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A novel principle for immunomodulator delivery with hematopoietic cells as vehicles Focus on soluble TNF-receptor gene expression, targeting to secretory lysosomes and regulated secretion

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A novel principle for immunomodulator delivery with hematopoietic cells as vehicles Focus on soluble TNF-receptor gene expression, targeting to secretory lysosomes and regulated secretion

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2005

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Sorting of soluble TNF-receptor for granule storage in hematopoietic cells as a principle for targeting of selected proteins to inflamed sites.
Blood. 102: 682-8, 2003

II Gao Y, Hansson M, Calafat J, Tapper H, and Olsson I.
Sorting soluble Tumor Necrosis Factor (TNF) receptor for storage and regulated secretion in hematopoietic cells.
J Leukoc Biol. 76: 876-885, 2004

III Gao Y, Tapper H, Olsson I, and Hansson M
Granule Targeting of Soluble Tumor Necrosis Factor (TNF) Receptor Expressed During Granulopoietic Maturation in Murine Bone Marrow Cells
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Selected Abbreviations

BPI	bactericidal/permeability-increasing protein
cDNA	complementary deoxyribonucleic acid
CTL	cytotoxic T lymphocyte
Endo-H	endoglycosidase H = Endo-β-N-acetylglucosaminidase H
ER	endoplasmic reticulum
fMLP-R	formylmethionine leucyl-phenylalanine receptor
G-CSF	granulocyte colony-stimulating factor
GFP	green fluorescent protein
IL	interleukin
kDa	kilodalton
LAMP	lysosome-associated membrane protein
LBP	lipopolysaccharide-binding protein
lgp 120	lysosomal glycoprotein 120 = LAMP-1
LPS	lipopolysaccharide
M-6-P	mannose 6-phosphate
MPO	myeloperoxidase
MPR	mannose 6-phosphate receptor
mRNA	messenger ribonucleic acid
NGAL	neutrophil gelatinase-associated lipocalin
N-glycanase	*N*-glycosidase F = Endo-acetyl-β-N-glucosaminidase F
NK cell	natural killer cell
N-linked	asparagine-linked
PCR	polymerase chain reaction
PMA	phorbol myristic acetate
PMN	polymorphonuclear neutrophil
RBL	rat basophilic leukemia/mast cell tumor line
RMCP-II	rat mast cell protease II

ROS	reactive oxygen species
sTNFR	soluble tumor necrosis factor receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TGN	trans-Golgi network
TNF-α	tumor necrosis factor α

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1. Introduction

Blood cells have many functions. Red cells transport oxygen. Platelets have a role in hemostasis. White blood cells/leukocytes play a role in host defense e.g. by granule storage and release of lytic and antimicrobial agents. Functional differences among white blood cells depend largely on cell-specific granule proteins the synthesis of which occurs at particular windows of hematopoietic differentiation.

Inflammation is a beneficial response to infection, but persistent inflammation can be harmful such as in autoimmune diseases. The pro-inflammatory cytokine TNF-α is a key factor in inflammation whose inhibition can fulfill an anti-inflammatory effect. Systemic anti-TNF treatment is beneficial in chronic inflammatory disorders but also associated with infectious complications. A local delivery of anti-inflammatory agents to the site of inflammation would diminish complications which are due to systemic effects. Therefore, the basic idea of this research is to explore the possibility of targeting the inflamed site by anti-inflammatory agents using storage granules, for example secretory lysosomes of hematopoietic cells as vehicles. Secretory lysosomes are lysosome-related organelles with both storage/degradative and secretory functions found mostly in hematopoietic cells. Their content is contained in these organelles until release by degranulation. Exogenous proteins have been shown previously to become targeted to secretory lysosomes after gene expression in hematopoietic cell lines, but the sorting mechanisms of luminal granule proteins are largely unknown. With focus on a non-hematopoietic protein, soluble TNF receptor, we asked whether targeting to secretory lysosomes was feasible after

expression in hematopoietic cells. In a longer perspective, the idea was to use hematopoietic cells as vehicles for targeting transgenic immunomodulators. To achieve this goal an immunomodulatory agent e.g. soluble TNF receptor must (i) be synthesized with a native conformation, (ii) be sorted to a cellular storage compartment, and (iii) be subjected to exocytosis after stimulation. My thesis will show how these obstacles have been overcome in vitro, but secretion in vivo of functional immunomodulator remains to be shown.

2. Background

Hematopoiesis

Every day, the body produces a large number of blood cells originating from hematopoietic stem cells. Both stem cell reproduction/proliferation/self-renewal/differentiation must be correctly fulfilled. Self-renewal and differentiation may take place at the same time by an asymmetric cell division resulting in an identical daughter cell and a multi-potential hematopoietic progenitor cell that goes to differentiation depending on regulatory signals [1].

Regulation of hematopoiesis is interactive. Intrinsic regulation is based on transcriptional factors that bind to short specific DNA sequences and affect gene expression positively or negatively. Combinations of transcriptional factors regulate the production of growth factors, cytokines and their receptors necessary for hematopoietic development. Transcriptional factors also govern the timing of cell-specific protein expression during differentiation [2].

Extrinsic regulation is carried out by cytokines. In fact, hematopoietic cells need cytokines all the time for survival. It is not clear if cytokines are able to drive a multi-potential progenitor cell to a specific lineage or only support survival. Anyway, cytokines bind to receptors on the cell surface and trigger signaling pathways that affect gene expression [2].

Innate immunity

Immunity has both nonspecific and specific components. Innate immunity is a set of disease-resistance mechanisms which are pathogen-unspecific. Neutrophils, mast cells, eosinophils, macrophages, NK cells, and dendritic cells all play an important role for innate immunity. In contrast to innate immunity, acquired immunity displays a high degree of specificity and remarkable memory. Lymphocytes and the antibodies they produce are the major agents of acquired immunity. However, acquired immunity does not respond directly when encountered with a pathogen. Innate immunity provides the first line of defense during a crucial period just after the host's exposure to a pathogen. In general, most of the microorganisms encountered by a healthy individual are readily cleared by innate immunity [3], the importance of which is shown by the fact that rare defects in this system are almost always lethal [4]. If an invading microorganism eludes the innate system, the acquired immunity is triggered. Actually, innate and acquired immunity do not operate separately but interact and cooperate in host defense.

While adaptive immunity recognizes specific structures, innate immunity recognizes conserved molecular structures shared by large groups of pathogens. For example, pathogen-associated molecular patterns are shared by different groups of microorganisms, double-stranded RNA is a character structure of RNA viruses [5]. Pathogen-associated molecular

patterns are recognized by various receptors on effector cells. The macrophage mannose receptor can interact with both gram-positive and gram-negative bacterial and fungal pathogens [4]. Interaction between pathogen-associated molecular patterns and receptors triggers phagocytosis, during which the plasma membrane expands around the microbe and forms a phagosome vesicle. The phagosome fuses with lysosome-related organelles, which release their antimicrobial agents and lytic enzymes leading to killing and digestion [6].

Effector cells

Neutrophils

Neutrophils are the first circulating blood cells to migrate to a site of inflammation. There, they fulfill a role by phagocytizing pathogens and exposing them to ROS and lytic proteins from the granules [7]. Neutrophils have four types of granules, which are synthesized sequentially during granulopoiesis (Fig.1): Proteolytically active primary/azurophil granules, proteolytically inactive secondary/specific and gelatinase granules as well as secretory vesicles. Primary granules are manufactured in myeloblasts and promyelocytes, specific granules in myelocytes and metamyelocytes, gelatinase granules in band cells, and secretory vesicles in segmented neutrophils [8, 9].

The primary granules contain MPO, lysozyme, BPI, defensins, and various serine proteases. *MPO* is involved in generation of oxygen/nitrogen intermediates [7]. *BPI* is cytotoxic to gram-negative bacteria [10]. The N-terminus of BPI is highly cationic and contains both antibacterial and endotoxin-neutralizing properties, and the C-terminus contributes to opsonizing gram-negative bacteria [10].

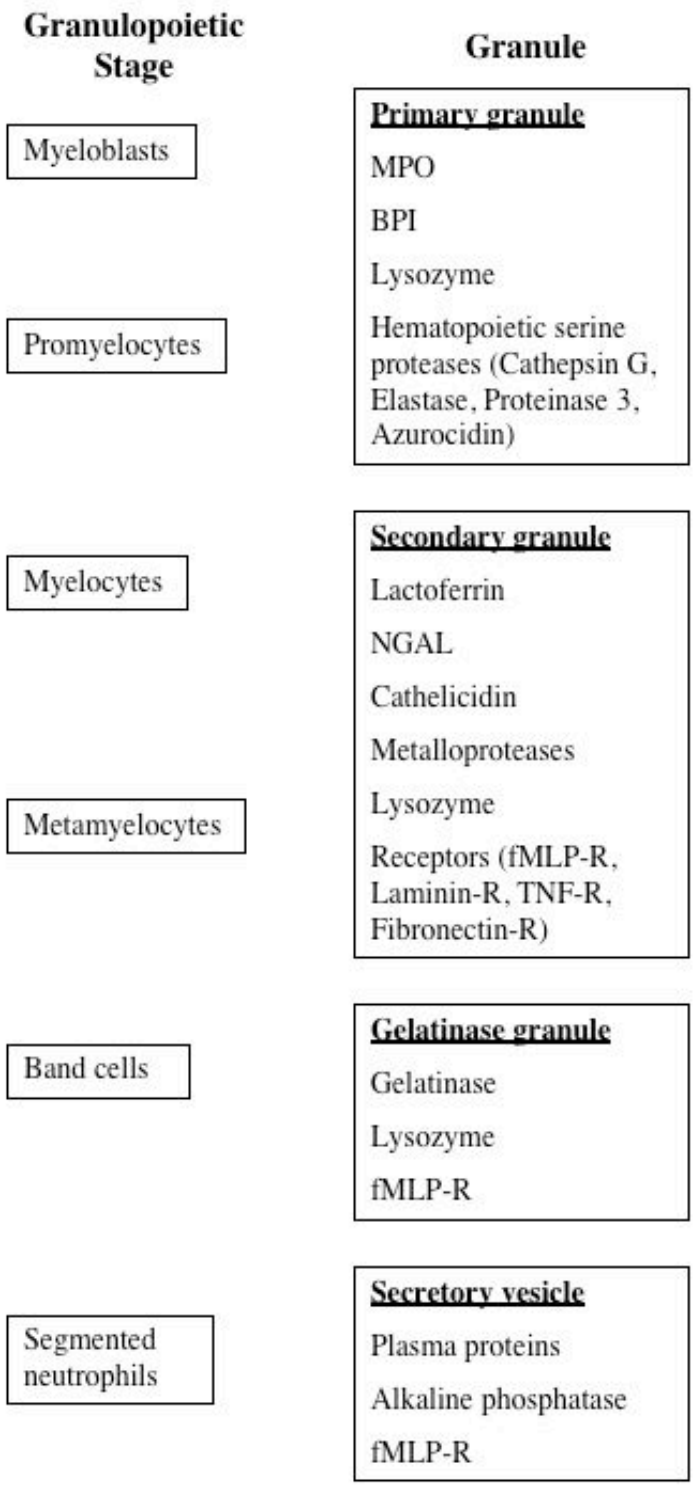

Fig.1. Characteristic contents of subsets of neutrophil granules formed during granulopoietic differentiation.

Lysozyme degrades bacterial peptidoglycans by cleaving the glycosidic bond of N-acetylglucosamine. The lysozyme effect might be strengthened by synergy with other antibiotic proteins such as lactoferrin and defensin [6]. A broad cytotoxic activity against bacteria, fungi, parasites, and viruses is promoted by defensins, which form multimeric transmembrane pores [6, 9]. Hematopoietic serine proteases including cathepsin G, elastase, and proteinase 3 are multifunctional cationic polypeptides with a role in host defense [9].

Secondary granules contain lactoferrin, NGAL, cathelicidins, metalloproteases and lysozymes. *Lactoferrin* is a member of the transferrin family of iron-binding proteins those deprive microorganisms of iron. Lactoferrin also binds LPS and thereby neutralizes endotoxic activity [6]. *NGAL* can participate in an antibacterial iron depletion strategy of the innate immune system [11]. *Cathelicidins*, which belong to the cathelin family, are stored intact in secondary granules. During degranulation of primary and secondary granules, the N-terminal antibacterial peptide is cleaved off by elastase originating from the primary granules [12]. *Metalloproteases*, including gelatinase/MMP-9 and collagenase/MMP-8, are capable of degrading the extracellular matrix during neutrophil migration and extravasation [9].

The segregation of neutrophil proteins into separate storage compartments could be necessary since some of these proteins may be unable to coexist. For example, primary granules store terminally processed proteins such as MPO, serine proteases, and other antibiotic proteins. In contrast, specific granules store metalloproteases, cathelicidins, and other antibiotic proteins as inactive proforms, which are

converted into active forms by proteases liberated from azurophil granules during degranulation [8].

NK cells

NK cells are lymphocytes with large lytic granules, which play a critical role in the early immune defense against viruses and tumors. These cells recognize virus-infected- and altered-self-cells through NK cell receptors, which detect the absence of self on targets rather than the presence of non-self [4]. When target cells are bound, degranulation occurs with release of the granule content including perforin and granzymes. *Perforin* is a pore-forming protein, and *granzyme* is a serine protease that induces apoptosis [13, 14]. Moreover, NK cells produce several cytokines including TNF-α and INF-γ, which have anti-tumor effects [15].

CTLs

CTLs are critical in recognition and elimination of virus-infected cells and tumor cells, and in graft-rejection [16]. Upon interaction with antigen-class I MHC complexes on target cells, CTL precursors express IL-2 receptors. Antigen-activated CTL precursors require IL-2 for proliferation and differentiation into cytotoxic cells. The T-cell receptors on a CTL interact with processed antigen-class I MHC complexes on a target cell leading to formation of a CTL/target cell conjugate [17] towards which the CTL granules are reoriented. Perforin and granzymes are released by exocytosis leading to target cell killing. In addition, FasL on CTLs interacts with target cell Fas to trigger apoptosis [18].

Mast cells

Mast cells are tissue dwelling cells and contain cytoplasmic granules with histamine and other pharmacologically active substances. They have a

pivotal role in allergic reactions and take part in innate and acquired immunity, wound healing, fibrosis, and autoimmune diseases [19]. Mast cells are mainly found in the host-environment interface such as the mucosa where microorganisms may penetrate. Therefore, mast cells constitute a first line in inflammation with early direct pathogen contact. Furthermore, mast cells store and secrete TNF-α, an important mediator of leukocyte recruitment [20].

In an allergic reaction plasma cells secrete IgE, which binds with high affinity to Fc receptors (FcεR) on mast cells and basophils leading to activation. Repeated exposure to the antigen crosslinks membrane bound IgE causing degranulation. Histamine, leukotrienes and prostaglandins are released and act on surrounding tissues. The principal effects, vasodilation and smooth-muscle contraction, may be either systemic or localized [19, 21, 22]. The activity of IgE depends on its ability to bind specific FcεR on mast cells and basophils. Two classes of FcεR have been identified. The first class is high-affinity receptor (FcεRI), which activates protein tyrosine kinases leading to mast cell degranulation. The other class of FcεR is low-affinity receptor (FcεRII), which might be involved in regulation of the IgE response [22].

Inflammation

Inflammation is a beneficial physiological response to infection and injury. Macrophages are attracted to the site of inflammation and secrete proinflammatory cytokines in particular IL-1 and TNF-α [23, 24]. This stimulates neutrophil production and mobilization to meet the needs. Vascular endothelial cells increase the expression of E- and P-selectin leading to neutrophil attachment followed by transendothelial migration

[9, 25]. Once in the tissue, the activated neutrophils migrate to the inflammation focus along gradients of chemoattractants. During this process, and for a short time, neutrophil genes for pro-apoptosis are down-regulated and genes for anti-apoptosis are up-regulated [26]. Genes for proteins involved in priming of degranulation are also up-regulated at this time [26]. These changes support the action of activated neutrophils at the site of inflammation. Increased numbers of immunoglobulin Fc and complement receptors are expressed supporting neutrophil phagocytosis of opsonized pathogens. ROS are produced which together with antibacterial granule constituents support pathogen killing [9, 25]. Large numbers of activated neutrophils can be recruited to an inflammatory site. The elimination of these cells by apoptosis may contribute to quick recovery from disease. Therefore, at the end, it is important that tissue neutrophils of the inflammatory process upregulated genes for apoptosis. Death receptor ligands (TNF, TRAIL), death receptors (TNFR1, Fas, TRAILR), and downstream messengers of death receptors (FADD, TRADD, caspases 8/10) are produced [27]. As a result, neutrophil apoptosis is increased leading to their phagocytosis by macrophages and elimination. In contrast, neutrophil persistence can give rise to tissue damage and result in chronic inflammation and autoimmune diseases [28].

TNF-α

TNF-α is multifunctional major proinflammatory cytokine [29]. First, it promotes PMN adherence [30] and phagocytosis [31], and has a key role in macrophage activation and killing [32]. But it has many other actions such as inhibiting clonogenic growth [33], inducing mitogenesis of fibroblasts [34], thymocytes [35], T- and B- cells [36, 37]. Furthermore,

this cytokine can induce tumor cell apoptosis [38] and become involved in cancer-associated cachexia [39].

TNF-α receptors

The biological activity of TNF is mediated by specific receptors. One is the 60 kDa TNF receptor (TNFR1) and another is the 75 kDa TNF receptor (TNFR2). The receptor (Fig.2) consists of an extracellular domain for TNF-binding, a transmembrane, and an intracellular domain [40-42]. A 30-kDa TNF binding protein (TNF-BP) was first identified in biological fluids [43-45]. This protein binds to TNF so as to inhibit its cell surface receptor binding thereby inhibiting its biological activity. For example, TNF-BP inhibits TNF cytotoxic activity [43, 44]. TNF-BP was shown to be identical with soluble TNF receptor [46] consisting of the extracellular part of the transmembrane receptor [40]. Thus, soluble TNF receptor is generated by proteolytic cleavage of the TNF receptor at the cell surface catalysed by a metalloproteinase [47] (Fig.2). Cleavage may also take place after receptor internalization followed by soluble TNF receptor secretion [40].

Receptor binding of TNF-α on the cell surface induces downstream signal transduction as a result of aggregation of the intracellular receptor domain and adapter protein recruitment [48]. The binding of the TNF receptor death domain to adapter proteins triggers the caspase cascade which leads to apoptosis [49, 50]. The TNF receptor death domain can also activate the nuclear factor-κB (NF-κB), which induces cellular resistance to TNF cytotoxicity [38].

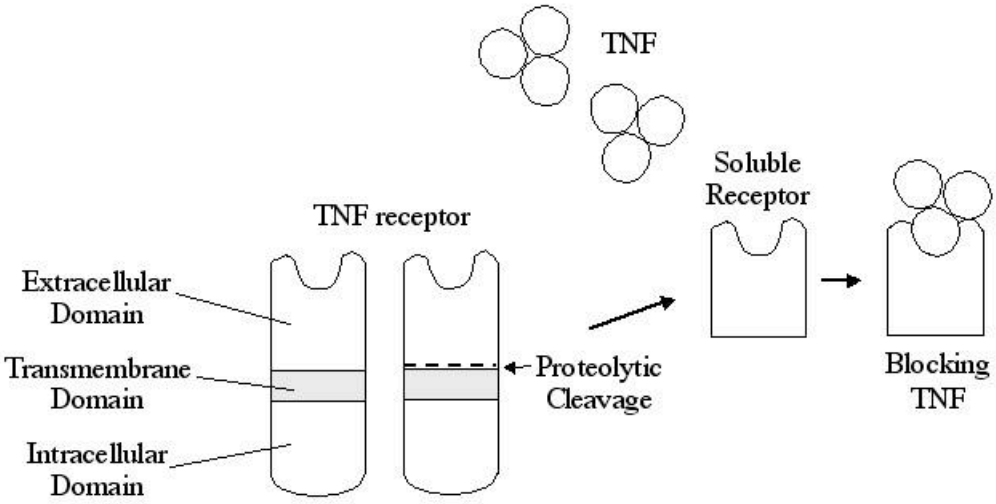

Fig. 2. Schematic formation of soluble TNF receptor. Soluble TNF receptor (TNF-binding protein) corresponds to the extracellular domain of TNF receptor and is released upon protelytic cleavage. Soluble receptor can bind and inactivate TNF, which is normally a trimeric molecule.

TNF-α has a major role in inflammatory disease. For example, TNF-α and IL-1 are produced in the synovial tissue of rheumatoid arthritis [51], and their blood concentration correlates with disease severity [52]. Not only TNF-α but also TNF receptors are up-regulated in the synovial cells [53, 54]. The resulting abnormal levels of TNF-α and TNF receptor are likely to play a major role in the pathogenesis of rheumatoid arthritis [55]. TNF-α induces inflammatory cell release of proteolytic and cytotoxic agents, which damage tissues [56]. Obviously, TNF-α is a major therapeutic target in rheumatoid arthritis and other chronic inflammation diseases in which TNF-α inhibition is beneficial [57]. Treatment with a murine-human chimeric antibody (infliximab) [58] and

a fusion protein between soluble human TNFR2 and human IgG1 (etanercept) [59-61] are used in rheumatoid arthritis. Systemic administration of anti-TNF-α antibody or the sTNFR fusion protein is anti-inflammatory and joint protective in rheumatoid arthritis [62]. Thus, anti-TNF-α treatment diminished the number of inflammatory cells in the joints [63]. Anti-TNF-α therapy is also beneficial in Crohn's disease [64]. However, systemic anti-TNF-α therapy is associated with infectious complications such as tuberculosis. A local delivery of an anti-TNF-α agent e.g. soluble TNF receptor using hematopoietic cells as vehicles is suggested as a possibility to overcome systemic side effects.

The protein secretory pathway

ER is the protein synthesis site where also quality control is carried out so that only properly folded proteins are exported. Improperly folded protein will be retained and degraded within proteasomes [65]. Newly synthesized proteins are exported to the Golgi complex in COPI- or COPII-coated vesicles [66]. The Golgi is organized into the cis-Golgi network, the Golgi stack, and the TGN. The Golgi is involved in post-translational protein modifications such as phosphorylation, complex oligosaccharide chain formation, and proteolytic processing [67]. The TGN is a sorting station for secretion and organelle targeting [68]. Protein secretion is either constitutive or regulated. In constitutive secretion, proteins are released by "default" after biosynthesis. In regulated secretion, stored proteins are released after stimulation e.g. by a calcium signal. As a result, microtubules rearrange and storage organelles move towards the cell membrane for exocytosis [68].

Secretory lysosomes

Secretory lysosomes are lysosome-like dual-function organelles that serve both as (i) degradative/storage, and (ii) secretory compartments [69]. They are generally found in hematopoietic cells but also in melanocytes [70]. The secretory lysosome is characterized by having a limiting membrane, a matrix, dense cores, and internal vesicles (Fig.3). Dense cores contain proteins stored in a condensed form for protection against the potential lytic environment [69]. The internal vesicles are formed by inward budding from the limiting membrane.

Secretory lysosomes have cell-specific functions. CTLs and NK cells recognize infected or tumorigenic cells and destroy them after cytolytic protein release from secretory lysosomes. Mast cells and basophils store histamine and serotonin, which are released to the exterior upon antigen activation. Neutrophils use secretory lysosomes for storage of antibacterial agents, which are released into phagosomes and to the exterior. Macrophages and dendritic cells degrade antigens and assemble peptides with MHC class II receptors in these organelles for antigen presentation [69, 71].

Proteins reach lysosomes both by biosynthetic and endocytic pathways. Certain soluble lysosome proteins, such as hydrolases, are modified during biosynthesis by the addition of a M-6-P moiety, which is recognized by the MPR system [72]. The phosphorylated proteins are transported from TGN to the late endosomes, dissociated from the receptors and delivered to the secretory lysosome. The MPRs are recycled to the TGN. Granzymes are also targeted to lysosomes by the MPR-system [73]. Lysosome membrane proteins, such as LAMP-1 and

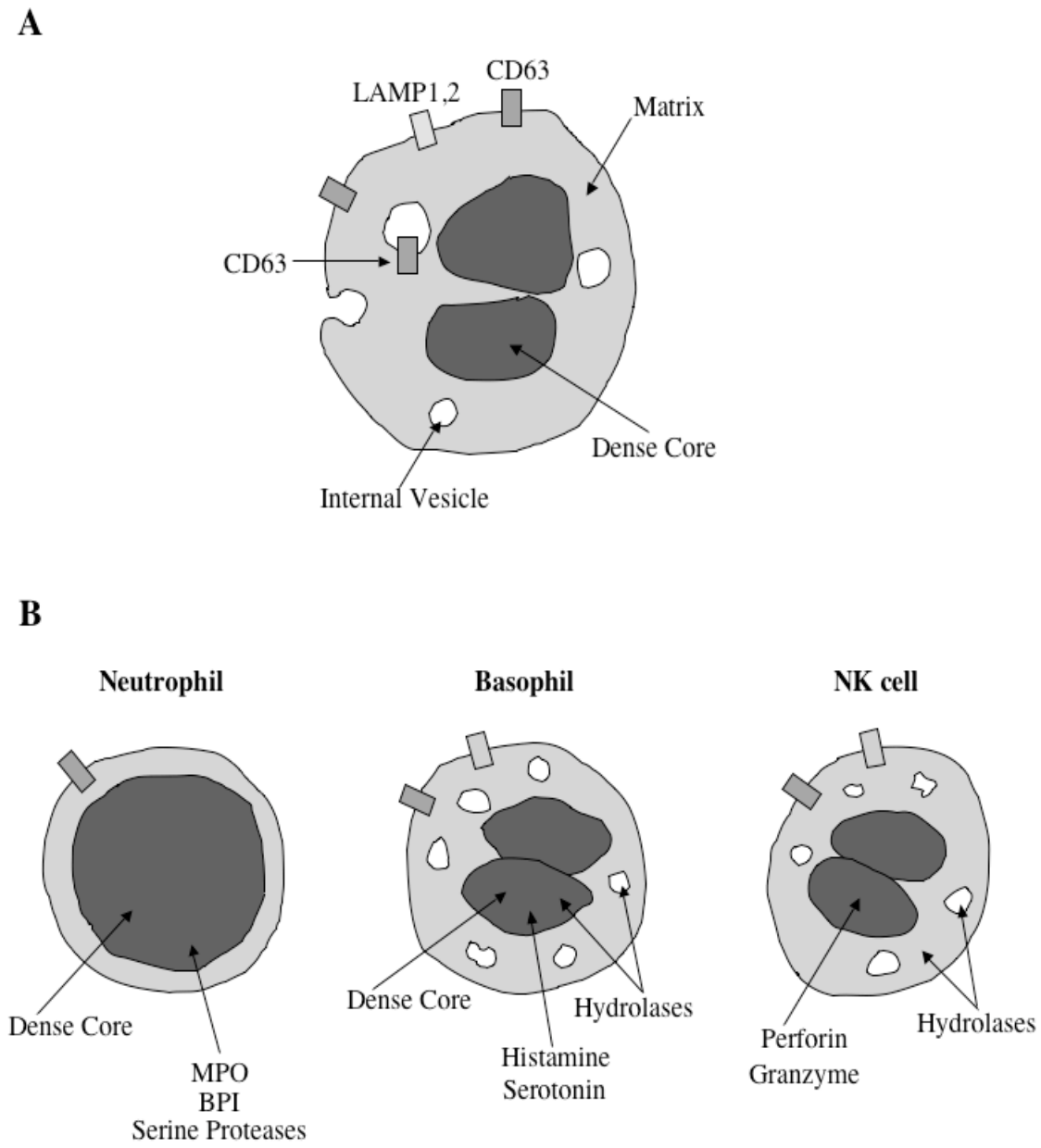

Fig. 3. Schematic structure of the secretory lysosome. Shown is a secretory lysosome (A) typical of hematopoietic cells (B) such as neutrophil, basophil, and NK cell. Stored proteins are tightly packed in dense cores and also present in a matrix. Internal vesicles are formed by inward budding and may have a lysosomal function.

LAMP-2, and CD63 (LAMP-3), contain a tyrosine-based sorting signal in the cytoplasmic tail, which is recognized by the sorting machinery leading to production of budding transport vesicles. These vesicles could reach secretory lysosomes either directly or indirectly. In the indirect route, the lysosome membrane proteins first travel the "default" pathway to the plasma membrane and become internalized through the endocytic pathway [69].

Regulated secretion of (secretory) lysosomes involves ligand binding of a cell-surface receptor leading to an increased intracellular calcium concentration and granule mobilization [74]. Once the secretory lysosome has docked at the plasma membrane, its content is released into the extracellular space [69].

The function of the secretory lysosome is impaired in several rare disorders. The Chediak-Higashi syndrome (CHS) is associated with neutropenia and defective natural killer cell function [75]. Giant lysosomes, melanosomes, MIICs, lytic granules, and azurophil granules are typical because of defective lysosome fission [69, 71]. The Hermansky-Pudlak syndrome 2 is characterized by oculocutaneous albinism and prolonged bleeding due to abnormal melanosomes and absence of dense granules from blood platelets [69, 71]. The Griscelli's syndrome shows defective CTL and melanocyte secretion [69, 71].

3. The present investigation

Since secretory lysosomes are dual-function organelles, serving both a degradative/storage and a secretory function, and are mostly found in

cells from hematopoietic lineages, we asked whether an exogenous protein such as a soluble TNF-α receptor could be expressed in hematopoietic cells and targeted to secretory lysosomes for storage and regulated secretion. TNF-α is a key factor in inflammation and inhibiting TNF-α by soluble TNFR1 is known to be beneficial as anti-inflammatory therapy. The idea occurred was of forming a basis of using circulating hematopoietic cells as vehicles for targeting sites of inflammation with therapeutically active agents. Soluble TNF receptor was explored as a model agent.

The specific questions were:

1. Can soluble TNF-receptor be expressed in hematopoietic cell lines and become targeted for secretory lysosome storage and secretion?

2. Can soluble TNF-receptor also be expressed in normal hematopoietic progenitor cells and become targeted for secretory lysosome storage and secretion?

4. Experimental considerations

Cell models

RBL-1 cells and RBL-2H3 cells were used to investigate sorting, storage, and secretion of granule proteins (Paper I and II). RBL-1 cells grow in suspension, whereas RBL-2H3 cells grow in adherent layers. RBL-2H3 cells, but not RBL-1 cells, show IgE receptor activation [76].

As RBL cells are transformed the results were confirmed in normal hematopoietic cells. Murine hematopoietic progenitor cells were chosen

because cells from an inbred mouse strain are genetically identical in contrast to cells from human beings. Furthermore, mouse models for inflammatory disorders can be used for in vivo studies [77].

Transfection

The constructs sTNFR1-tm-pcDNA3, sTNFR1-tm-Y-pcDNA3, and sTNFR1-tm-Y-EGFP-pcDNA3 were expressed in RBL-1 cells and RBL-2H3 cells. The cDNA for a fusion protein was constructed by spliced overlap extension of polymerase chain reactions where cDNA of the corresponding normal protein was used as template [78, 79]. The cDNA was cloned into the eukaryotic expression vector pcDNA3, which is driven by a virus promoter and containing antibiotic-resistance gene. Stable transfectants were established after introduction of the expression vector into targeted cells by eletroporation. Antibiotic-resistant cell clones were selected, expanded and screened by biosynthetic radiolabeling.

Retroviral transduction

Retroviral vectors can give stable and efficient transduction of a gene into primary cells including hematopoietic progenitor cells. The retroviral vector MIG [80] based on the murine stem cell virus (MSCV) and containing an internal ribosomal entry site, and the gene for enhanced green fluorescent protein (GFP), was used for transducing hematopoietic progenitor cells. MIG contains the retroviral long terminal repeat from MSCV with viral promoter, enhancer, and polyadenylation signals to enhance expression in stem cells [80]. To produce infectious viral particles from the retroviral plasmid, the viral structural proteins encoded by *gag*, *pol*, and *env* are supplied in *trans* by packaging cell lines.

Ecotropic retroviral producing cells (GP + E86) were used as packaging cells in my experiments.

Cell proliferation is necessary for retroviral infection as mitosis is required for entry of the viral integration complex into the nucleus. Therefore, progenitor cells were incubated with cytokines to be forced into cell cycling before and during transduction. Early acting cytokines, including SCF, flt3 ligand, and thrombopoietin, were used to induce proliferation, and G-CSF was used to induce granulocyte differentiation [2, 81].

Biosynthetic radiolabeling and immunoprecipitation

Proteins were radiolabeled during biosynthesis through the incorporation of a radioactive amino acid (pulse radiolabeling). The processing of newly synthesized protein was then followed by chase of the radiolabel in a medium devoid of radioactive amino acids and analyzed at selected time intervals. This is useful for studying the fate of a protein synthesized *de novo*. Complete lysis before immunoprecipitation is important for maximum protein recovery.

Subcellular fractionation

After cell disruption and nucleus removal the post-nuclear supernatant was layered on a Percoll solution for centrifugation and gradient formation. Percoll is isotonic so proteins will equilibrate at their true density. Light density organelles, such as ER and Golgi, accumulate in the upper part of the gradient, whereas high density organelles such as granules accumulate in the lower part of the gradient. When biosynthetic radiolabeling and chase of the radiolabel precedes the Percoll separation, the distribution difference between pulse and chase can show a

subcellular shift of the protein of interest. A disadvantage is that organelles with identical density can not be separated on a density gradient.

Indirect immunofluorescence staining

The cells were fixed, permeabilized, and incubated with specific antibody followed by fluorescent secondary antibody. If the antigen is intracellular a permeabilization step is needed, which will make small holes in the plasma membrane and intracellular membranes so that the antibody can reach the subcellular antigen by travel through these holes. At the same time, permeabilization should be properly controlled so that there will be minimal organelle content leakage and mixing. A large range titration of both first and secondary antibodies is necessary to reduce background and autofluorescence. Moreover, with multicolor staining, there should be no other reaction (cross-reaction) besides the intended first-secondary antibody reaction.

Immunoelectron microscopy

This method is useful for subcellular localization. Cells are fixed and cut into ultrathin cryosections, incubated with a specific antibody followed by a secondary antibody conjugated with gold particles. It is crucial that cells are kept intact by efficient but gentle fixation to lower non-specific staining. The advantage of electron microscopy is high resolution to tell not only which cellular compartment a protein is located in but also where in the compartment. Immunoelectron microscopy gave confirmation of results from subcellular fractionation and immunofluorescence microscopy. Therefore, this technique was of crucial importance in this work.

5. Discussion and conclusions

Targeting proteins to secretory lysosomes for storage requires protein passage through ER-Golgi, escaping constitutive secretion, and surviving the lytic granule environment.

Overcoming ER retention and constitutive secretion

Not all proteins that are synthesized in the ER will be exported. Conformation-based quality control keeps misfolded proteins in ER for degradation in proteasomes because of abnormal folding [65]. Anchoring sTNFR1 through a transmembrane domain (tm) facilitated ER-export. However, sTNFR1-tm was not retained but constitutively secreted. The sTNFR1-tm traversed the mid-Golgi as judged by acquisition of complex glycan side chains, which takes place at this location. However, the lack of secretory lysosome targeting suggested a need for a sorting signal.

Achieving secretory lysosome targeting in cell lines

Sorting pathways of secretory lysosome luminal proteins are still largely unknown except for a MPR-dependent endosomal pathway important for lysosomal hydrolases [72]. Sorting of transmembrane proteins, such as CD63/LAMP-3, to secretory lysosomes involves a cytosolic signal. Our strategy was to take advantage of the sorting signal (Y) present in CD63 and incorporate it for sTNFR1 targeting by creating the construct sTNFR1-tm-Y. This was successful as both sTNFR1-tm-Y and sTNFR1-tm-Y-egfp became targeted to secretory lysosomes, but the sorting route of these two proteins was not identical (Fig.4). Targeting of sTNFR1-tm-

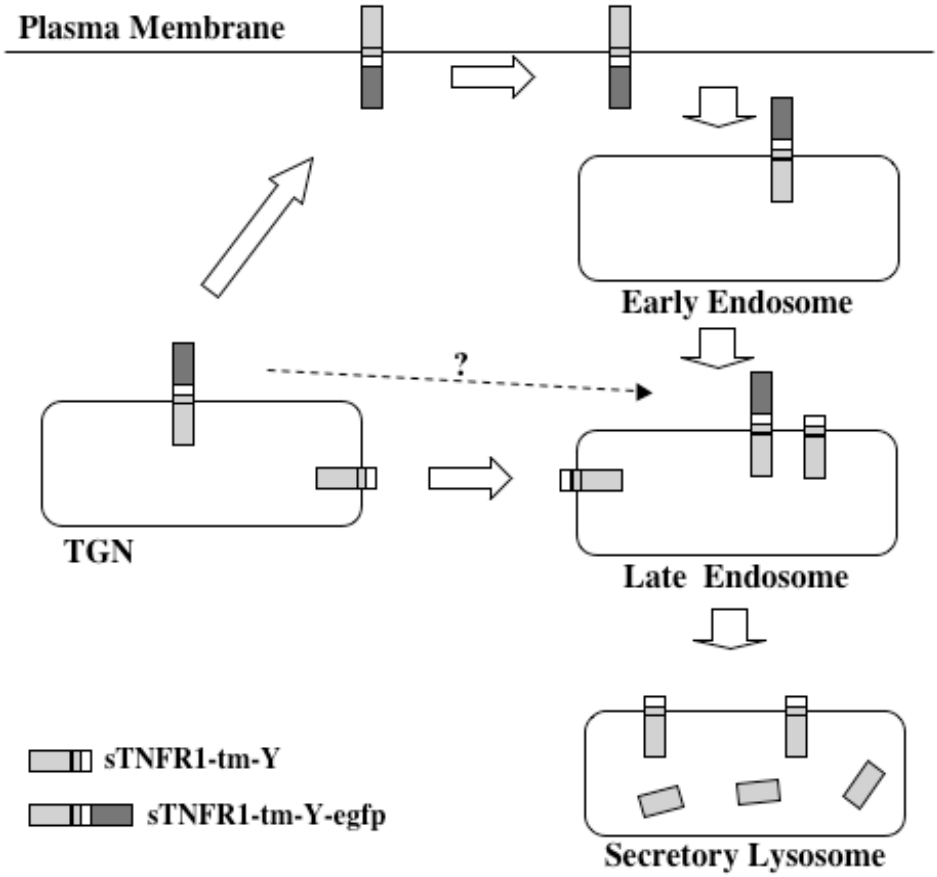

Fig.4. Targeting of sTNFR1-tm-Y and sTNFR1-tm-Y-egfp to secretory lysosomes. sTNFR1-tm-Y is sorted from TGN directly to late endosomes followed by secretory lysosome targeting. sTNFR1-tm-Y-egfp is, at least in part, routed to plasma membrane. After internalization at the plasma membrane, sTNFR1-tm-Y-egfp is assumed be sorted to endosomes, where egfp might be released. The resulting sTNFR1-tm-Y would be targeted to secretory lysosomes. There is no ruling out of the possibility that sTNFR1-tm-Y-egfp can also be sorted from TGN directly to late endosomes without a plasma membrane detour.

Y to secretory lysosomes was “direct”, whereas targeting of sTNFR1-tm-Y-egfp to secretory lysosomes was, at least in part, “indirect” involving a detour to the plasma membrane (Paper I and II).

The sorting signal employed is a tyrosine-base sequence (YXXΦ, Y stands for tyrosine; X stands for any amino acid; and Φ stands for an amino acid with a bulky hydrophobic side chain) for lysosome targeting. This sequence is involved in coat recruitment, membrane invagination and transport vesicle formation at the TGN and at the plasma membrane [82]. The spacing of the sorting signal relative to the membrane is an important determinant [83]. In case of sTNFR1-tm-Y, the YXXΦ motif was located at the C-terminus to facilitate lysosome targeting at the TGN and/or endosome presumably mediated by an AP-3 complex. The AP-3 complex has been found to be associated with both the Golgi region and structures corresponding to post-TGN sorting compartments [84]. Therefore, AP-3 might be involved in trafficking sTNFR1-tm-Y from TGN to late endosomes, and from them to secretory lysosomes. The YXXΦ motif of sTNFR1-tm-Y-egfp is not at the C-terminus but hidden by egfp. The lack of exposure of the sorting motif may prevent “direct” targeting to secretory lysosomes. However, we can not rule out that sTNFR1-tm-Y-egfp was trafficking a “direct” route too. In any case, sTNFR1-tm-Y-egfp might be carried from TGN to the plasma membrane by transport vesicles. However, at the plasma membrane the YXXΦ motif might still facilitate endocytosis supported by an AP-2 dependent pathway [82]. LAMPs can be targeted to lysosomes through an indirect pathway over the plasma membrane where they are internalized and delivered to the endocytic pathway by an AP-2 complex [85]. Furthermore, internalized LAMPs are recognized by AP-3 complexes and

delivered to lysosomes [86]. The sorting YXXΦ motif that is hidden in sTNFR1-tm-Y-egfp might not be able to mediate efficient AP-3 binding at TGN leading to sTNFR1-tm-Y-egfp trafficking to the plasma membrane, at least in part. At the plasma membrane, sTNFR1-tm-Y-egfp internalization might occur because the hidden YXXΦ motif or another motif could still function as an internalization signal facilitated by AP-2. Proteolysis of sTNFR1-tm-Y-egfp might occur in endosomes and result in sTNFR1-tm-Y generation after egfp release. The YXXΦ motif would now be available for AP-3 binding and delivery to secretory lysosomes. However, it has not yet been possible to study in detail the fate of sTNFR1-tm-Y-egfp after its internalization.

Secretory lysosome targeting of sTNFR1-tm-Y was verified by results from subcellular fractionation, immunoelectron microscopy and immunofluorescence microscopy. Both sTNFR1-tm-Y and sTNFR1 were observed in the densest fractions containing secretory lysosomes. Transmembrane sTNFR1-tm-Y might remain in the limiting membrane of secretory lysosomes or be carried into internal vesicles formed by inward budding. Partial proteolysis of sTNFR1-tm-Y might therefore occur both at the limiting membrane and in internal vesicles resulting in sTNFR1 release to the matrix (Fig.5.). In support of this, both sTNFR1-tm-Y and sTNFR1 were detected in secretory lysosomes by subcellular fractionation, suggesting that generation of sTNFR1 from sTNFR1-tm-Y might occur in secretory lysosomes. A TNFR1 signal was detected both on and inside the limiting membrane by immunoelectron microscopy. The sTNFR1 might even be generated in late endosomes and become sorted to secretory lysosomes by the fusion between late endosomes and secretory lysosomes [87]. Secretory lysosomes have a proteolytic environment, but sTNFR1-tm-Y and/or sTNFR1 were found to be as

stable as endogenous granule protein (paper I). Thus sTNFR1 accumulated in secretory lysosomes at the same efficiency as endogenous protein. The sTNFR1 might be protected similar to endogenous proteins either in dense cores or by a polyanionic proteoglycan matrix [88].

Achieving regulated secretion in cell lines

Calcium-mediated regulated secretion is characteristic of secretory lysosomes (Fig.5). A calcium signal can be triggered either by chemicals (e.g. a calcium-ionophore) or by a physiological stimulus (e.g. crosslinking of IgE receptor). Calcium-ionophore induced sTNFR1 secretion from RBL-2H3 cells expressing sTNFR1-tm-Y. Degranulation was verified by the secretion of an endogenous granule protein – RMCP-II. Crosslinking of IgE receptor also induced secretion of sTNFR1 from sTNFR1-tm-Y expressing RBL-2H3 cells. Collectively, our data suggested that secretory lysosome targeted sTNFR1-tm-Y was released by regulated secretion.

PMA-activation of protein kinase C does not induce regulated secretion [89]. In support of this, PMA induced neither sTNFR1 nor endogenous granule protein secretion in RBL-2H3 cells. However, PMA gave rise to ectodomain shedding of sTNFR1. In this case, results from subcellular fractionation after PMA activation showed that sTNFR1 was most likely released from non-secretory lysosome sites. In conclusion, results from subcellular fractionation after incubation with calcium-ionophore showed sTNFR1 to be released mostly from secretory lysosomes consistent with regulated calcium-induced secretion.

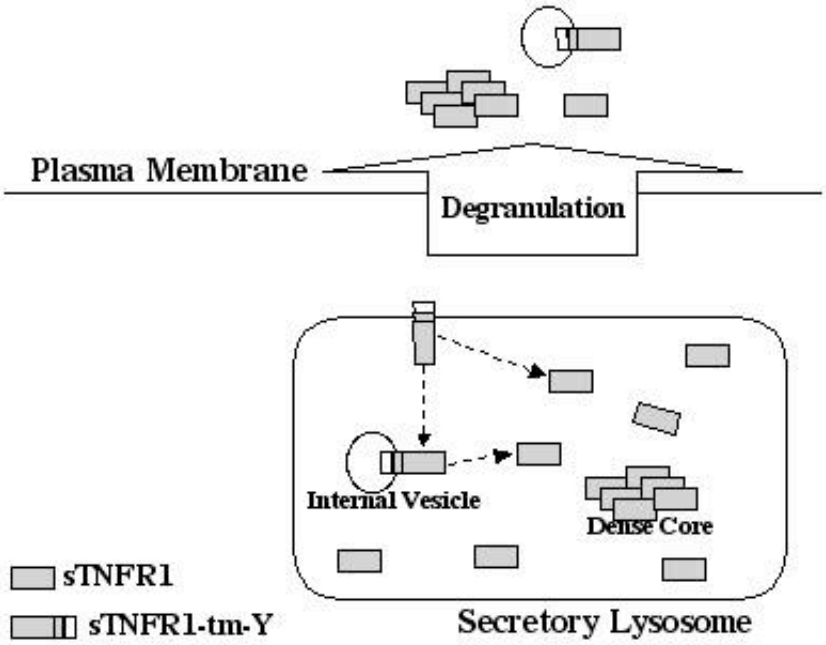

Fig.5. Release of sTNFR1 from sTNFR1-tm-Y. The sTNFR1-tm-Y might remain in the limiting membrane of secretory lysosomes or be carried into internal vesicles formed by inward budding. Partial proteolysis of sTNFR1-tm-Y can occur both at the limiting membrane and in internal vesicles resulting in sTNFR1 release to the matrix. The sTNFR1 could be protected by becoming part of dense cores. During degranulation, sTNFR1 and other secretory lysosome content will be released to the extracellular environment.

Achieving expression and granule targeting in normal hematopoietic progenitor cells

sTNFR1-tm-Y was expressed in normal murine hematopoietic cells, which were induced into granulopoietic differentiation by cytokines. At an early stage of differentiation the culture was dominated by precursor

cells. Later, the culture was dominated by granulocytes and macrophage-like cells.

sTNFR1-tm-Y was co-transduced with the egfp gene in a retroviral vector. EGFP expression was a measure of transduction efficiency. Flow cytometry results showed that about 50% cells expressed EGFP indicating a transduction efficiency of 50%. The EGFP intensity of the transduced cells was constant during terminal differentiation consistent with a long half-life of the EGFP protein (Paper III).

The sTNFR1-tm-Y biosynthesis was high at early granulopoietic differentiation and decreased at maturation. This is consistent with an overall decrease of protein synthesis towards cell maturation [12]. No secretion of newly synthesized sTNFR1-tm-Y and/or sTNFR1 was observed. This suggests efficient retention of newly synthesized sTNFR1-tm-Y. The steady level of sTNFR1-tm-Y/sTNFR1 was highest at early differentiation with a gradual decrease along cell maturation. This suggests that sTNFR1-tm-Y/sTNFR1 synthesized in immature granulocytes persisted to some extent into mature granulocytes. The decrease might due to protein degradation or dilution due to cell divisions (Paper III).

Indeed, sTNFR1-tm-Y was found be targeted to primary/azurophil granules during differentiation (Fig.6.). This was verified by immunoelectron microscopy of a colocalization between sTNFR1-tm-Y and the primary granule marker MPO (Paper III). Targeting was supposed to be facilitated by the sorting signal incorporated in sTNFR1-tm-Y. Possible targeting to secondary granules was suggested by a partial

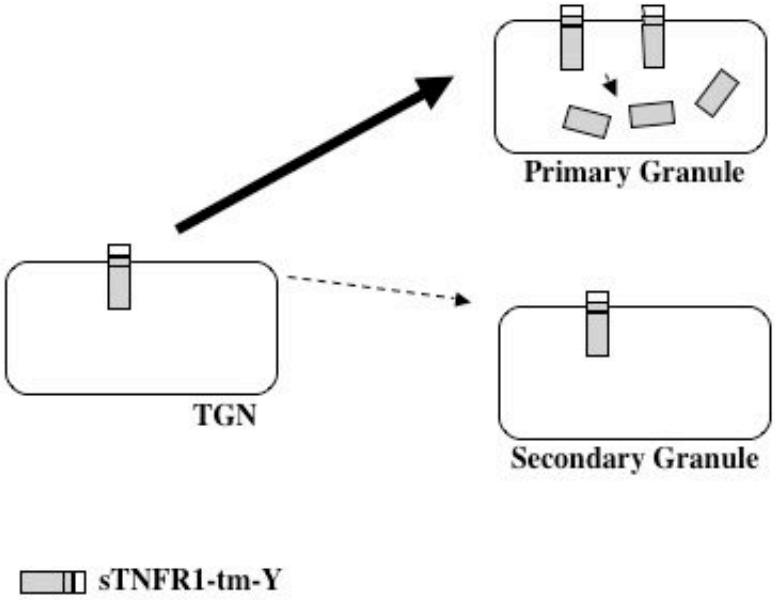

Fig.6. Targeting of sTNFR1-tm-Y to primary and possibly to secondary granules. sTNFR1-tm-Y expressed in hematopoietic progenitor cells was targeted to primary granules during granulopoietic differentiation and processed to sTNFR1. Furthermore, slight targeting of sTNFR1-tm-Y into secondary granules was suggested.

colocalization between sTNFR1-tm-Y and NGAL, a marker of secondary granule. This results was however uncertain since the sorting signal (Y) was designed for lysosome-like organelles. It is possible that there is an overlap between primary and secondary granule targeting when the latter granules begin to form. That is to say that earlier formed secondary granules retain an ability to respond to Y-mediated targeting. Otherwise, targeting mechanisms for secondary granules are unknown.

Proteins stored in secretory lysosomes should be transferred to phagosomes and extracellular environment by regulated secretion [90]. The finding of regulated secretion of sTNFR1-tm-Y/sTNFR1 into phagosomes was therefore important. The results showed that sTNFR1-tm-Y expressed in normal progenitor cells should be targeted to secretory lysosomes and released by regulated secretion.

General conclusions

Our results have shown that it is possible to target sTNFR1 for storage in secretory lysosomes of hematopoietic cell and induce secretion by degranulation. Importantly, it was possible to express sTNFR1 in normal hematopoietic progenitor cells and achieve sorting to secretory lysosomes and perhaps other organelles during granulopoietic differentiation. Taking advantage of a sorting signal should be generally applicable for transmembrane protein targeting to secretory lysosomes of hematopoietic precursors cells during granule biogenesis. A local deposition of cytokines and soluble cytokine receptors in the inflammation process should strengthen specific effects and diminish systemic effects. Therefore, our results support a general concept that hematopoietic granules, the normal storage site of antimicrobial and other agents with a role in innate immunity, might be used as vehicles for the deposition of therapeutically active agents, such as sTNFR1, at sites of inflammation and malignancy during degranulation. This should be possible to apply *in vivo* after *ex vivo* gene transfer in progenitor cells followed by cell infusion.

Summary

The goal of this research is to use hematopoietic cells as vehicles for targeting transgenic immunomodulators to the inflammatory sites. The

work presented was focused on the anti-inflammatory soluble TNF receptor. A transmembrane soluble TNF receptor (sTNFR1) construct was expressed in hematopoietic cell lines. ER export was facilitated by addition of a transmembrane (tm) sequence, and constitutive secretion was overcome by incorporating a tyrosine-base cytosolic sorting signal (Y) taken from CD63. This signal directed sTNFR1-tm-Y from the trans-Golgi network (TGN) to secretory lysosomes, followed by generation of membrane-free soluble sTNFR1. The secretion of sTNFR1 could be triggered by a calcium-ionophore or by crosslinking of the IgE receptors on mast cells leading to degranulation. In contrast, sTNFR1-tm-Y-egfp in which the sorting signal (Y) was hidden by egfp was, at least in part, transported from TGN to the plasma membrane. This was followed by endocytosis and possible secretory lysosome targeting. PKC-activation with phorbol ester induced ectodomain shedding of sTNFR1 from sTNFR1-tm-Y-egfp. In addition to cell lines, the soluble TNF receptor construct sTNFR1-tm-Y was also successfully expressed in normal murine hematopoietic progenitor cells. During granulopoietic differentiation sTNFR1-tm-Y targeting was achieved to primary granules followed by proteolytic generation of sTNFR1. Finally, our work has shown that an immunomodulatory agent such as soluble TNF receptor can be synthesized with a native conformation, be targeted to a storage compartment, and become secreted upon stimulation.

6. Future perspectives

This work is assumed be extended by *ex vivo* transfer of the gene for sTNFR1-tm-Y in hematopoietic progenitor cells followed by *in vivo*

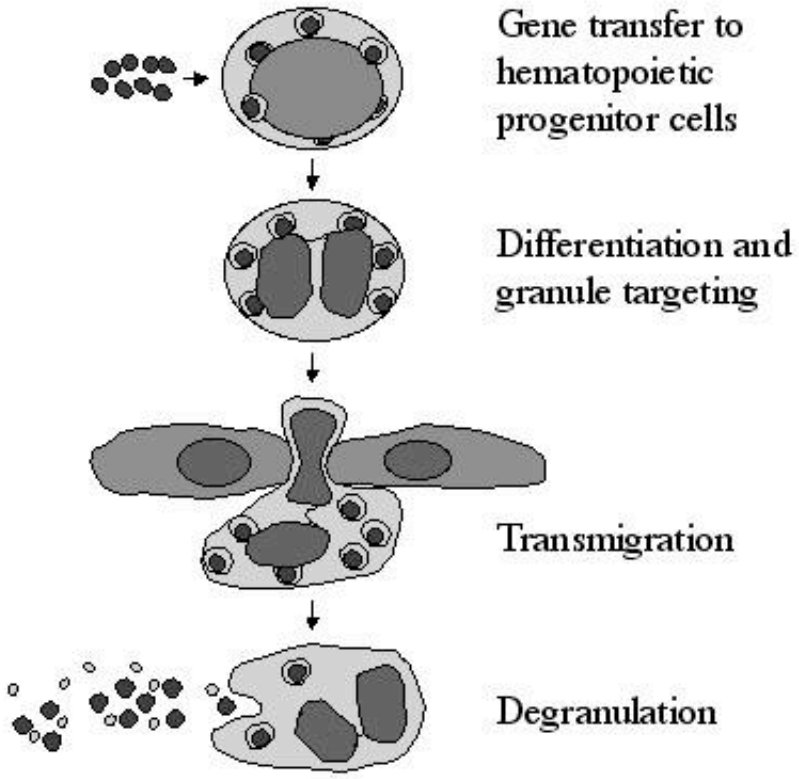

Fig.7. Model of targeting proteins to inflamed and malignant sites with secretory lysosomes of hematopoietic cells as vehicles. Protein with anti-inflammatory potential such as sTNFR modified with a secretory lysosome targeting signal is genetically transferred to hematopoietic progenitor cells. During myeloid differentiation, transgenic protein is targeted to granules for storage. Activated hematopoietic cells migrate to the site of inflammation and degranulate with release of effector protein.

experiments. To begin with, these cells will be used for cell therapy in mice with inflammatory disorders such as rheumatoid arthritis (Fig. 7). Hematopoietic progenitor cells will be retrovirally transduced *ex vivo* with the TNFR1-tm-Y gene, and transplanted to mice with antigen-

induced arthritis or other inflammatory disorder. Murine hematopoiesis will be reconstructed with hematopoietic progenitor cells that have an anti-inflammatory potential. Consonant with the findings in this thesis, the immunomodulatory protein sTNFR1-tm-Y is assumed be targeted to secretory lysosomes of hematopoietic inflammatory effector cells during their maturation. These effetor cells should become attracted to the site of inflammation e.g. joint synovial and release soluble TNF receptor. Local inhibition of TNF-α might be achieved (Fig.7). Besides soluble TNF receptor, soluble IL-1 receptor is a candidate for this type of potential gene therapy [91, 92]. A chimeric protein of soluble IL-1 receptor and soluble TNF receptor can also be envisaged for targeting to secretory lysosomes.

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