of molecules such as TNF [9] and histamine [43] leads to upregulation of adhesion

molecules, and enhancement of vascular permeability. These effects on the vasculature, as well as secreted mast cell proteases, are critical in the chemotactic recruitment of neutrophils and other effector cells to a site of infection. In addition, the mast cells produce a variety of chemotactic lipid mediators and chemokines that are important as key stimulators for cell migration out of the vasculature. Mast cell activation during infection is dependent on complement-mediated signals as well as interactions with selected TLRs and other receptor systems [44].

Inflammation

The inflammatory response

The inflammatory response, initiated by the innate immune system, is a defence mechanism aiming to fight invading infectious agents. The acute inflammation is characterized by the rapid recruitment of effector cells and by the massive release of effector molecules and inflammatory mediators such as antimicrobial peptides, cytokines and chemokines [6]. Thus, the inflammatory process comprises a complex network of exogenous and endogenous mediators, interacting as stimulators or inhibitors to enhance the operation [15]. The process of an inflammatory response is also initiated in case of tissue damage, where it is crucial for the elimination of potentially harmful agents that may cause *septicemia* [45].

Tissue damage in inflammation

Even though the inflammatory response is an indispensable part of an individuals' defence against infections, its actions may also confer negative effects on the tissues of the host.

Neutrophils, which constitute a considerable part of the inflammation process, have been reported to cause tissue damage by extracellular release of destructive granule constituents and oxygen metabolites during phagosome formation. These toxic molecules, which cannot discriminate between invading microorganisms and host tissue structures, may also be released extracellularly upon encounter with immunocomplex- or antibody-coated particles too large to engulf. Moreover, the activated neutrophil produces hypochlorous acid, which can cause cell dysfunction and necrosis by ATP depletion, and inactivates the specific inhibitor of neutrophil elastase, α-1-antitrypsin, leading to uncontrolled proteolytic activity of elastase [46].

In addition, the onset of inflammation has been reported to interfere with the wound healing process, comprising unbeneficial effects like slowing of the repair and induction of excessive fibrosis [45].

Thus, once the task of microbial killing is completed, rapid resolution of the acute inflammation is of significant importance, since persistent inflammation may lead to severe tissue damage and organ failure. Successful termination of an inflammatory response involves the release of anti-inflammatory and reparative cytokines, programmed leukocyte death by apoptosis and clearance of the dead cells by phagocytes. The latter do subsequently leave the site of inflammation through the lymphatics. This highly coordinated “resolution program” has been suggested to be initiated by events occurring already during the first few hours of an acute inflammatory response [23].

There is a broad range of disorders caused by an “over-active” immune system. Failure in the mechanisms regulating the clearance of inflammation, and components of genetic prevalence are factors that may contribute to the development of chronic inflammatory diseases as well as autoimmune diseases.

The tumour necrosis factor (TNF) superfamily

Cytokines of the tumour necrosis factor (TNF) superfamily play a major role as pro-inflammatory mediators. The soluble and/or membrane-bound TNF ligands interact with one or more specific membrane bound or soluble receptor of the TNF receptor superfamily, of which the majority are expressed by immune cells [47].

Figure 2 shows generation of soluble TNF receptor by proteolytic cleavage of the extracellular part of the TNF receptor at the cell surface. The receptor processing has been demonstrated to involve both serine proteases and metalloproteases [48].

TNF ligand interactions with transmembrane TNF receptors **(Figure 2)** leads to formation of receptor trimer and activation of multiple signal transduction pathways [49], which may trigger intracellular processes affecting the homeostasis of the immune system. This includes processes such as cell proliferation, survival, differentiation and apoptosis. These activities are beneficial for the host in inflammation and protective immune responses in infectious diseases, and also play important roles in secondary lymphoid organogenesis [47].

However, TNF is also one of the most prominent mediators in the induction of inflammatory responses of the innate immune system. It activates processes including cytokine production, expression and activation of adhesion molecules and stimulates growth. These are processes that need to be carefully controlled in order to avoid tissue damage [47]. The activity of TNF has been shown to cause host damaging effects in disorders like fever syndromes, *sepsis*, and in autoimmune diseases like *inflammatory bowel disease* and *rheumatoid arthritis* [47, 50].

Blockade of the superfamily member TNFα, in order to neutralize its activation **(Figure 2)**, has proven to be very successful as a therapeutic approach in the treatment of several immune-mediated inflammatory diseases [51].

The drugs used in the clinical practice in order to block TNFα are biological protein-based drugs, consisting of either monoclonal anti-TNFα antibodies or modified high affinity TNFα receptors. TNF receptor dimers that have been linked to the Fc portion of IgG, have been demonstrated to be effective inhibitors of the trimeric TNFα. Also chimerized monoclonal antibodies of high affinity have been proven efficient to block TNFα. Thus, these therapeutic molecules act as physiological inhibitors but are engineered as pharmaceutical inhibitors to increase their efficacy [52].

However, TNFα blockade does not provide a permanent cure, and may need combinatorial therapies [50]. Moreover, systemic release of TNF activity blocking agents is associated with an increased risk to develop opportunistic infections including reactivation of tuberculosis [53].

Therefore, local inhibition of TNF would provide a mean to overcome systemic side effects. The use of haematopoietic cells as vehicles for delivery of an anti-TNFα agent, e.g. a soluble TNF receptor, to inflamed sites might be a concept for future anti-inflammatory therapy [54].

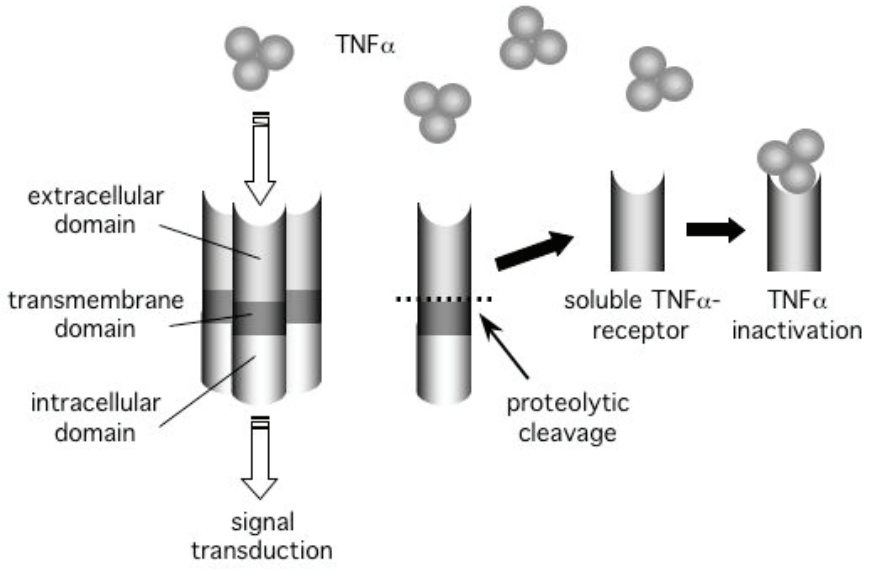

Figure 2. ***Schematic picture of TNFα receptor activation and soluble TNFα receptor formation.*** *Trimeric TNFα binding to TNFα receptor induces receptor trimer formation and signal transduction. The extracellular domain of the TNFα receptor can be released by proteolytic cleavage to generate soluble TNFα receptor. The soluble receptor can bind and inactivate the TNFα molecules.*

The secretory pathway and protein quality control

The transport route used by newly synthesized proteins to reach the cell membrane or the extracellular space is termed the secretory pathway [55]. For cargo transport into the cell, termed endocytosis [56], which may include internalization of plasma membrane receptors, pinocytosis or phagocytosis, the cell uses the endocytic pathway. In addition to cargo transport, endocytic compartments have been reported to participate in the sorting of proteins in the biosynthetic route of polarized cells, suggesting a linkage, at various points, of these two transport routes [57].

The typical secretory and endosomal pathways of a non-polarized cell are depicted in **Figure 3**.

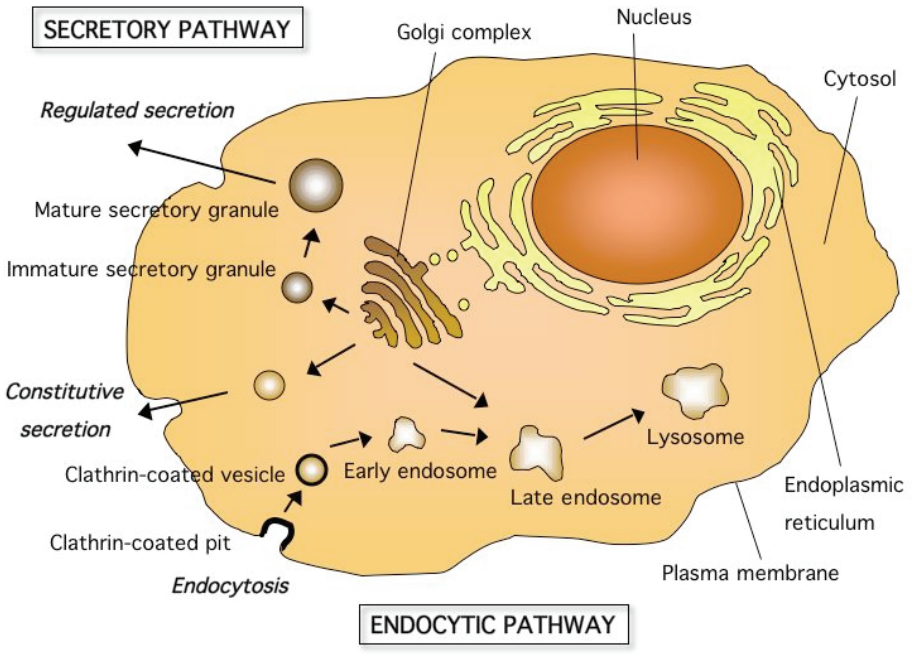

Figure 3. ***Schematic picture of a typical secretory cell, showing the secretory and endocytic pathways.*** *Proteins destined for regulated secretion are packaged into an immature granule, which eventually form a mature granule. After fusion with the plasma membrane, soluble proteins are secreted to the extracellular space and membrane proteins are retained in the plasma membrane. Proteins destined for the plasma membrane or the extracellular space, lacking signals for retention or sorting, are delivered to the plasma membrane by constitutive secretion. Proteins at the plasma membrane destined for degradation are internalized by the formation of a clathrin-coated pit, which becomes a clathrin-coated vesicle delivering its content into an early endosome, which matures into a late endosome. When the late endosome has matured into a lysosome, by regulated fusion events, the content will be subject to lysosomal degradation.*

The endoplasmic reticulum (ER)

The large organelle termed the endoplasmic reticulum (ER) is considered to be the entrance into the secretory pathway. Here, secretory proteins are synthesized on membrane bound ribosomes and translocated in an unfolded state into the ER lumen via an aqueous channel termed the Sec61 translocon [58]. The proteins are then subjected to posttranslational modifications such as folding and maturation processes. These processes are tightly controlled by proofreading systems. The complete protein must typically have reached a correctly folded conformation, which corresponds to the most energetically favourable state and is generally called the ”native” conformation [59].

As a part of the modification process, N-linked oligosaccharides are added cotranslationally to the growing nascent polypeptides. These firstly added “core glycans” are relatively simple and homogenous and play common roles in promoting protein folding, quality control as well as some sorting events. As the glycoproteins move through the ER, the glycans are subjected to trimming by glycosidases and mannosidases. When the folded protein reach the Golgi complex, the glycan chains undergo further trimming and terminal glycosylation, leading to the formation of mature glycoproteins with complex N-linked glycans [60, 61].

Protein quality control in the ER

In order to obtain its final conformation, the protein interacts with a number of ER-resident molecular chaperones and folding enzymes [62], which assist the folding process. However, these chaperones do also have the ability to distinguish between properly and incompletely folded proteins and dispatching incorrectly folded proteins for destruction. Together with other ”conformation-sensor molecules”, selectively

”tagging” misfolded proteins, the chaperones constitute the final checkpoints of the proof-reading system called quality control. Primary protein quality control is a retention-based system, which distinguishes between native and non-native protein conformations on the basis of the exposure of hydrophobic regions, unpaired cysteine residues and the tendency to aggregate. BiP [63], calnexin and calreticulin [64] are some important proteins involved in this process. Secondary protein quality control is often cell type specific mechanisms involved in the regulation of ER retention and export [59]. Properly folded and modified proteins that pass the quality controls are allowed to exit the ER and enter the secretory pathway via specific ER exit sites. These consist of small membrane clusters or buds coated with the coatomer protein II (COPII) coat [59, 65].

Endoplasmic reticulum associated degradation (ERAD)

The concentration of unfolded proteins in the ER is sensed by an unfolded protein response system. When the level of unfolded or misfolded proteins rises above a certain level this system induces a signalling cascade, which leads to an upregulated expression of proteins required for protein folding and quality control. The system is also tightly connected to the endoplasmic reticulum associated degradation (ERAD) system, where proteins that fail are retro-translocated from the ER to the cytosol, a process involving polyubiquitinylation by the cytoplasmic ubiquitinylation machinery [66, 67]. Proteins tagged with ubiquitin are subsequently targeted to the cytosolic proteolytic enzyme complex termed the 26S proteasome [68], for degradation. The polyubiquitin chain is then hydrolyzed into single ubiquitin and recycled for participation in a new protein degradation process. The protein quality control and the ERAD system is necessary for the maintenance of a healthy cellular

metabolism and life in many different organisms [66]. Recent findings indicate that the substrates for ERAD do not exclusively consist of misfolded proteins. The ERAD system may also be involved in the regulated turnover of ER-resident enzymes and used by human pathogenic viruses. Thus, the ERAD system might be considered as an important mechanism affecting cell physiology in many different aspects such as protein folding and transport, metabolic regulation, immune response and the ubiquitin-proteasome system dependent degradation [69].

The Golgi complex

The Golgi complex, which is considered to be the central station along the secretory pathway, consists of flattened membranous compartments with dilated rims, termed cisternae. These biochemically distinct compartments are organised in the form of a stack comprising the *cis*, *medial* and *trans* cisternae, which are interconnected by tubular networks. The number of cisternae (three to eight are most common) varies between different cell types. In the Golgi compartments, newly synthesized proteins and lipids, received from the ER, are subjected to posttranslational modifications prior to distribution to the endosomal/lysosomal system and to the plasma membrane [70]. Importantly, the glycan chains added to the glycoproteins in the ER, undergo further trimming of mannoses. In many cases complex N-linked glycans are produced by the addition of new sugars in order to create structural diversification and give the mature proteins novel functions, displayed by the different sugars. The different post-translational modifications are carried out by membrane-bound Golgi-resident proteins, such as glycosyltransferases, glycosidases, lectins, permeases, transport receptors and proteolytic enzymes [60].

The maintenance of the Golgi subcompartmentalized structure and the transport of cargo molecules between the different cisternae is subject in an ongoing debate. It is not known, whether the anterograde traffic (the forward transport of cargo) through the Golgi complex is mediated by vesicles/tubules, or the process of cisternal progression/maturation. The different models are however not mutually exclusive and both vesicle-, tubule-, and cisternal-mediated cargo transport could be important in anterograde traffic through the Golgi complex [71, 72].

The trans-Golgi network (TGN)

The *trans*-most cisternae of the Golgi stack, is associated with a tubular-reticular structure termed the *trans*-Golgi network (TGN), which is the exit pole of the complex. Here, terminal glycosylation as well as cleavage of protein precursors into their mature forms by pro-protein convertases (like furin [73]) takes place. The transport proteins are then packaged into membrane carriers and directed to their final cellular destinations, like the plasma membrane or the endosomal/lysosomal system [70].

Post-ER protein quality control

Although the protein quality control in the ER and the ERAD system seem to be efficient in protecting the cell from accumulation of unfolded or misfolded proteins, there might be some escape from these processes. Certain cases of retrieval of misfolded proteins from the Golgi complex to the ER have actually been reported [74-76]. This protein retrieval pathway may, in some cases, be required for an efficient ERAD system, or act as a "back-up system" in case the ER retention capacity for a protein should be saturated. Resident chaperones and folding enzymes are not found in the mammalian Golgi complex, indicating a lack of protein folding capacity in this organelle. However, some enzymes involved in

the protein folding machinery in the ER are also found in the ER-Golgi intermediate compartment (ERGIC) [77] indicating the possibility of some glycoprotein folding to be localized to this compartment [59].
Most of the reported protein quality control events in mammalian cells occur in the ER. However, several studies in yeast cells have suggested protein quality control to also take place in post-ER compartments [78] such as the Golgi complex [79, 80].

Secretion

Constitutive secretion

The secretory pathway of eukaryotic cells comprises a series of distinct steps for anterograde (forward) transport of proteins between the membrane compartments along this route. Some retrograde (backward) transport is however also involved in order to maintain a steady state composition of certain proteins. The transport from the TGN to the plasma membrane is the last step for many of the secreted proteins. Proteins destined for the plasma membrane or the extracellular space, lacking sorting- or retention-signals for granule storage, are delivered to the plasma membrane by constitutive secretion. This secretory route does not require any specific stimulus, and is therefore also termed the “default secretory pathway”. It exists in all eukaryotic cells and is responsible for the arrival of critical proteins at the plasma membrane. These are proteins involved in the cell’s interaction with the environment and regulate processes like the uptake of nutrients needed for cell growth and survival. This delivery does however need regulatory mechanisms to ensure that the appearance of specific proteins at the cell surface is coupled to

intracellular signalling, cellular differentiation, organ development and cell growth [81].

Regulated secretion

Professional secretory cells, like endocrine and neuroendocrine cells, and specialized haematopoietic cells are, in addition to constitutive secretion, also capable of regulated secretion. These cells have mechanisms for protein retrieval and storage of secretory proteins in storage compartments, such as the neutrophil granules [32], and secretory lysosomes [40]. The stored proteins may then, upon cell stimulation, be released to the cell exterior or into a forming phagolysosome [32].

The exocytotic machinery of cells possessing secretory lysosomes have been suggested to differ in some aspects from the general regulated secretory mechanisms [82]. In the case of neutrophils, they posses several types of granules, which differ in their response to elevation of intracellular Ca^{2+} levels, i.e. their tendency for secretion. Moreover, the exocytotic machinery of neutrophils seems to, in addition to Ca^{2+}-dependent mechanisms, be controlled by regulatory steps initiated by binding of a cell surface receptor [83].

Secretory lysosomes

Composition and function

Secretory lysosomes are found in many different cell types of the immune system as well as in other cell types, such as melanocytes, osteoclasts and renal tubular cells [40]. This organelle serves as the endpoint of the endocytic pathway where extracellular particles and proteins are degraded by proteases and lipases, but it is also a secretory organelle.

Secretory lysosomes can therefore be described as modified lysosomes that, in addition to lysosomal hydrolases required for protein degradation, also contain specialized secretory proteins. Unlike conventional lysosomes [84], they are able to undergo regulated secretion and release the stored specialized secretory proteins in response to external stimuli. The pH is important in the control of the dual function of this organelle. An acidic pH is required for the function of the lysosomal hydrolases, whereas the secretory proteins function at neutral pH after secretion [85]. Morphologically, conventional and secretory lysosomes can appear similar, although secretory lysosomes may have diverse types of structures, including both electron dense cores and multivesicular regions. Both organelles have the resident membrane proteins CD63 [86] and the lysosomal-associated membrane proteins LAMP-1 and LAMP-2 [87], as well as the receptors involved in the recognition and delivery of both cytosolic and membrane bound material to the lysosome [40]. Secretory lysosomes/azurophil granules of neutrophils lack LAMP-1 and –2 [88], but contain CD63 [39].

Secretory lysosomes move back and forth along microtubules by means of kinesin- and dynein-based motors. Lysosomal secretion generally occurs at the plasma membrane where the membrane fusion event is mediated by specific proteins. Several proteins from the SNARE family [89], regulated by proteins including small GTPases from the Rab family [90], have been shown to be involved in the fusion machinery. Rab27a has been suggested to play a crucial role in the detachment of secretory lysosomes from the microtubules. However, the proteins involved in the secretion machinery may vary between different cell types, according to the requirements for lysosome delivery and the way in which secretion occurs. Mutations in genes encoding proteins involved in the function of secretory lysosomes may lead to deficiencies as the *Chediak-Higashi*

syndrome [91], the *Griscelli syndrome* [92, 93] and the *Hermansky-Pudlak* syndrome [94, 95], characterized by immunodeficiency and hypopigmentation [40, 85].

Targeting to secretory lysosomes

Proteins in the TGN that are destined for the secretory lysosomes are targeted by different mechanisms **(Figure 4)**. In the TGN, the lysosomal hydrolases, like some other transmembrane proteins, are subjected to oligosaccharide chain modifications involving the addition of mannose 6-phosphate (M6P) groups, allowing them to be recognized by transmembrane mannose 6-phospate receptors (MPRs). The MPR-lysosomal complexes are then transported from the TGN to endosomal compartments, where the MPRs dissociate and return to the TGN, and the lysosomal hydrolases are delivered to the lysosomes [96, 97].

Recognition of sorting signals, contained in the cytoplasmic domain of cargo molecules, by vesicle coat proteins is of importance for correct protein sorting. Vesicle coat proteins, like clathrin [98] and adaptor molecules (APs) [99] have been demonstrated to be involved in the formation of transport vesicles. Clathrin/AP-1 coated vesicles have been suggested to be responsible for the sorting of lysosomal hydrolases, whereas clathrin/AP-2 coated vesicles have been demonstrated to mediate clathrin-dependent endocytosis at the plasma membrane. Direct transport of some lysosomal proteins from the TGN to the lysosomes, is suggested to be mediated by AP-3 [97].

The recruitment of adaptor complexes to the cargo molecules is dependent of the recognition of conserved amino acid sequences that constitute specific sorting-motifs. Two well-characterized classes of sorting signals, involved in endosomal targeting and internalization from

the plasma membrane, are the tyrosine-based and the dileucine-based sorting motifs [97, 100].

With the exception of the serine proteases granzyme A and granzyme B, found in lytic granules of CTLs and NK-cells, which are targeted by the MPR system [101], the mechanisms for targeting of soluble proteins to secretory lysosomes of haematopoietic cells are not known.

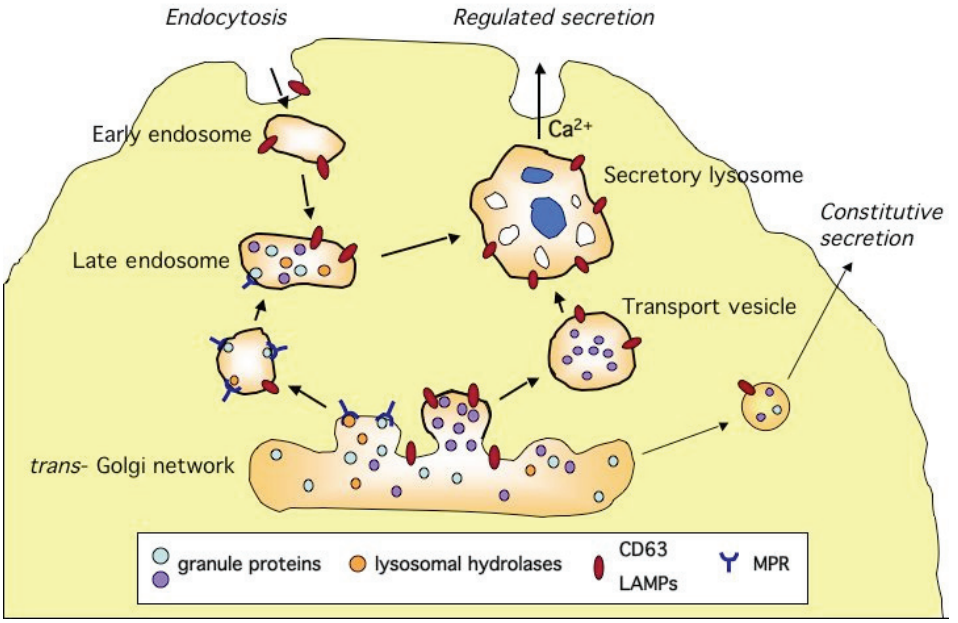

Figure 4. ***Schematic picture of protein targeting to secretory lysosomes in the trans-Golgi-network.*** *Lysosomal hydrolases bind to the mannose 6-phosphate receptor (MPR) and are targeted to the secretory lysosome via the endosomal system. Other transmembrane proteins, like CD63 and the lysosomal-associated membrane proteins (LAMPs), have sorting signals in their cytoplasmic domains that are recognized by coat proteins involved in the formation of transport vesicles. Proteins lacking retention- or sorting-signals are secreted by constitutive secretion. The targeting mechanisms for luminal granule proteins are largely unknown.*

The von Willebrand factor (VWF)

The fact that the von Willebrand factor (VWF) is a spontaneously aggregating protein that is normally targeted for storage with subsequent release by regulated secretion, makes it suitable for studies on the effect of protein multimerization on targeting for storage.
In addition, VWF has been expressed in a number of cells other than endothelial cells and megakaryocytes [102-107], and has been observed to form storage granules also in heterologous cells [107].

Biosynthesis and function

The VWF is a multifunctional plasma protein, synthesized by endothelial cells and megakaryocytes, that plays a critical role in the normal process of blood coagulation. After signal peptide cleavage and glycosylation in the ER, the pro-protein dimerizes prior to transport to the Golgi complex, where post-translational modifications, including the generation of complex sugars and further glycosylation, takes place. Simultaneously, the pro-VWF dimers polymerize into multimers that are subsequently transported to the TGN, where endoproteolytic cleavage of the propeptide and further multimerization occur. These processes yield mature VWF multimers and VWF propeptide dimers [108], which remain non-covalently associated, at least intracellularly [109]. However, mature VWF and the VWF propeptide have been reported to have distinct biological functions. Mature VWF is involved in the adhesion and aggregation of blood platelets at sites of vascular injury and functions as a chaperone protein for coagulation factor VIII. The VWF propeptide has been suggested to be involved as an inflammatory mediator [108].

Abnormality or deficiency of the VWF, usually caused by mutations within the VWF gene, leads to bleeding disorders, as seen in the autosomal inherited *von Willebrand's disease* [110].

Targeting of the von Willebrand factor

The VWF differs from many other endothelial proteins in that it can be secreted from the cell by both constitutive and regulated secretion [108]. However, only incompletely polymerized and processed, i.e. functionally incompetent protein, is secreted by the constitutive pathway [111]. The mature functional multimeric VWF released by regulated secretion is stored, together with its propeptide, in endothelial cell-specific elongated rod-shaped organelles termed Weibel-Palade bodies [112], or in round-shaped alpha-granules of megakaryocytes [113].

VWF expression has been shown to be necessary but not sufficient for the formation of Weibel-Palade bodies, in which the VWF multimers become densely packaged in several longitudinal arranged tubules. These structures are surrounded by a limiting membrane, containing the leukocyte receptor P-selectin and, like secretory lysosomes of haematopoietic cells, the membrane protein CD63 [114]. In addition to mature VWF and the VWF propeptide, the Weibel-Palade bodies contain endothelin, important in vasoconstriction, and the cytokine IL-8 [108].

The Weibel-Palade bodies are, like secretory lysosomes, able to undergo regulated secretion upon cell stimulation. Thrombin, formed locally at sites of vascular damage, histamine and epinephrine are some agents that have been shown to trigger the mechanism of Weibel-Palade body exocytosis in endothelial cells. The exocytosis mechanism has been suggested to involve Ca^{2+}-rising agents and cAMP pathway activating agents, possibly acting in synergy [108, 114].

The multimerization of VWF, which is dependent on a functional VWF propeptide [104, 115], has been suggested to be important for the formation of Weibel-Palade bodies. However, VWF multimerization alone has been demonstrated to be insufficient for the formation of storage granules [114]. Instead, an intact propeptide, functioning as an intracellular chaperone possibly containing sorting signal(s), and its non-covalent association with the VWF, has been shown to be important for VWF targeting, suggesting VWF multimerization and storage to be two independent processes [116, 117].

The present investigation

Aim

The general objective of my work has been to increase the knowledge about mechanisms underlying targeting of proteins to secretory lysosomes of haematopoietic cells.

Specific aims

I. To determine whether ligand-induced aggregation would affect targeting to secretory lysosomes.
Specific question: Does aggregation of proteins in the Golgi complex facilitate their sorting for storage in secretory lysosomes?

II. To determine whether soluble monomeric and oligomeric non-native proteins can be targeted to the cell surface and become internalized or not.
Specific question: Does protein sorting involving the plasma membrane require a native conformation?

III. To determine whether normal multimerized von Willebrand factor (VWF) and multimerization-defective VWF are differently targeted to secretory lysosomes.
Specific question: Will multimerized VWF be sorted for storage in the secretory lysosomes or trigger formation of Weibel-Palade-like organelles when constitutively expressed in haematopoietic cells?

IV. To determine whether a cytosolic sorting sequence can target a non-haematopoietic protein to secretory lysosomes.

Specific question: Is it possible to target an exogenous protein to the secretory lysosomes of haematopoietic cells?

Experimental considerations

Here, the principles, advantages and disadvantages of the most important methods are covered briefly. For more detailed descriptions, see **Appendix**.

Cell models

The mucosal mast cell-resembling Rat Basophilic Leukemia cell line RBL-1 [118], and the murine myeloblast-like cell line 32D c13 [119] were chosen for stable expression of cDNA constructs.

The intracellular granule content of RBL-1 cells, like the azurophil granule content of human neutrophils, is dominated by lysosomal enzymes and serine proteases [118]. These proteins are packaged in the same compartment, which also contains the lysosomal-associated membrane proteins (LAMPs) [120], suggesting the RBL-1 granule to be a lysosome-related organelle.

32D-cells contain cytoplasmic granules that have an abnormal vacuole-like appearance [119, 121]. The granules do also carry the lysosomal marker LAMP-1 [122, 123], indicating lysosomal resemblance, and are able to fuse with newly synthesized phagosomes with subsequent release of their contents [119], making them resemble degranulating azurophil granules.

The mast cell line RBL-2H3, used in immunofluorescence microscopy experiments, grow in adherent layers and can be grown directly on cover slips prior to immunostaining. This may reduce the possible appearance of arte-facts caused by the attachment of cells to poly-lysine.

In conclusion, immortalized cell lines do not correspond perfectly to their normal counterparts and, in addition, over-expression of a protein in a cell line may contribute to misleading results, not applicable to normal cells. Therefore, it is important to verify the present results, obtained in studies with cell lines, in experiments with neutrophil precursor cells. This has been done with the findings in **paper IV** (see **Appendix**) [124].

Transfections

All cDNA constructs used in this present investigation **(papers I-IV)** were stably transfected into the cells and subjected to monoclonal selection and expansion. This was, in contrast to transient transfection or polyclonal selection, preferable in our studies since large numbers of cells, expressing the proteins of interest, were required for a long period of time.

Biosynthetic radiolabelling and immunoprecipitation

These methods in combination are useful for time measurements of ongoing processes like protein synthesis, intracellular protein transport, processing and secretion. In contrast to the immunoblotting method, which measures the "steady state", biosynthetic radiolabelling allows studies of *de novo* synthesized proteins. Complete lysis of the cells, is however important for successful immunoprecipitation of the radiolabelled proteins.

Subcellular fractionation

Subcellular fractionation on a Percoll gradient is a useful method in comparison studies of subcellular protein distribution after radiolabelling and chase of the radiolabel. A disadvantage is that different organelles with identical density cannot be separated on a density gradient. It is therefore preferable to include a co-localization marker in the experiment, or confirm the obtained results by studies using microscopy techniques.

Immunoelectron microscopy

The advantage of this method is that high-resolution pictures of intracellular structures are obtained. These can be used to determine the subcellular localization of a protein of interest, also within an individual compartment. Moreover, the eventual co-localization with endogenous proteins can be studied. However, it is crucial that the cells are kept intact by fixation and that specific antibody binding is obtained. It is preferable to combine this method with other methods like subcellular fractionation and, in some cases, other microscopy techniques.

Flowcytometry

The immunostainings preceding the flowcytometry analyses were performed at a temperature not exceeding 15ºC in order to avoid endocytosis of cell surface located proteins, which might give a misleading result. The granulocytic cells in our experiments were gated according to forward and side scatter to exclude dead cells from the analyses. With regard to unspecific antibody binding and cell survival, it is preferable to confirm the obtained results by microscopy techniques visualizing the cell surface/intracellular localization of the labelled proteins.

Immunofluorescence microscopy

In accordance to the flowcytometry experiments (see above), the immunostainings preceding the analyses were performed at maximum 15ºC, in order to avoid endocytosis. The advantage of this method is that successfully immunofluorescence-labelled proteins can be easily visualized, and their cell surface- versus intracellular localization can be determined.

However, the expression of stably transfected protein constructs, as well as the specificity and efficiency of the immunofluorescence labelling, may vary depending on the cell line and the protein, rendering quantitative comparisons difficult. This method is therefore preferentially combined with other, more objective quantitative analyses. In **paper II,** the obtained results were confirmed by flowcytometry and protein biotinylation, and in **paper IV** this method was combined with subcellular fractionation and immunoelectron microscopy.

General discussion and concluding remarks

The detailed results from the present investigation will not be described here. The reader is referred to the original papers in the **Appendix** for the presentation of the primary data. Instead, general questions concerning the obtained results will be discussed.

1. Does aggregation of proteins facilitate their sorting for storage in secretory lysosomes?

(papers I and III)

The effect of protein aggregation on sorting for storage was investigated because of its proposed role in targeting of soluble protein hormones in exocrine and endocrine cells [125, 126].

Two basic models for sorting into secretory granules have previously been suggested in such cells: sorting-for-entry, which is a passive process that sorts proteins into the forming immature secretory granule, and sorting-by-retention, an active process operating in the immature secretory granule [127].

In exocrine and endocrine cells, which like neutrophils have storage granules whose content can be released by regulated secretion, protein aggregation has been suggested to be involved in both basic models mentioned here. Whether the protein aggregation occurs passively or is actively controlled by the cell is however not clear, and although aggregation is important, the formation of secretory granules has been suggested to require more than protein precipitation [125, 126].

In **paper I**, we asked whether artificially induced aggregation could affect targeting to secretory lysosomes in haematopoietic cells.

This was investigated by using intracellularly controlled oligomerization of hybrid proteins **(Figure 5)**, reversibly induced by the lipid soluble and cell-permeable drug FK506 [128]. One hybrid protein consisted of neutrophil elastase (ELA) fused with FK506 binding protein (FKBP) resulting in the hybrid protein ELA-$(FKBP)_3$, which was induced to aggregate by the addition of FK506 dimeric ligand. Another construct contained human growth hormone (hGH) fused with an FKBP variant (FKBP*) to form $(FKBP^*)_4$-FCS-hGH, which aggregates spontaneously and can be cleaved by furin in the TGN. The aggregates dissociate upon addition of monomeric ligand.

The results indicated no targeting of the hybrid proteins to the secretory lysosomes of the cell lines tested. Instead, both ELA-$(FKBP)_3$ and $(FKBP^*)_4$-FCS-hGH were eliminated from the cell, in both aggregated and non-aggregated form, by constitutive secretion. Also free hGH,

released by furin in the Golgi complex, was constitutively secreted. In contrast, transgenic expression of full length ELA, in these cells, performed by Gullberg et al. [35], showed targeting to secretory lysosomes, in addition to constitutive secretion of proteolytically unprocessed ELA.

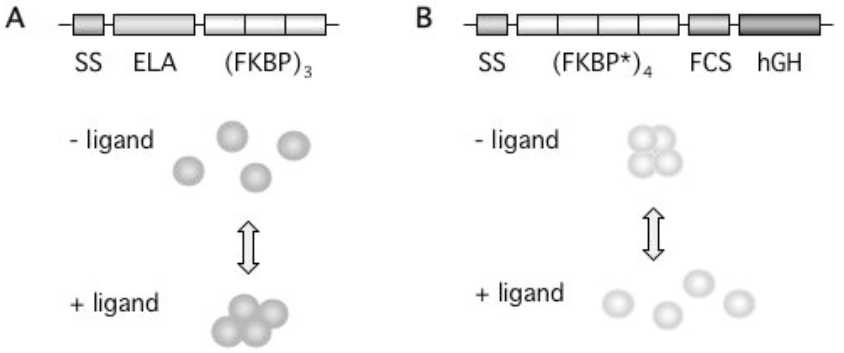

Figure 5. ***Schematic picture of the cDNA constructs encoding ELA-(FKBP)$_3$ (A) and (FKBP*)$_4$-FCS-hGH (B) and ligand-induced aggregation/dissociation.*** *(A) Presence of ligand (+ ligand) leads to formation of protein aggregates. (B) In the absence of ligand (- ligand) the hybrid protein aggregates spontaneously. In the presence of ligand (+ ligand) the aggregates dissociate. Abbreviations: SS; signal sequence, ELA; elastase, FKBP; FK506 binding protein, FKBP*; mutated FK506 binding protein, FCS; furin cleavage site, hGH; human growth hormone.*

From this we concluded that protein aggregation is unlikely to be the sole mechanism for targeting of soluble proteins to the secretory lysosomes of haematopoietic cells. Rather, targeting seemed to rely on structural requirements such as correct processing and folding. An important observation was that the hybrid proteins, which are unlikely to have a native conformation, in contrast to many other non-native proteins, were constitutively secreted instead of being subjected to ER-associated degradation.

To further elucidate the role of protein aggregation in secretory lysosome targeting, we expressed the plasma protein von Willebrand factor (VWF) in haematopoietic cells (**paper III**). VWF is normally expressed in endothelial cells and megakaryocytes. After processing steps including multimerization and propeptide removal, it is sorted for storage as very high molecular weight multimers [109, 129] in Weibel-Palade bodies of endothelial cells [112] and platelet granules [113].

VWF was chosen because it is a spontaneously aggregating protein that is normally targeted for storage with subsequent release by regulated secretion. In addition, it can be expressed in a number of cells other than endothelial cells and megakaryocytes [102-107], and has the ability to form storage granules also upon expression in heterologous cells [107].

Here, we compared the targeting of wild type (wt) VWF and a VWF propeptide mutant with a multimerization defect (VWF_m) [130] **(Figure 6)**.

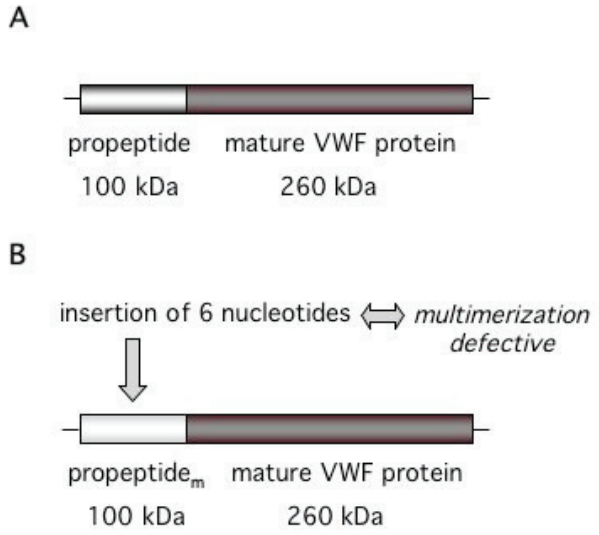

Figure 6. ***Schematic picture of the cDNA constructs encoding the VWF proteins used in the investigation.*** *(A) shows wild type pro-VWF and (B) VWF containing a mutation in the propeptide, which renders the protein multimerization defective. Abbreviations: VWF; von Willebrand factor, propeptide$_m$; mutated propeptide.*

Our results indicated targeting of VWF, but no Weibel-Palade body formation. Although not strictly quantitatively comparable, the lack of detectable VWF_m in secretory lysosomes, in contrast to detectable VWF, suggests a requirement for protein multimerization for targeting.
This conclusion seems to be in contrast to the previous one (**paper I**), suggesting protein aggregation not to be crucial for targeting.

However, on the basis of the observations in **paper I** we speculate on a novel protein quality control system in the secretory pathway. Others have suggested post-ER quality control involving retrograde transport of non-native proteins from the Golgi back to the ER and the ER-associated degradation pathway, alternatively targeting into the endosomal system for lysosomal degradation [131]. Our observations suggest an alternative quality control in which proteins with non-native conformation may be prevented from targeting to secretory lysosomes and instead of being targeted for degradation become eliminated by constitutive secretion. This implies that the ER protein quality control is not perfect but can allow escape of non-native proteins to the Golgi complex, as was actually noticed previously [38, 132]. In addition, previous results from our laboratory indicate targeting for storage of exogenous proteins expressed in myeloid cells, suggesting the targeting mechanisms to be cell-specific and not protein-specific with restriction to endogenous proteins [132, 133]. The present results indicate however that targeting may also be conformation-dependent.
Noteworthy is also that although protein aggregation is not sufficient for sorting-for-entry of proteins in the TGN, into the regulated secretory pathway, it cannot be excluded that the indicated protein stabilizing effect of aggregation might have a role in sorting-by-retention in the granules. In the latter sorting model, proteins that are concentrated into granules are

retained to different extents, suggesting the existence of a sorting mechanism in this organelle. Studies in exocrine and endocrine cells have indicated protein aggregation to be involved in the sorting-by-retention mechanism [125, 126]. However, sorting-for-entry into the forming granules is a prerequisite for studies of this possibility in haematopoietic cells.

Concerning the conclusion in **paper III**, the suggestion of a novel type of post-ER protein quality control may lead to an alternative interpretation of the results. Thus, the results may imply that the VWF_m was not targeted to the secretory lysosomes because of a non-native conformation. Instead, unlike the VWF, VWF_m was constitutively secreted. In support of this suggestion, it has been demonstrated that VWF multimerization is not a prerequisite for storage of VWF in the mouse pituitary tumour cell line AtT-20 [115]. Rather, structurally competent VWF and a propeptide capable of non-covalent association with the mature VWF for co-traffic to storage granules, is suggested to be required for VWF storage [115].

2. Does protein sorting involving the plasma membrane require a native conformation?

(paper II)

Recent investigations in our laboratory suggested an indirect pathway for targeting of neutrophil proELA to secretory lysosomes, involving the plasma membrane [134]. Therefore, our work on the hybrid protein ELA-$(FKBP)_3$ was extended to examine whether the chimera also could be targeted to the cell membrane and become internalized or not (**paper II**). We used the same reversible aggregation system as in **paper I**, described briefly in ***paragraph 1*** and **Figure 5** above. The results indicated that

ELA-$(FKBP)_3$ was present on the cell surface in both aggregated and non-aggregated form, but in contrast to proELA, it was not internalized in the cell lines tested.

Our findings, indicating endosomal reuptake of cell surface localized proELA, is consistent with the proposed indirect targeting route via the plasma membrane, and also in accordance with the suggested requirement for an intact C-terminal propeptide [134]. Yet, ELA-$(FKBP)_3$, which also should contain an intact C-terminal propeptide was excluded from internalization. Instead, the protein was constitutively secreted in both monomeric and oligomeric form.

This might indicate that ELA-$(FKBP)_3$, which is a chimeric protein and thereby unlikely to expose a native conformation, is excluded from endosomal uptake because of a failure in conformational requirements. In support of this, the mechanism of endocytosis has been demonstrated to be a highly controlled and regulated process, dependent on specific signalling molecules, specific receptors, transmembrane regions and interactions with adaptor molecules, and is not likely to occur passively [56]. Therefore, ELA-$(FKBP)_3$ in both monomeric and oligomeric form may be excluded from internalization because of a failure in interacting with a specific receptor or an adaptor molecule, probably depending on a non-native conformation.

Moreover, neutrophil ELA has previously been suggested to be capable of, in addition to the luminal conformation, also adopt a transmembrane configuration, where the C-terminus extrudes into the cytoplasm [135].
The fact that our results indicated the cell surface located ELA-$(FKBP)_3$ to be insensitive to ionic strength and low pH, may support the possibility

that the fusion protein may be incorporated as a transmembrane protein in the cell membrane. However, the fact that ELA-$(FKBP)_3$ in our experiments could be detected by an anti-proELA antibody directed against the C-terminus, suggesting this part to be exposed on the outer surface, contradicts this possibility. Nevertheless, our data also indicated binding of anti-ELA and anti-FKBP antibodies to the cell surface located ELA-$(FKBP)_3$, which is likely to require an exposure of a large portion of the fusion protein on the cell surface.

This implies that the proposed existence of a proELA transmembrane configuration with the C-terminal on the outer surface of the cell membrane cannot be excluded, provided that the ELA protein contains two transmembrane domains, which has been suggested by Benson et al. [135].

Taken together, the results from this investigation suggest a difference in protein targeting between a native and a non-native protein, which is most likely to depend on conformational differences. The results in **papers I** and **III** led us to speculate on a post-ER protein quality control, possibly operating in the Golgi complex. The findings in this paper may lead to speculations about the existence of a protein quality control operating at the plasma membrane, rescuing proteins for internalization or discarding them by constitutive secretion.

3. Is it possible to target exogenous proteins to the secretory lysosomes of haematopoietic cells?

(paper IV)

We asked whether it is possible to target an exogenous protein to secretory lysosomes in haematopoietic cells for storage and subsequent release by regulated secretion. This could be useful as a therapeutic

principle using secretory lysosomes of haematopoietic cells as vehicles for therapeutic proteins to be released at inflamed sites [54].

The protein used in the investigation was the soluble TNF receptor form (sTNFR1), which was chosen because TNF is a very potent mediator in inflammatory reactions. Furthermore, blockade of TNF, in order to neutralize its activation, has proven to be successful as a therapeutic approach in the treatment of certain immune-mediated inflammatory diseases [50].

Since sTNFR1 is retained in the ER, not being a normal gene product when expressed in haematopoietic cell lines [132], the protein was expressed with a transmembrane (tm) domain from TNFR1, in order to avoid ER retention. In addition, in order to get sorting for storage in secretory lysosomes, and thereby prevent constitutive secretion, a cytosolic tyrosine sorting signal (SIRSGYEVM) from CD63 [136], here termed Y, was incorporated, resulting in the protein sTNFR1-tm-Y **(Figure 7)**.

Figure 7. ***Schematic picture of the proteins used in the investigation.*** *(A) shows sTNFR1-tm and (B) sTNFR1-tm-Y. Abbreviations: sTNFR1; soluble tumour necrosis factor receptor 1, tm; transmembrane domain, Y; cytosolic tyrosine sorting signal from CD63 (SIRSGYEVM).*

Our results showed that targeting of an exogenous protein to haematopoietic storage organelles is possible to achieve. However, addition of the transmembrane part was required in order to avoid ER retention followed by degradation, and instead get transport to the Golgi complex. The sTNFR1-tm was observed to be excluded from sorting in the TGN and constitutively secreted, which may indicate a post-ER protein quality control where failure to pass leads to constitutive secretion, as suggested above (**papers I-III**). The addition of a cytosolic sorting signal for secretory lysosomes taken from the transmembrane CD63 protein (Y) allowed the protein sTNFR1-tm-Y to be targeted to secretory lysosomes. This demonstrates that sorting signals for transmembrane proteins can be utilized for targeting of exogenous proteins to secretory lysosomes.

After targeting the protein was, in part, released by endogenous proteolytic activity to generate a soluble protein. In addition, RBL-1 cells could be stimulated to secrete both sTNFR1-tm-Y and sTNFR1, suggesting that sTNFR1-tm-Y is rather stable in the proteolytic environment of the secretory lysosome, except for the loss of the tm-Y fragment. The secretory proteins are usually stored in dense cores and may thereby resist proteolysis. In addition, a proteoglycan matrix may protect the stored proteins against degradation [137]. It could be that stimulation of the RBL cells leads to secretion of the membrane-bound sTNFR1-tm-Y from the secretory lysosome by the formation of small internal membrane vesicles termed exosomes [138]. Importantly, the sTNFR1 was secreted from the cell.

Taken together, the results in **paper IV** suggest that the granule subsets of haematopoietic cells might be used as carriers for pharmacologically

active molecules to be delivered into phagosomes, malignant processes and inflamed tissue [54]. The approach utilized here may have a general application. A prerequisite for successful use is however the clearance of the gene product by both an ER protein quality control and possible post-ER protein quality control systems.

4. Could lack of targeting for storage be due to failure in post-ER protein quality controls?

It is well documented that newly synthesized proteins in the ER are subjected to a quality control prior to ER export [59]. Unfolded or misfolded proteins are recognized by an unfolded protein response system, which is tightly connected to the endoplasmic reticulum associated degradation (ERAD) system [69], where proteins that fail are retro-translocated from the ER to the cytosol. Here, the proteins are tagged with ubiquitin and subsequently targeted to the cytosolic proteasome for degradation [66, 69].

However, previous results from our laboratory have suggested this protein quality control not to be perfect, as escape of non-native proteins to the Golgi complex could be observed [38, 132]. In support of this, certain cases of retrieval of misfolded proteins from the Golgi complex to the ER have actually been reported [74-76].

In addition, the *in vitro* observation of constitutively secreted incompletely polymerized and processed von Willebrand factor, i.e. functionally incompetent protein [108, 139] may be in support of this. The fact that these dysfunctional proteins were not intracellularly degraded, but instead released from the cell by constitutive secretion fits with our observations of the constitutively secreted hybrid proteins ELA-$(FKBP)_3$ (**paper I** and **II**), $(FKBP^*)_4$-FCS-hGH (**paper I**) and the

mutated protein VWF_m (**paper III**), which were all excluded from targeting and instead eliminated from the cell by constitutive secretion.
The escape of these non-native proteins from ERAD suggests a "leaky" ER protein quality control, possibly requiring specific retention signals in the proteins.

Moreover, the lack of targeting of the above proteins indicate a requirement for specific sorting signals in the Golgi complex, suggesting the sorting process to be an active and not a passive process, and an ability of discrepancy between different proteins in this organelle.
Therefore, we speculate on the existence of a post-ER protein quality control possibly operating in the Golgi complex, which discard proteins with non-native conformations from the cell by constitutive secretion.

Our observations that a cell surface located native protein, in contrast to a non-native cell surface located protein, was internalized (**paper II**), indicated special requirements for endosomal reuptake. This extends our speculation about a post-ER protein quality control to include a possible existence of a protein quality control operating at the plasma membrane, rescuing proteins for internalization or discarding them by constitutive secretion.

Since the protein quality control in the ER has been reported not to be perfect, but allow some escape of unfolded or misfolded proteins [131], probably because of a saturation of the retention capacity or lack of protein retention signals, one cannot exclude the possibility of a post-ER protein quality control existing as a "back-up system" to protect the cell from accumulation of dysfunctional proteins.

Evidence for the existence of a post-ER protein quality control, especially operating in the Golgi complex where it targets misfolded proteins for vacuolar degradation, has been observed in yeast [78-80].

Concluding remarks

To summarize, the results from the present investigation suggest that aggregation of proteins does not facilitate their sorting-for-entry into forming granules destined for the secretory lysosomes of haematopoietic cells. Rather, a native conformation seems to be required in order to avoid elimination from the cell by constitutive secretion.

In addition, a native conformation was indicated to be a requirement for endosomal uptake of cell surface located proteins. This leads to suggestions about protein quality controls existing at post-ER levels in the secretory pathway, possibly operating in the Golgi complex and at the plasma membrane. The results indicated the suggested post-ER protein quality controls to, instead of targeting failing proteins for intracellular degradation as is observed in ER protein quality control, eliminate the proteins from the cell by constitutive secretion.

Moreover, targeting for storage of soluble TNF receptor to secretory lysosomes of haematopoietic cells was achieved. This may be useful in the development of a concept involving secretory lysosomes of haematopoietic cells as vehicles for targeting of therapeutic molecules for local release at sites of persistent inflammation.

Populärvetenskaplig sammanfattning

(Summary in Swedish)

Upplagring av proteiner i vita blodkroppar

Kroppens immunförsvar, vars uppgift är att skydda oss mot infektioner, består av ett antal olika celltyper som till stor del finns i blodet. Immunförsvaret kan indelas i två delar; ett medfött försvar som är likadant hos alla individer och ett försvar som byggs upp under individens livstid. Det senare utformas olika beroende på vilka bakterier och virus som individen utsätts för. En viktig celltyp i det medfödda immunförsvaret är de s.k. neutrofilerna som tillhör de vita blodkropparna. Deras uppgift är att vara bland de första immuncellerna på plats och skydda kroppen i det akuta skedet, då kroppen får ovälkommet besök av potentiellt farliga bakterier. Neutrofilerna transporteras med blodet till infektionsstället och vid ankomsten lämnar de blodkärlen och går ut i vävnaden där de ”äter upp” inkräktarna. Inuti neutrofilerna oskadliggörs sedan bakterierna av ett antal bakteriedödande proteiner som finns lagrade i små blåsor, s.k. granula, i cellen. Dessa proteiner kan dock ibland komma ut och orsakar då skador på den omkringliggande vävnaden. Neutrofilen kan även stimuleras av faktorer i den inflammatoriska processen att utsöndra de toxiska granula-proteinerna till omgivningen. Upplagringen av de bakteriedödande proteinerna måste vara väl reglerad så att dessa kan frisättas vid rätt tillfälle och på rätt plats för att undvika vävnadsskada. I mitt arbete har jag därför koncentrerat mig på att studera hur transport och upplagring av proteiner i granula går till, i syfte att hitta de styrande mekanismerna. Dessa skulle i så fall kanske kunna påverkas för att undvika ogynnsam inflammation.

Dels har jag studerat effekten av aggregation, dvs. sammanhopning av proteiner, för transporten av nytillverkade proteiner till granula. Aggregation skulle kunna vara ett sätt att fylla granula med innehåll.
I studien undersöktes både naturligt och modifierat protein. Resultaten visade dock att endast naturligt protein kunde lagras och att aggregation av proteiner därmed inte tycks vara tillräckligt som mekanism för att styra proteinerna till granula. Istället utsöndrades de icke-naturliga proteinerna från cellen, kanske ett sätt för naturlig eliminering av felaktiga proteiner.

Sedan undersöktes upptaget av proteiner på cellens yta för transport till granula, med avsikt att studera skillnader i transport via cellmembranet mellan naturligt och icke naturligt protein. Resultaten visade att ett i cellen naturligt protein kunde tas in från ytan för upplagring i granula, medan ett modifierat protein inte togs upp utan istället utsöndrades.

Sammantaget antyder resultaten att proteiner måste passera en ”kvalitetskontroll”, som ”underkänner” och sorterar bort onaturliga eller felaktiga proteiner från att upplagras i granula. En sådan kvalitetskontroll har redan rapporterats i en struktur i cellen som kallas det *endoplasmatiska retiklet*, där icke godkända proteiner skickas till nedbrytning i s.k. *proteasomer* inuti cellen. Resultaten från dessa experiment tyder dock på att proteinerna kan utsättas för kvalitetskontroll även i en struktur kallad *Golgi-apparaten*, samt på cellens yta, dvs. på det s.k. *cellmembranet*. Kvalitetskontroll av proteiner i dessa strukturer, som dessutom inte tycks skicka felaktiga proteiner till nedbrytning utan istället utsöndrar dem från cellen, finns inte rapporterad tidigare. Kvalitetskontroll i Golgi-apparaten har dock föreslagits existera i jästceller.

Som ovan nämnts, kan frisättning av bakteriedödande proteiner vid en inflammatorisk reaktion orsaka vävnadsskador. Det är därför viktigt att inflammationsprocessen regleras ordentligt och att den avslutas så snart de invaderade mikroorganismerna oskadliggjorts. En icke fungerande reglering av det inflammatoriska försvaret kan i kombination med ärftliga faktorer bidra till uppkomst av kronisk inflammatorisk sjukdom såsom *reumatisk ledsjukdom.*

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Ett sätt skulle kunna vara att, med hjälp av genteknik, använda sig av neutrofila granula för upplagring av anti-inflammatoriska proteiner. Dessa skulle då kunna styras direkt till inflammationsstället och där frisätta de inflammationsdämpande proteinerna.

Därför undersöktes möjligheten att förse ett protein, som normalt inte upplagras i neutrofila granula, med en ”upplagringssignal” från ett annat protein som normalt styrs till granula. Här valdes det att försöka lagra upp ett protein som kan oskadliggöra så kallad tumörnekrotisk faktor (TNF), som har stor inblandning i den vävnadsförstörelse som kan uppstå vid inflammation. Resultaten visade att proteinet transporterades till granula för upplagring där. Detta antyder att neutrofila granula skulle kunna användas som bärare av terapeutiska proteiner för att lokalt dämpa inflammatoriska processer med avsikt att minska vävnadsskador. Detta koncept skulle även kunna tillämpas på andra celler i immunförsvaret som också innehåller granula vars innehåll kan utsöndras i en inflammationsprocess.

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