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Isabelle SoRa NAEGELEN
Born on 16th December 1984 in Seoul (South Korea)

REGULATION OF NEUTROPHIL CYTOKINE RELEASE AND DEGRANULATION DURING INFLAMMATION: ROLE OF SNARE FUSION PROTEINS

Dissertation Defense Committee

Prof. Dr. Paul HEUSCHLING, Chairman
Université du Luxembourg, Life Sciences Research Unit

Prof. Dr. Nicolas DEMAUREX, Vice Chairman
Université de Genève, Faculté de Médecine

Prof. Dr. Eric J. TSCHIRHART, Dissertation Supervisor
Université du Luxembourg, Life Sciences Research Unit

Prof. Dr. Florence NIEDERGANG
Université Paris Descartes, INSERM, Institut Cochin

Prof. Dr. Marie-Hélène PACLET
Université Joseph Fourier, CHU Grenoble, GREPI

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Abstract

Neutrophil granulocytes are the first effector cells to be recruited to sites of infection. They rapidly release granule proteins and pro-inflammatory cytokines to efficiently kill intruding pathogens and recruit other immune cells. In exocytotic events, specific interactions of so-called soluble *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins lead to the formation of complexes in order to mediate membrane fusion. Because of the excessive release of neutrophil-derived mediators leading to the development of chronic inflammatory disorders, we aimed in the present scientific work to investigate in more details the **regulatory processes of mediator release in neutrophils during inflammation, with emphasis on SNARE proteins**. The main objectives were *i)* to characterize the release of pro-inflammatory mediators, *ii)* to profile SNARE expression, *iii)* to determine the functional role of SNAREs and SNARE complexes in cytokine release and degranulation, and *iv)* to identify the intracellular localization of SNAREs.

To characterize the pro-inflammatory response in neutrophils in regard to exocytosis, extensive kinetic studies were performed in a first step on LPS-stimulated primary neutrophils. A novel linear fitting approach was created to correlate the relationship between granule proteins and cytokines secreted to the inflammatory site.
In a second step, SNARE expression levels were determined by whole-transcript analysis and the similar profiles in primary neutrophils as well as DMSO-differentiated HL-60 cells (dHL-60 cells), a neutrophil cell model, were underlined. Using an RNAi strategy, the SNARE syntaxin 3 (STX3) was identified as an essential actor in the release of the cytokines IL-1α, IL-1β, IL-12b, and CCL4. It was also involved in MMP-9 exocytosis from gelatinase granules where it could partly be localized. The SNARE SNAP29, which shares common localization with STX3, functionally affects the release of IL-12b, CCL2 and IL-8 as well as MMP-9, and represents a potential candidate to form cognate complexes with STX3. The knockdown of VAMP3, another SNARE candidate, showed deregulated secretion of IL-12b, CCL4, IL-8 as well as MMP-9. However, VAMP3 was located at the plasma membrane and was thus excluded as being part of the STX3-SNAP29 complex.

Our findings provide first evidence that SNARE fusion proteins are involved in the release of IL-12b, IL-1α, IL-1β, CCL4, IL-8, and CCL2 in a neutrophil-like cell model. The impact of SNAREs on gelatinase degranulation led us to hypothesize that cytokines might be packaged in these granules before subsequent exocytosis.

List of Abbreviations

Akt	protein kinase B
AP-1	activator protein-1
ATP	adenosine triphosphate
C5a	complement component 5a
CCL2	MCP-1
CCL3	MIP1α
CCL4	MIP1β
CCL5	RANTES
CD11b	integrin αM chain
CD13	aminopeptidase N
CD14	co-receptor to TLR4
CD15	3-fucosyl-N-acetyl-lactosamine
CD16	FcγRIII receptor
CD18	integrin β2
CD35	CR1, complement receptor 1
CD45	leukocyte tyrosine kinase
CD63	granulophysin
CD66b	CEACAM8, NCA-95, CD67
C/EBPβ	CCAAT-enhancer-binding protein β
CFTR	cystic fibrosis transmembrane conductance regulator
CK	casein kinase
COP	coat protein
CR3	complement receptor 3, CD11b/CD18
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
ELR	glutamic acid-leucine-arginine
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ERK	extracellular signal-regulated kinase
Fc	fragment crystallizable
fMLF	formyl-methionyl-leucyl phenylalanine
GCSF	granulocyte colony stimulating factor
GDP	guanine diphosphate
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione S-transferase
GTP	guanine triphosphate
GTPase	guanine triphosphatase
H_2O_2	hydrogen peroxide
HL-60	human promyelocytic leukemia cells
HOCl	hypochlorous acid

ICAM	intercellular adhesion molecule
IFNγ	interferon γ
IκB	inhibitor of κB
IKK	IκB kinase
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factor
JNK	c-jun N-terminal kinase
LBP	LPS-binding protein
LDL	low-density lipoprotein
LPS	bacterial lipopolysaccharide from *E. coli*
LTF	lactoferrin
Mac-1	macrophage receptor 1
Mal	MyD88-adapter like
MAPK	mitogen-activated protein kinase
MD-2	myeloid differentiation factor-2
MMP-9	matrix metalloproteinase-9, gelatinase B
MPO	myeloperoxidase
MyD88	myeloid differentiation primary response gene 88
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGAL	neutrophil gelatinase-associated lipocalin
NSF	*N*-ethylmaleimide-sensitive fusion protein
NK	natural killer
NOX2	NADPH oxidase
O_2^-	superoxide
PAF	platelet-activating factor
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PMN	polymorphonuclear leukocytes
PRR	pattern recognition receptors
Rab	ras genes from rat brain
RIG	retinoic acid-inducible gene
RIP	receptor interacting protein
ROS	reactive oxygen species
RP	reserve pool
RRP	readily releasable pool
SM	Sec1/Munc18

SNAK	STX-associated kinase
SNAP	soluble NSF attachment protein
SNAP23	23-kDa synaptosome-associated protein
SNARE	soluble *N*-ethylmaleimide sensitive factor attachment protein receptor
SRP	slowly releasable pool
STED	stimulated emission depletion
STX	syntaxin
Syk	spleen tyrosine kinase
SYT	synaptotagmin
TAB	TAK-binding protein
TACE	TNF-converting enzyme
TAT	transactivator of transcription
TAK	transforming growth factor β-activated kinase
TGFβ1	transforming growth factor β
T_H	T helper lymphocyte
TIR	toll/interleukin-1 receptor
TLR4	toll-like receptor 4
TN	tetranectin
TNFα	tumor necrosis factor α
TRAF	TNF receptor associated factor
t-SNARE	target SNARE
UPP	unprimed pool
VAMP	vesicle-associated membrane protein, synaptobrevin
VCAM	vascular cell adhesion protein
v-SNARE	vesicle SNARE

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Introduction

I Generalities

1. Inflammation and neutrophils

Acute inflammation (latin *inflammare* = to set on fire) defines the necessary processes by which any tissue of the body responds rapidly to a local injury in order to protect from aggression or disease. Harmful stimuli that lead to tissue injury can be of very different nature: *i)* physical (mechanical trauma, thermal injury, radiation), *ii)* chemical (acids, bases, heavy metals, toxins), *iii)* infections (bacterial, fungal or viral), or *iv)* immunological reactions (hypersensitivity, allergic or autoimmune) (Merched, 2012).

At the site of tissue injury, inflammatory processes are set in motion to neutralize the initiating stimulus, remove and repair damaged tissue (Medzhitov and Janeway, 1997). Inflammation can be characterized by four major cardinal signs that have been described by the Roman physician Cornelius Celsus (1st century A.D.): *rubor et tumor cum calore et dolore*. Redness (lat. *rubor*) and heat (lat. *calor*) of the inflamed tissue are observed due to an increased blood flow and cell accumulation in the vessel leading to localized hyperaemia. Swelling (lat. *tumor*) of the inflamed tissue is elicited by the vasodilatation of the blood vessels that increases vascular permeability thereby promoting leakage of humoral components (*i.e.* plasma-containing antibodies, complement components) (Cohnheim, 1873). Pain (lat. *dolor*) results from initial tissue damage or the release of inflammatory mediators that raises the sensitivity of pain receptors and might induce hypersensitivity (hyperalgesia). A fifth cardinal sign, proposed by the Greek Galen of Pergamon (2nd century A.D.), consists of functional disturbance (lat. *functio laesa*) that refers to loss of mobility in the joint, induced by swelling and pain of the inflamed tissue.

Tissue contamination with pathogens results in the recruitment of high numbers of inflammatory cells to the infection site, including resident macrophages, which recognize pathogens and are able to release pro-inflammatory mediators with distinct properties. For example, the pyrogenic cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNFα) mediate the expression of cell adhesion molecules by endothelial cells (Pober and Cotran, 1990) whereas interleukin-8 (IL-8) massively recruits leukocytes to the inflammatory site (Turk, 1994). Among these cells, neutrophils represent 40 to 70% of all peripheral blood leukocytes (Nathan, 2006) and are the most prominent leukocytes recruited rapidly towards sites of acute infection (Williams and Hellewell, 1992). Also known as polymorphonuclear (PMN) leukocytes, they are involved in the early recognition of invading pathogens (viruses, fungi, bacteria, protozoa) and represent the first line of innate immune defense (Nathan, 2006).

II Origin and development of neutrophils

In order to ensure immediate mobilization for the acute response to pathogen exposure, neutrophils circulate as dormant/resting cells in the blood stream or are present in marginating pools in the tissues (Cascao *et al.*, 2009; Borregaard, 2010). Under steady-state conditions, neutrophils have a short half-life of only 6 to 10 hours in the blood, and a survival rate of one to two days in the marginating tissue pools due to their rapid clearance by constitutive apoptosis (Mollinedo, 2003; Tsukahara *et al.*, 2003). Therefore, neutrophils are constantly generated in the bone marrow from multi-potent hematopoietic stem cells and progenitor cells. Each day, the production of more than 10^{11} neutrophils is regulated by colony-stimulating factors, such as G-CSF and GM-CSF (Anderlini *et al.*, 1996). In case of acute inflammation, this production rate is increased to more than 10^{12} neutrophils *per* day (Williams and Hellewell, 1992).

1. Granulopoiesis

The stages of neutrophil maturation during granulopoiesis are associated to morphological alterations and notably to the sequential formation of differential granule subsets (Cascao *et al.*, 2009). Several morphological stages of cell maturation can be distinguished: myeloblast, promyelocyte, myelocyte, metamyelocyte, non-segmented band cell and segmented neutrophils, which are terminally differentiated and display a multi-lobed chromatin-dense nucleus (*Figure 1*).

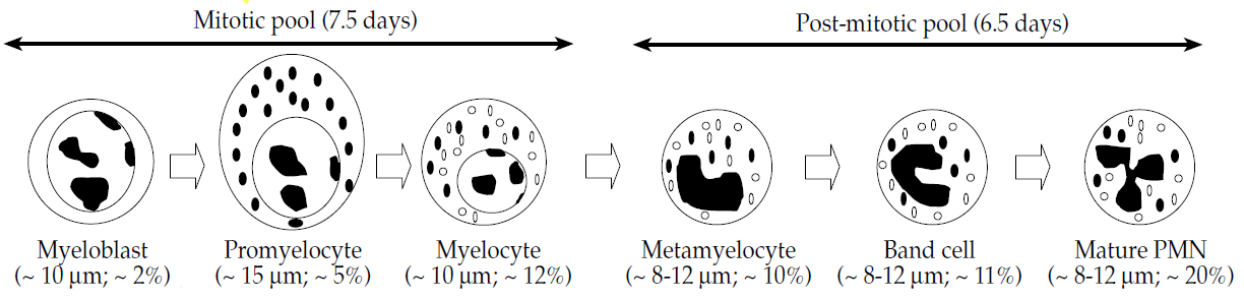

Figure 1 *Morphological stages of neutrophil maturation in the bone marrow (Mollinedo, 2003; modified): Myeloblast, promyelocyte, myelocyte, metamyelocytes, band cell, and mature polymorphonuclear (PMN) leukocyte. Approximate cell size and relative percentage of each maturation stage relative to the total population in the bone marrow are given. Neutrophils, derived from hematopoietic stem cells in the bone marrow, spend about 7.5 days in the mitotic pool, and 6.5 days in the post-mitotic pool.*

The initiation of granule synthesis is a hallmark of the transition between myeloblast to promyelocyte. In the early promyelocytic stage, immature transport vesicles arise from the Golgi apparatus and fuse together (Hartmann *et al.*, 1995). Both myeloblasts and promyelocytes stain positive for peroxidase meaning that azurophil (primary) granules are formed at these stages (Borregaard *et al.*, 1995b). The synthesis of specific (secondary) granules, originally identified as peroxidase-negative granules, is initiated at the stages myelocyte and metamyelocyte (Bainton *et al.*, 1971). Gelatinase (tertiary) granules are formed in band cells and segmented mature neutrophils (Kjeldsen *et al.*, 1992; Borregaard, 1997; Faurschou and Borregaard, 2003). In the very late stages of neutrophil development, secretory vesicles (quaternary granules) are formed by endocytosis (Karlsson and Dahlgren, 2002) and contain plasma proteins, such as albumin (Faurschou and Borregaard, 2003).

In a time frame of about two weeks, over 300 antimicrobial and proteolytic proteins are distributed in a hierarchical order within four types of neutrophil granules. According to the *targeting-by-timing hypothesis*, the proteins synthesized during neutrophil development are attributed to the different granule types in a strictly time-dependent manner (Le Cabec *et al.*, 1996; Arnljots *et al.*, 1998). The resulting structural and functional heterogeneity of the granules derives from differential gene expression, highly regulated by the combination of myeloid transcription factors present at define stages of cell development (Egesten *et al.*, 1994; Borregaard *et al.*, 1995a; Borregaard, 1997). Representing up to 15% of the cell volume, neutrophil granules have traditionally been classified as peroxidase-positive and peroxidase-negative on the basis of their myeloperoxidase (MPO) content (Bainton and Farquhar, 1968). Later on, they were divided into three subsets: granules that contain lactoferrin (LTF) but no gelatinase (MMP-9) (15%), granules that contain LTF and MMP-9 (60%), and granules that contain MMP-9 but no LTF (25% of peroxidase-negative granules) (Arnljots *et al.*, 1998). Many granule proteins are not solely found in one single granule type, thereby showing that neutrophil granules rather represent a *continuum* than strictly separate granule types (Rorvig *et al.*, 2013).

Mature neutrophils are displaced from the bone marrow into the blood circulation as fully functionally active cells with a pool of granules rapidly mobilizable upon appropriate stimulation (Klingauf and Neher, 1997).

III Neutrophil recruitment

As a consequence of tissue injury, tissue-resident macrophages release pyrogenic cytokines, which act on the endothelial cell wall and also on the circulating neutrophils in the blood stream. On one side, endothelial cells are activated and upregulate the expression of selectin adhesion molecules. On the other side, circulating neutrophils experience “priming”, a mechanism by which resting cells undergo the initial shape change and polarization necessary for migration to the site of inflammation (Coffer and Koenderman, 1997). Neutrophils are recruited along a gradient of soluble factors, *i.e.* bacterial agonists (*e.g.* formyl-methionyl-leucyl phenylalanine (fMLF)) or inflammatory mediators (*e.g.* complement component 5a (C5a), platelet-activating factor (PAF), IL-8, or leukotriene B4), all leading to chemotaxis of the neutrophils towards the site of tissue injury (Muller *et al.*, 2002; Ley *et al.*, 2007; Scheiermann *et al.*, 2012).

During recruitment to the inflammatory site, neutrophils go through multiple steps involving *i)* rolling, *ii)* activation and adherence to endothelium, and *iii)* diapedesis through the vascular barrier. The rolling phase is mainly mediated by low-affinity interactions between L-selectin and sialyl-Lewis X on neutrophils, and P- and E-selectin on inflamed endothelial cells. The integrin LFA-1 is the principal adhesion molecule on neutrophils that mediates transition from rolling to adhesion on the endothelial cell surface (Phillipson *et al.*, 2006). Firm adhesion of the leukocyte to the vessel wall is mediated *via* the β_2-integrin family, and intercellular adhesion molecule (ICAM) 1 and vascular cell adhesion molecule (VCAM) 1 expressed by endothelial cells (Phillipson *et al.*, 2006; Cascao *et al.*, 2009). To exit the vascular system, neutrophils cross the inflamed vascular barrier with increased permeability *via* diapedesis (Beutler, 2004). This can occur through paracellular transmigration at endothelial junctions or transcytosis at non-junctional locations.

IV Neutrophil-mediated defense during inflammation

1. Innate defense mechanisms

Once arrived at the inflammatory site, neutrophils are exposed to higher concentrations of the soluble agonists and subsequently undergo different steps of activation. To protect hosts from invading pathogens, activated neutrophils ensure an efficient killing of a broad range of infecting microorganisms by concomitantly activated processes originally classified into oxidative and enzymatic (non-oxidative) processes (Smith, 1994) (*Figure 2*).

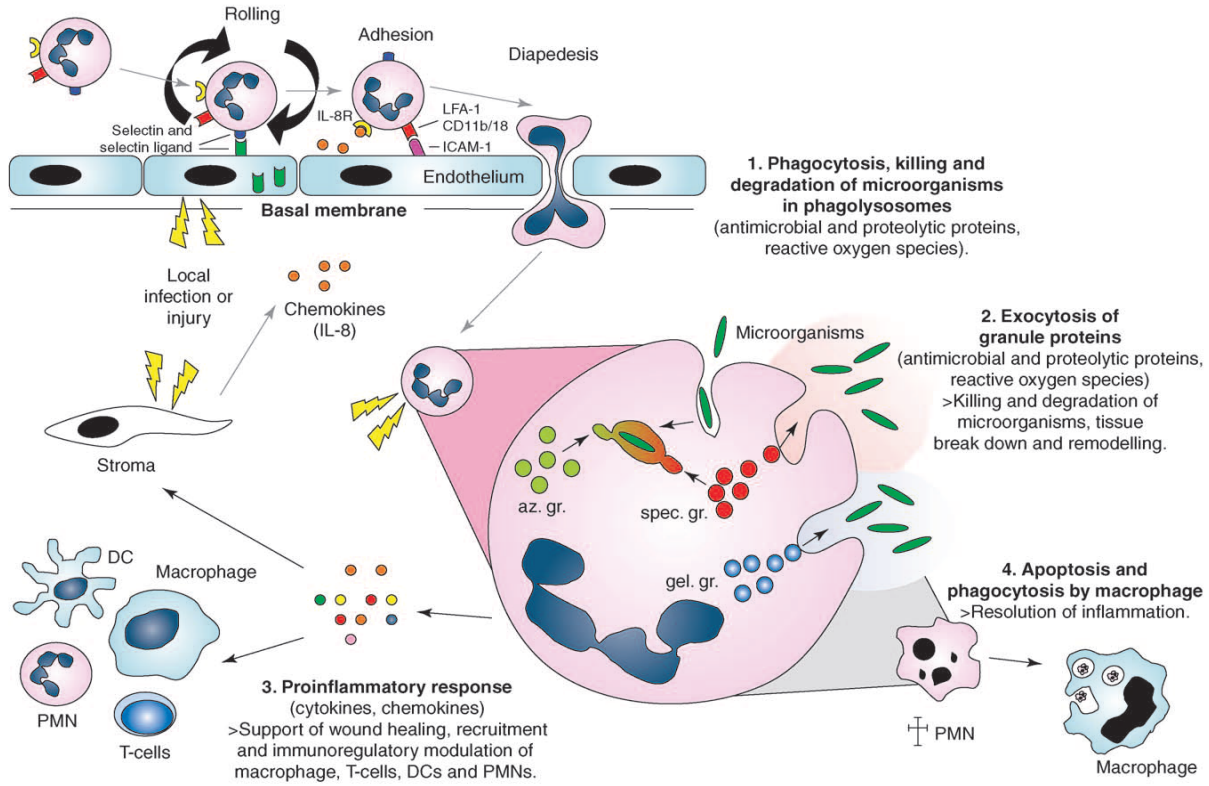

Figure 2 *Innate[1] immune defense mechanisms mediated by neutrophils. Upon tissue injury, neutrophils are recruited to the inflammatory site via series of interactions with the endothelial cell wall along a gradient of chemoattractants. Once arrived at the "combat field", neutrophils become activated and execute different functions in order to protect the host against pathogens (1-3). Eventually, neutrophils commit apoptosis and are phagocytosed by macrophages for subsequent resolution of inflammation (4). Abbreviations: az. gr., azurophil granules; DC, dendritic cells; gel. gr., gelatinase granules; ICAM-1, intercellular adhesion molecule 1; LFA-1, leukocyte function-associated molecule 1; PMN, polymorphonuclear leukocyte; spec. gr., specific granules (Theilgaard-Monch et al., 2006).*

[1] Elie Metchnikoff (Nobel Prize in Physiology or Medicine, 1908) discovered that inflammation is a protective process of immunity, a host-mediated defense mechanism. He proved the existence of phagocytes while he was studying starfish larvae.

1.1 Phagocytosis

Neutrophils are known to be professional phagocytes that recognize pathogens, immune complexes and other particles, either directly or indirectly *via* soluble molecules. Direct recognition occurs mainly through pattern receptors (*e.g.* CD14, toll-like receptors) while indirect recognition can be mediated by Fc (fragment crystallizable) or complement receptors binding to IgG coated- or iC3b-opsonized particles (Underhill and Ozinsky, 2002; Jutras and Desjardins, 2005). By binding to the pathogen *via* surface receptors, actin filaments are polymerized in neutrophils. This leads to changes in the cytoskeleton structure and enables neutrophils to entirely engulf pathogens, as well as apoptotic cells (Greenberg and Grinstein, 2002). Upon formation of phagosomes, fusion occurs with lysosomes that lead to the acidification of the maturating phagolysosomes. Then, the intracellular fusion of granules with phagolysosomes leads to the release of antimicrobial enzymes upon which encapsulated pathogens are proteolytically digested (Pitt *et al.*, 1992; Desjardins *et al.*, 1997; Jahraus *et al.*, 1998; Greenberg and Grinstein, 2002; Jutras and Desjardins, 2005). The lowering of the pH to 4-5 inside the phagolysosome allows enzymes such as cathepsins to be highly active.

1.2 Production of reactive oxygen species

The process of phagocytosis is directly associated to the generation of reactive oxygen species (ROS) by activation of the NADPH oxidase (NOX2) situated at the phagosomal membrane (Bass *et al.*, 1987; DeLeo *et al.*, 1998). Originally described by Rossi *et al.* in 1972, NOX2 is a multicomponent enzyme that consists of the membrane-bound proteins $gp91^{phox}$ (cytochrome b_{558}) and $p22^{phox}$, as well as the cytosolic subunits $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$ (Borregaard and Tauber, 1984) and the GTP-bound form of Rac (Bokoch and Diebold, 2002). Upon cell activation, phosphorylated cytosolic components are recruited to the membrane and Rac exchanges GDP for GTP, thus leading to the complex assembly of NOX2 (Segal *et al.*, 1980). NOX2 oxidizes NADPH and reduces O_2 to generate the superoxide radical ($O_2^{\bullet -}$), a substrate for the production of hydrogen peroxide (H_2O_2) inside the phagosome (Babior *et al.*, 1973).

H_2O_2 in combination with chloride ions (Cl^-) in the presence of MPO results in the formation of hypochlorous acid (HOCl). HOCl acts as a potent microbicial agent that ruptures the cell wall of ingested bacteria (Winterbourn *et al.*, 1985; Smith, 1994; Dale *et al.*, 2008; Klebanoff *et al.*, 2013). The transport of Cl^- into the phagosome is highly dependent on the recruitment of the cystic fibrosis transmembrane conductance regulator (CFTR) into the phagosomal membrane.

Moreover, fusion of azurophil granules with the phagosome provides the enzyme MPO, which in its turn catalyzes the reaction between H_2O_2 and Cl^-, and yields generation of HOCl.
NOX2 can also be situated at the plasma membrane and thus release ROS into the inflammatory tissue environment. By this mechanism, ROS act as essential signaling molecules to mediate amplification of the pro-inflammatory immune response (Finkel, 1998). However, excessively released ROS can also affect healthy surrounding tissue and thus, contribute to neutrophil-mediated tissue injury.

1.3 Degranulation

The ability of leukocytes to generate and secrete proteins that have destructive activity has been known since the 1860s (Segal, 2005; Wallach *et al.*, 2014). Besides the intracellular fusion of granules with the phagolysosome, mentioned above, neutrophils release the different types of granules also to the extracellular environment. Under pro-inflammatory conditions, granule contents are released in a reversed chronological order compared to their synthesis according to the *formed-first-released-last model* (Sengelov *et al.*, 1993; Borregaard, 1997; Pellme *et al.*, 2006). The rate and extent of secretion of each type of granules are remarkably different and vary depending on the activation stimulus (Kjeldsen *et al.*, 1994). While exocytosis of secretory vesicles can occur within minutes, the release of azurophil and specific granules requires longer time periods. In 1993, Sengeløv *et al.* have shown that different types of granules are mobilized to the plasma membrane by different Ca^{2+} requirements for secretion (Sengelov *et al.*, 1993). The induced increase in cytosolic Ca^{2+} levels, known as “calcium transient”, that induce half-maximal marker release (half maximal effective concentration, EC_{50}) of the granules are: 140 nM for secretory vesicles, 250 nM for gelatinase granules, 550 nM for specific granules, and 680 nM for azurophil granules. Remarkably, a higher threshold for exocytosis of gelatinase granules is needed than for secretory vesicles (Kjeldsen *et al.*, 1992; Kjeldsen *et al.*, 1993). Also, a higher threshold for specific degranulation is needed than for gelatinase degranulation, and azurophil granules can only be mobilized partially (Sengelov *et al.*, 1993; Nusse *et al.*, 1998; Faurschou *et al.*, 2002).

The different granule types are time-dependently involved in the different stages of neutrophil recruitment and mechanisms of bacterial killing. Secretory vesicles are the most easily mobilized granules (Kobayashi and Robinson, 1991) and are mobilized when neutrophils adhere to the activated endothelial wall (Sengelov *et al.*, 1993; Sengelov *et al.*, 1994; Sengelov *et al.*, 1995). Gelatinase granules facilitate the adhesion of neutrophils to the endothelial wall

and their migration through basement membranes (Mollinedo *et al.*, 1991; Kjeldsen *et al.*, 1993; Delclaux *et al.*, 1996). Specific and azurophil granules are necessary for the neutrophil to migrate through tissue (Sengelov *et al.*, 1995) and participate in the antimicrobial activities of neutrophils at the site of inflammation (*Figure 3*).

The extracellular release of granule proteins is not only limited to help the neutrophil in its own functions but have recently been discovered to modulate also the recruitment of other immune cells to the inflammatory site, notably monocytes (Soehnlein *et al.*, 2009).

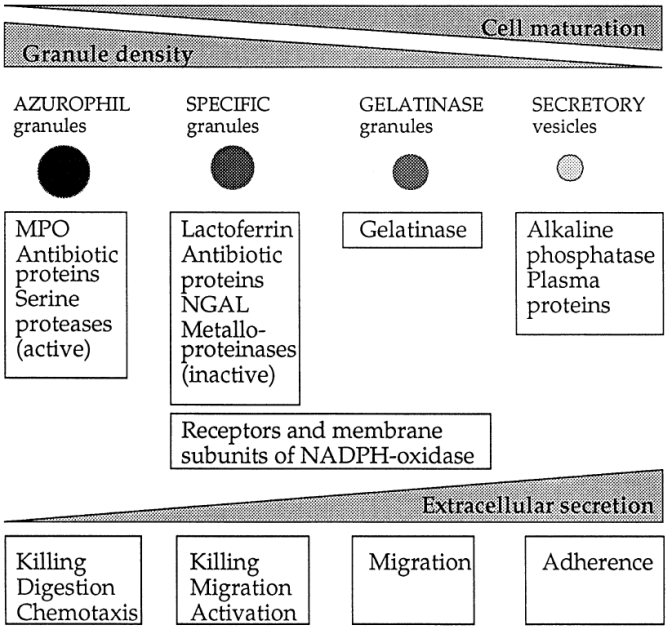

Figure 3 *Neutrophil granules (Gullberg et al., 1999). The four types of cytoplasmic granules display structural and functional heterogeneity. They differ in density, size and cargo protein content, as well as in the ease of mobilization to the extracellular milieu. Also, distinct functions are associated with the granule content.*

1.3.1 Downstream signaling to granule mobilization

Several kinases have been identified to be crucial for the exocytosis of neutrophil granules. First, ERK and p38 MAPK proteins are rapidly phosphorylated and activated upon cell stimulation (Grinstein and Furuya, 1992; Torres *et al.*, 1993; Thompson *et al.*, 1993; Krump *et al.*, 1997; Nick *et al.*, 1997; Meshki *et al.*, 2004). Second, the spleen tyrosine kinase (Syk) is activated (Yan *et al.*, 1997). Third, the three Src family members expressed in neutrophils, Hck, Fgr, and Lyn, are activated by fMLF receptor stimulation. Interestingly, different granule populations appear to be associated with different Src kinases. Following cell activation, Hck translocates to the azurophil granule population (Mohn *et al.*, 1995) whereas Fgr becomes associated with specific granules (Gutkind and Robbins, 1989). The selective recruitment of Src

kinases indicates that different signaling pathways exist in neutrophils that induce the release of each granule population (Lacy, 2006). Rho proteins represent the link to the actin cytoskeleton (Ridley, 2001) in the exocytosis for secretory vesicles, gelatinase granules, and specific granules (Jog *et al.*, 2007). Ral guanine triphosphatase (GTPase) controls specific granule release (Chen *et al.*, 2011), and the phosphatidylinositol 3-kinase (PI3K)-induced Rho GTPase Rac2 is essential in the regulation of azurophil degranulation (Abdel-Latif *et al.*, 2004; Mitchell *et al.*, 2008).

1.3.2 Neutrophil granule subsets

1.3.2.1 Secretory vesicles

Secretory vesicles mainly represent a pool of various membrane-associated receptors, such as CD13 (aminopeptidase N, Kanayama *et al.*, 1995), CD14 (glycoprotein), CD16 (FcγRIII receptor, Ravetch and Perussia, 1989), CD18 (β_2 integrin, Walzog *et al.*, 1994), complement receptor 1 (CR1, CD35, Sengelov *et al.*, 1994; Kumar *et al.*, 1997), CD45 (leukocyte tyrosine kinase) and fMLF receptor (Borregaard *et al.*, 1987). Therefore, the mobilization of secretory vesicles to the plasma membrane prepares the neutrophil for *outside-in* signaling and renders it highly responsive to its environment.

A characteristic protein carried by secretory vesicles is alkaline phosphatase**,** a phosphatidylinositol-linked membrane glycoprotein that is shown to inactivate bacterial lipopolysaccharide (Bentala *et al.*, 2002; Koyama *et al.*, 2002). Due to the presence of alkaline phosphatase in secretory vesicles, these granules are also known as “phosphasomes”. Plasma proteins such as albumin and tetranectin (Nielsen *et al.*, 1997) are also characteristic for secretory vesicles that are formed by endocytosis (Karlsson and Dahlgren, 2002).

1.3.2.2 Gelatinase granules

Gelatinase granules contain a glycoprotein member of the integrin family, CD11b that is the 185 kDa α-chain of the macrophage receptor 1 (Mac-1). The 95 kDa β-chain belongs to CD18 that interacts with ICAM and VCAM for the adhesion of the leukocyte to the vascular wall. The CD11b/CD18 complex (CR3, Gahmberg, 1997) resides in two different subcellular pools, similar to CR1 (Sengelov *et al.*, 1994). Gelatinase granules were originally detected by the presence of MMP-9 (Kjeldsen *et al.*, 1993; Kjeldsen *et al.*, 1994). MMP-9, also known as gelatinase B, is a zinc-containing endopeptidase, and member of the Metzincin family. Stored as a latent inactive protein, it is activated by oxidants and proteases (Witko-Sarsat and Descamps-Latscha, 1994). Its major role consists of modifying or degrading the extracellular matrix and basement

membranes but leads also to specific processing and release of cytokines, *i.e.* IL-1β, TNFα, IL-8 (Vaday *et al.*, 2001).

1.3.2.3 Specific granules

About 4600 specific granules *per* cell (Nusse and Lindau, 1988) represent the main reservoir of the catalytic subunit of NOX2, cytochrome b_{558} (Karlsson and Dahlgren, 2002). By fusion of specific granules with the forming phagosomes, cytochrome b_{558} is assembled with cytosolic subunits resulting in an active NOX2 and production of ROS in the phagolysosome (Borregaard and Cowland, 1997). Specific granules express CD66b (CEACAM8, NCA-95, CD67) (Ducker and Skubitz, 1992; Beauchemin *et al.*, 1999) as well as CD15 (3-fucosyl-N-acetyl-lactosamine, Nakayama *et al.*, 2001).

Characteristic proteins carried by specific granules are binding proteins, proteolytic enzymes, and serine proteases. Collagenase facilitates the neutrophil's migration steps by cleaving collagen into two distinct and specific peptide fragments. LTF and lipocalin-2 both interfere with the iron metabolism of bacteria by binding iron directly or analog proteins of bacteria (siderophores) (Caccavo *et al.*, 2002; Farnaud and Evans, 2003). While lysozyme leads to the destruction of the bacterial cell envelope components, cathelicidin (LL-37 peptide) (Sorensen *et al.*, 2001; Wang *et al.*, 2004) inhibits the bacterial growth at micromolar concentrations (Turner *et al.*, 1998).

1.3.2.4 Azurophil granules

Azurophil granules, also known as peroxidase-positive granules, have a mean diameter of 260 nm (Borregaard *et al.*, 1995a). Each human neutrophil contains about 1300 azurophil granules (Nusse and Lindau, 1988), that are called to be the cell-type specific secretory lysosomes representing both, a degradative and secretory compartment (Nusse and Lindau, 1988; Lollike *et al.*, 2002; Blott and Griffiths, 2002). In this type of granules, CD63 was first identified as granulophysin and is one of the major lysosomal membrane proteins and member of the tetraspanin superfamily (Horejsi and Vlcek, 1991; Kuijpers *et al.*, 1991; Niessen and Verhoeven, 1992; Cham *et al.*, 1994; Abdel-Latif *et al.*, 2004). CD63 signaling triggers a transient activation signal that requires extracellular Ca^{2+} and regulates adhesive activity and surface expression of Mac-1 (Kuijpers *et al.*, 1991; Berditchevski *et al.*, 1997).

The protein characteristic for this granule type is MPO (Arnljots *et al.*, 1998). Accounting for 5% of the dry weight of the neutrophil (Segal, 2005), MPO is a heme containing glycoprotein

(Klebanoff, 1967; Lubbert and Koeffler, 1988) and ligand for Mac-1 (Borregaard and Cowland, 1997).

Azurophil granules contain various proteolytic and saccharolytic enzymes (Smith, 1994), all of which 30 to 50% are defensins (Ganz, 1987). Defensins are cationic arginine-rich granule peptides that can elicit the destruction of cell envelope components of bacteria (Ganz, 2003). For example, human β-defensin-2 can aggregate to generate “channel-like” pores leading to disruption of membrane integrity and function, and ultimately causing the lysis of pathogens (Hoover *et al.*, 2000; Sahl *et al.*, 2005). Serine proteases such as elastase, cathepsin G and proteinase 3 are implicated in antimicrobial defense by degrading engulfed microorganisms inside the phagolysosomes of neutrophils (Belaaouaj *et al.*, 1998; Belaaouaj *et al.*, 2000; Reeves *et al.*, 2002; Weinrauch *et al.*, 2002; Segal, 2005), and the proteolytic modulation of cytokines (Wessels *et al.*, 2010).

1.4 Neutrophil extracellular traps

Over the last years, a new form of cell death different from apoptosis and necrosis has been discovered in neutrophils. Under the last action in a neutrophil’s life, so-called NETosis, neutrophil extracellular traps (NETs) with granule and cytosolic proteins are released by the cell in order to sequester bacteria (Brinkmann *et al.*, 2004). These NETs represent chromatin fibers that are decondensed in association with citrullination of the core histones by conversions of histone arginine to citrulline residues by peptidylarginine deiminase 4 (Neeli *et al.*, 2008; Wang *et al.*, 2009). The main components of NETs are the core histones H2A, H2B, H3, and H4, which together account for about 70% of the protein mass (Urban *et al.*, 2009). To enable granule and cytoplasmic proteins to mix with the chromatin, pores are formed in the nuclear membrane and secretory vesicle walls. About 24 neutrophil proteins have been associated with NETs (Urban *et al.*, 2009). Among them were S100A8/A9 (MRP8/MRP14) proteins representing 40% of neutrophil cytosolic proteins and are thus the most prominent NET components (Murthy *et al.*, 1993). Also cationic DNA-binding proteins (histones, defensins, elastase, proteinase 3, heparin-binding protein, cathepsin G, lactoferrin, MPO) were found to be associated with NETs. Elastase released from azurophil granules degrades histones and synergizes with MPO to drive chromatin decondensation essential for NET formation (Papayannopoulos *et al.*, 2010). The fact that granule proteins are released with the NETs might hinder proteases from diffusing and inducing damage to healthy tissue adjacent to the inflammatory environment. In this sense, NETs could serve as physical barrier that limits the spread of the pathogens.

2. Neutrophils as link between innate and adaptive immunity

Traditionally, neutrophils have solely been considered as storage for preformed antimicrobial peptides and proteolytic enzymes for direct killing of pathogens. For many decades, monocytes and macrophages have been assumed to be the most important sources of cytokines that are classified into interleukins, colony-stimulating factors, interferons, tumor necrosis factors, growth factors, and chemokines.

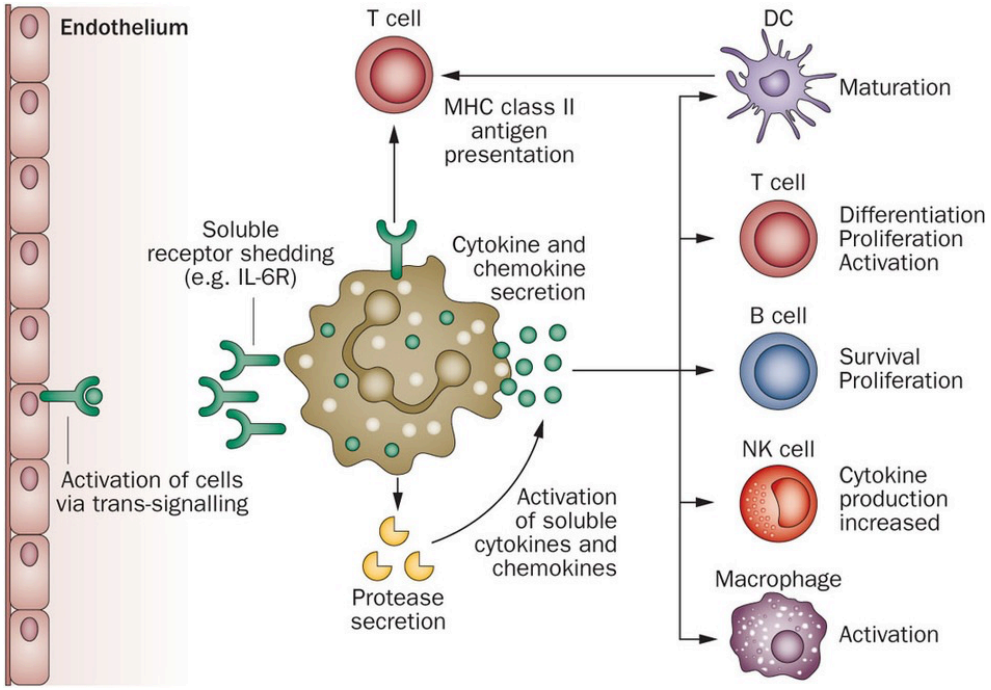

Figure 4 *Activated neutrophils communicate with various innate and adaptive immune cells through cytokine secretion (Wright et al., 2014). Abbreviations: MHC, major histocompatibility complex; DC, dendritic cell; NK natural killer.*

However, in the last decade, *de novo* expression of proteins has been detected in neutrophils (Theilgaard-Monch *et al.*, 2006). Often underestimated, neutrophils are now also recognized as an important source of cytokines[2] since they are the first effector cells vastly recruited to the inflammatory site that are able to communicate effectively with other immune cells (Detmers *et al.*, 1990; Xing *et al.*, 1994; Scapini *et al.*, 2000; Denkers *et al.*, 2003). The inducible production of cytokines by neutrophils is usually preceded by, and largely dependent on a previous accumulation of the corresponding mRNA transcripts (Bazzoni *et al.*, 1991). Although

[2] At the beginning of the 20th century, Henry Dale was the first to describe that cells are able to secrete soluble messengers that drive and coordinate inflammation *via* activation of cell surface receptors. At the inflammatory site, multiple cellular sources are releasing mediators in order to communicate with multiple targets and exerting multiple functions (Gabay and Kushner, 1999).

neutrophils have low transcriptional activity, data have pointed out that cytokine expression can be regulated at the level of mRNA stability in addition to a post-transcriptional mechanism of regulation. The secretion of these soluble messengers that drive and coordinate the onset and resolution of inflammatory processes in a time-regulated and sequential manner provide the neutrophils with the essential ability to modulate and amplify innate as well as adaptive[3] immune responses (Nathan, 2006; Soehnlein *et al.*, 2008) (*Figure 4*).

2.1 Neutrophil pro-inflammatory cytokine response

The capacity of neutrophils to produce cytokines has primarily been limited to IL-8 due to its constitutive release (Altstaedt *et al.*, 1996). Upon treatment with diverse agonists, also other pro-inflammatory cytokines have been detected in neutrophil supernatants. In previous reports, neutrophils have been described to produce pro-inflammatory cytokines, *i.e.* CXC chemokines (*e.g.* CXCL1-6), CC chemokines (*e.g.* CCL2, CCL3, CCL4), pro-inflammatory cytokines (*e.g.* IL-1α, IL-1β, IL-6), as well as immunoregulatory cytokines (*e.g.* IL-12) (Mantovani *et al.*, 2011). However, to date, the production of several neutrophil-derived cytokines, *i.e.* IL-6 and CCL2, has been subject to controversy due to the constantly raised question of neutrophil purity in primary cell preparations (Tamassia *et al.*, 2014).

2.1.1 Downstream signaling to cytokine production

The neutrophil-derived cytokines, mentioned above, are all under the control of the gene activator nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB, which is at the end of a signaling pathway, amongst other agonists mainly induced by bacterial lipopolysaccharide (LPS). Being a major structural component of the outer membrane of gram-negative microbes, LPS is known to be an endotoxin to induce inflammation that may result in endotoxic shock (Rietschel and Brade, 1992). Pfeiffer *et al.* first reported the principal biological effects of bacterial endotoxin (Pfeiffer *et al.*, 1996). A simplified overview of LPS-induced signaling pathways is illustrated in *Figure 5*.

Upon host infection, LPS molecules have been described to bind to LPS-binding proteins (LBP) in plasma. The resulting LPS-LBP complex interacts with the glycoprotein CD14 on the cell membrane to form a ternary complex (Couturier *et al.*, 1991; Troelstra *et al.*, 1997; Troelstra *et al.*, 1999). Exceptionally, binding of LPS to CD14 might not be LBP-dependent with higher LPS concentrations of 100 ng/mL (Troelstra *et al.*, 1997). In this case, LBP seems not to be essential

[3] Paul Ehrlich (Nobel Prize in Physiology or Medicine, 1908) discovered that adaptive immunity detects molecular components (antigens) that are specific to individual pathogens and thus, distinguishes between self from non-self.

for LPS-mediated activation but rather to accelerate the downstream signaling (Tsukamoto *et al.*, 2010). The ternary LPS-LBP-CD14 complex transfers LPS to another non-anchored protein, myeloid differentiation factor-2 (MD-2), which in turn associates with the pattern recognition receptors (PRR) called toll[4] -like receptor 4 (TLR4) (Poltorak *et al.*, 1998). Docking of LPS to the TLR4 complex leads to receptor dimerisation and recruitment of adaptor proteins MyD88-adapter like (Mal) and myeloid differentiation primary response gene 88 (MyD88) *via* interactions with toll/interleukin-1 receptor (TIR) domains (Akashi *et al.*, 2003).

The MyD88-dependent pathway leads to the activation of the IL-1 receptor associated kinase (IRAK) 4 and subsequently to IRAK1, guiding to the activation and ubiquitinylation of TNF receptor associated factor 6 (TRAF6) (Li *et al.*, 2002). Ubiquitinated TRAF6 in turn activates the transcription factor interferon regulatory factor (IRF) 5, and also interacts with the TAK-binding protein (TAB) 1, 2, and 3 complex, which in turn activates transforming growth factor-β-activated kinase 1 (TAK1). TAK1 activates inhibitor of κB (IκB) kinases (IKKs), which phosphorylate IκBs. The IKK complex containing either IKKα or IKKβ kinase phosphorylates IκBα or IκBβ when activated by protein kinase B (Akt), receptor interacting protein (RIP) or other upstream kinases. Hence, phosphorylated IκBs are degraded by the ubiquitin-proteosome and the free NF-κB translocates to the nucleus (McDonald *et al.*, 1997). The transcription factor NF-κB traditionally refers to the ubiquitous, constitutive and inducible heterodimer p50/p65, which is an anti-apoptotic gene regulator. Regulated by phosphorylation and acetylation, the p65 (RelA) subunit provides gene activator activity for a wide variety of genes in the nucleus, most prominently genes of inflammatory cytokines (McDonald *et al.*, 1997; Beutler *et al.*, 2001; Caroff *et al.*, 2002; Pasare and Medzhitov, 2005). Phosphorylated p65 promotes interaction with the DNA, while acetylated p65 determines strength and duration of gene activation. After gene activation, p65 eventually releases the DNA and binds to newly synthesized IκBα. The IκBα: NF-κB (p50/p65) complex translocates back to the cytoplasm.

Activation of gene expression can also derive from mitogen-activated protein kinase (MAPK) signaling pathways induced by LPS (Nick *et al.*, 1999; Fessler *et al.*, 2002; Ear *et al.*, 2005; Cloutier *et al.*, 2009; Fortin *et al.*, 2011). In this part of the pathway, TAK1 generally activates also p38 (Nick *et al.*, 1999), c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). In neutrophils, JNK is overwhelmingly cytoplasmic and does not translocate to the nucleus (Cloutier *et al.*, 2003), with the exception of adherent neutophils in which JNK can

[4] The *toll* gene has originally been identified as being essential for dorsal-ventral polarity in embryonic development in *Drosophila monocytogenes*. The discovery of toll receptor-deficiency in adult Drosophila leading to the development of fungal infections has been awarded with the Nobel Prize in Physiology or Medicine 2011 to Bruce A. Beutler and Jules A. Hoffmann (Lemaitre *et al.*, 1996).

be moderately activated (Avdi *et al.*, 2001; Cloutier *et al.*, 2003). MAP kinases mediate the activation of transcription factor AP-1. Another transcription factor, IRF5, associates with MyD88 and TRAF6, becomes activated and translocates into the nucleus. AP-1, NF-κB and IRF5 all lead to the activation of inflammatory cytokine gene synthesis.

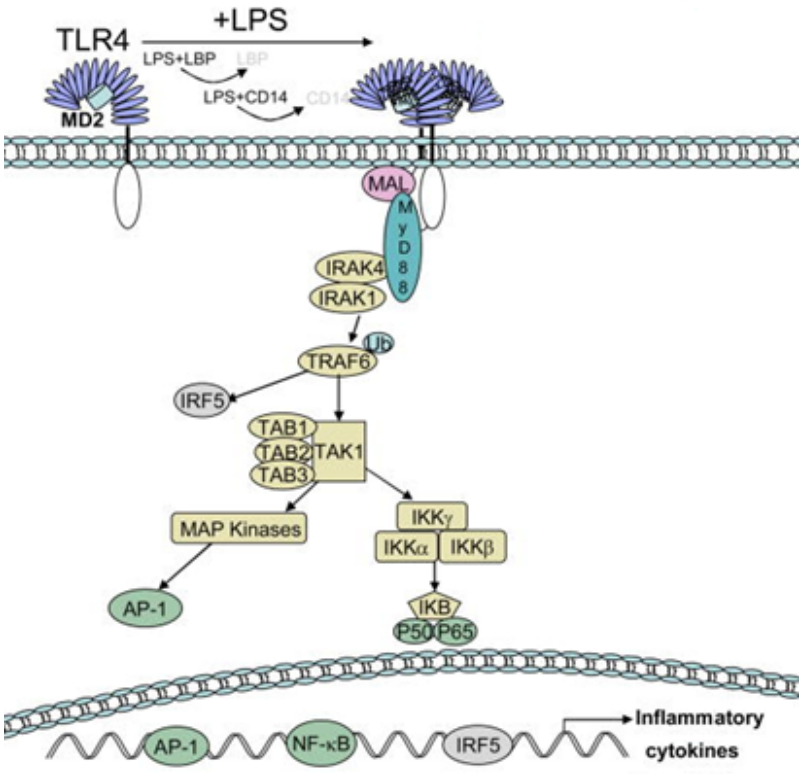

Figure 5 *Simplified view of the LPS-induced TLR4 signaling pathways (Casella and Mitchell, 2008, modified). Only MyD88-dependent signaling is depicted, since MyD88-independent signaling has been shown not to be mobilized in neutrophils (Tamassia et al., 2007). The transcription factors AP-1, NF-κB and IRF-5 together stimulate production of inflammatory cytokines.*

2.1.2 Neutrophil-derived cytokine response

2.1.2.1 Interleukin-8

The CXC chemokine IL-8 is known to be the major chemoattractant for neutrophils (Oppenheim *et al.*, 1991; Bazzoni *et al.*, 1991; Cassatella *et al.*, 1992; Baggiolini and Clark-Lewis, 1992; Takahashi *et al.*, 1993; Baggiolini *et al.*, 1994). Also, IL-8 accumulates and activates monocytes, macrophages and lymphocytes.

As a member of the CXC chemokine α-subfamily, IL-8 possesses a glutamic acid-leucine-arginine (ELR) motif preceding the first two cysteines (Scapini *et al.*, 2000). In neutrophils, the constitutive expression of IL-8 as a 99 amino acid precursor results from regulated

transcriptional (*e.g.* CCAAT-enhancer-binding protein β (C/EBPβ), Cloutier *et al.*, 2009), and/or post-transcriptional steps (Scapini *et al.*, 2000). On the post-translational level, it undergoes processing by neutrophil elastase, cathepsin G and proteinase-3 (Padrines *et al.*, 1994). After proteolytic cleavage, it can exist in two different isoforms and is secreted into the extracellular milieu either from intracellular storage upon CD66b crosslinking, or *de novo* production (Strieter *et al.*, 1992; Baggiolini and Clark-Lewis, 1992; Padrines *et al.*, 1994; Baggiolini *et al.*, 1995). IL-8 secretion occurs by the standard canonical pathway (*Figure 6A*) in two phases: an early one (20 to 30% of the total IL-8), directly induced by LPS itself and that can be inhibited by interferon-gamma (IFNγ), and a late, prolonged one, due to LPS-stimulated release of endogenous mediators, including TNFα and IL-1β (Cassatella *et al.*, 1993).

2.1.2.2 Interleukin-12

Interleukin-12 (IL-12) was identified as a heterodimeric glycosylated protein consisting of two subunits, a 40 kDa heavy chain (p40) and a 35 kDa light chain (p35). While p40 is homologous to the extracellular domains of members of the hematopoietic cytokine-receptor family, p35 is rate-limiting for the generation of the bioactive heterodimeric form of IL-12 (p70) (Carra *et al.*, 2000). Both genes need to be expressed and processed by cleavage in a coordinated fashion by the same cell in order to produce biologically active IL-12 (Carra *et al.*, 2000; Trinchieri *et al.*, 2003). The dimerization of p35 and p40 takes place in the endoplasmic reticulum (ER) (Carra *et al.*, 2000; Murphy *et al.*, 2000) and secretion occurs *via* the classical or canonical pathway (Duitman *et al.*, 2011) (*Figure 6B*). In this pathway, proteins are translocated across the ER through the Golgi apparatus to the plasma membrane, either directly or indirectly through primary lysosomes or secretory granules (Stanley and Lacy, 2010). While the production and secretion of IL-12 in murine neutrophils has been proven in several studies (Romani *et al.*, 1997; Bliss *et al.*, 1999; Petrofsky and Bermudez, 1999), the ability of human neutrophils to release this cytokine is still under debate (Wessels *et al.*, 2010).

IL-12 is known to be a key mediator of T_H1 responses and thus, bridges innate and adaptive immunity against infection (Muller *et al.*, 2009). Stimulation with IL-12 results in increased IL-8 production and release in neutrophils (Ethuin *et al.*, 2001) and production of IFNγ (Ethuin *et al.*, 2004).

2.1.2.3 Tumor necrosis factor α

The potent and multifunctional early response cytokine TNFα is produced (Hehlgans and Pfeffer, 2005) upon gene activation by the transcription factors NF-κB, or AP-1 (Gaestel *et al.*,

2009). Although TNFα is not expressed by neutrophils circulating in the bloodstream, its expression is rapidly and transiently inducible by LPS. Levels of mRNA are detected 60 min after stimulation and reach maximal amounts upon 4 h (McColl *et al.*, 1992). The TNFα precursor protein travels through the ER-Golgi compartments as type II transmembrane proteins. In the Golgi, it transiently accumulates before being delivered to the plasma membrane (Manderson *et al.*, 2007; Horiuchi *et al.*, 2010). TNFα is localized to secretory granules and to the plasma membrane in resting and activated cells, respectively. At the cell surface, pro-TNFα is rapidly cleaved by metalloproteinases, *e.g.* the TNF-converting enzyme (TACE) (Barbara *et al.*, 1996). The released ectodomain represents the soluble and mature TNFα cytokine (Black *et al.*, 1997). Many studies indicate that both the plasma membrane-bound as well as the soluble TNFα forms of the cytokine are biologically active (Hehlgans and Pfeffer, 2005) and are able to act in different signaling pathways, *e.g.* TNFα-induced survival pathway (Pimentel-Muinos and Seed, 1999). Non-stimulated neutrophils have been shown to secrete very low TNFα (30-60 pg/mL), whereas levels augmented to 160-190 pg/mL upon stimulation (Djeu *et al.*, 1990). The early release of TNFα is required to contribute to rapid vasodilatation, recruit mainly monocytes and neutrophils to the infection site, and potentially activate T lymphocytes (Cassatella *et al.*, 1993; Onnheim *et al.*, 2008; Muller *et al.*, 2009) (*Figure 6C*).

2.1.2.4 Interleukin-1α and interleukin-1β

From the β-trefoil interleukin-1 (IL-1) family, IL-1α and IL-1β are the most studied members in inflammation and immunity to infections (Lord *et al.*, 1991). Effective and rapid IL-1 mRNA synthesis requires three signals: firstly, IKKβ-, p38- or casein kinase (CK) 2-dependent signaling pathways need to be activated by TLRs, nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene (RIG)-like helicases. Secondly, the inflammasome needs to be activated to induce pro-IL-1 cleavage. Existing as a biologically inactive precursor on free ribosomes in the cytoplasm, pro-IL-1 processing into active fragments depends on cleavage by enzymes (*e.g.* IL-1β converting enzyme (Thornberry *et al.*, 1992) or caspase-1 (Franchi *et al.*, 2009)) inside the cell or upon secretion (*e.g.* neutrophil proteinase 3, elastase, and cathepsin G (Coeshott *et al.*, 1999; Netea *et al.*, 2010). Thirdly, the nucleotide P2X7 receptor needs to be activated by extracellular adenosine triphosphate (ATP, Qu *et al.*, 2007; Gaestel *et al.*, 2009; Qu *et al.*, 2009).

IL-1α and IL-1β are both secreted by a non-canonical pathway (Rubartelli *et al.*, 1990; Lord *et al.*, 1991) (*Figure 6D*). As “leaderless” proteins, they lack the conventional hydrophobic signal sequence (Halban and Irminger, 1994; Park and Loh, 2008) and are therefore released

independently from the ER and Golgi apparatus (Nickel, 2003; Prudovsky *et al.*, 2003). Their secretion pathway has been postulated to occur by *i)* exocytosis of secretory lysosomes, a process that requires a functional microtubule network (Andrei *et al.*, 1999; Andrei *et al.*, 2004; Carta *et al.*, 2006), *ii)* shedding of plasma membrane microvesicles (MacKenzie *et al.*, 2001; Nickel, 2003; Bianco *et al.*, 2005; Pizzirani *et al.*, 2007), or *iii)* guided release *via* specialized transporters (Hamon *et al.*, 1997; Marty *et al.*, 2005; Brough and Rothwell, 2007). The secretion of IL-1β by human neutrophils is still discussed controversially (Wessels *et al.*, 2010).

IL-1α and IL-1β both *i)* stimulate acute phase proteins, *e.g.* C-reactive protein, whose function is to localize the inflammation and to support the immune system (Gabay and Kushner, 1999), *ii)* signal endothelial cells to induce vasodilatation and upregulation of adhesion molecules that aids the migration of neutrophils, monocytes, and lymphocytes, *iii)* activate mainly monocytes, macrophages and neutrophils, and *iv)* co-stimulate adaptive cellular responses mediated by T helper (T_H) lymphocytes.

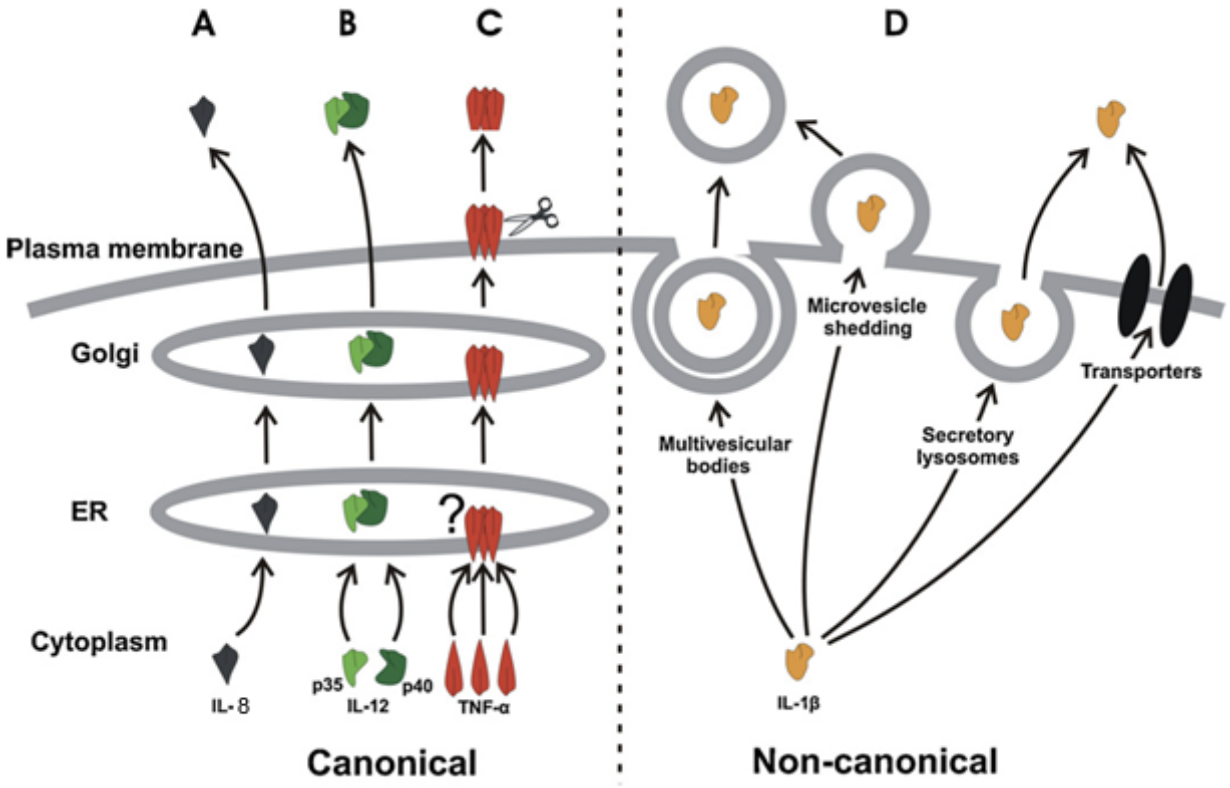

Figure 6 *Canonical and non-canonical pathways of cytokine secretion. (A) The standard pathway, (B) the unit interaction pathway, (C) the membrane-deposit pathway, and (D) non-canonical pathway of cytokine secretion (Duitman et al., 2011, modified).*

2.1.2.5 Interleukin-6

Interleukin-6 (IL-6) is one of the cytokines that has been seen as a sign of monocyte contamination in neutrophil preparations (Cassatella, 1995) and is still under debate as being a neutrophil-derived cytokine. IL-6 gene expression has been reported to be induced *via* the NF-κB pathway (Libermann and Baltimore, 1990). The functional form of IL-6 is a homodimer. Different secretion pathways for IL-6 have been postulated: *i)* independent from secretory granule release in mast cells (Kandere-Grzybowska *et al.*, 2003), *ii)* in secretory vesicles in eosinophils (Melo *et al.*, 2005), and *iii)* by constitutive exocytosis in macrophages (Stow *et al.*, 2009).

During inflammation, IL-6 is an important regulator of the transition from neutrophil to monocyte recruitment (Kaplanski *et al.*, 2003) and orchestrates chemokine production (Fielding *et al.*, 2008). IL-6 is one of the few cytokines that can act in a cytokine-receptor complex for paracrine signaling (Jones *et al.*, 2001). When the IL-6 receptor at the cell surface is cleaved by proteases, the soluble receptor (sIL-6R) and IL-6 form complexes that bind to gp130 and thus induce signaling cascades (Jablonska *et al.*, 1999). IL-6 also has systemic effects by *i)* inhibiting the expression of TNFα (Xing *et al.*, 1994), *ii)* stimulating the production of acute-phase proteins *iii)* inducing differentiation of B lymphocytes towards antibody-producing plasma cells, and *iv)* supporting T_H2 and T_H17 cell differentiation (Muller *et al.*, 2009).

2.1.2.6 Chemokine (C-C motif) ligand 2, 3, 4 and 5

CCL2 (MCP-1) is the first discovered human CC chemokine (Deshmane *et al.*, 2009). A higher level of CCL2 attracts monocytes (Baggiolini *et al.*, 1994) and augments the T_H lymphocyte response (Theilgaard-Monch *et al.*, 2004; Yoshimura and Takahashi, 2007; Deshmane *et al.*, 2009). In freshly isolated neutrophils, CCL2 has been claimed to be poorly produced upon induction with various stimuli (Van Damme *et al.*, 1994).

CCL3 (MIP1α) and CCL4 (MIP1β) (Kasama *et al.*, 1993; Kasama *et al.*, 1994) are chemoattractants for monocytes and subpopulations of T lymphocytes (Theilgaard-Monch *et al.*, 2004). Upon proteolysis of CCL3 by neutrophil serine proteases (Ryu *et al.*, 2005), mature CCL3 can also activate effector functions in macrophages, neutrophils (Oppenheim *et al.*, 1991), eosinophils, basophils, immature dendritic cells (Schroder *et al.*, 2006) and NK cells (Scapini *et al.*, 2000). CCL4 also elicits chemotaxis of dendritic cells and T_H1 lymphocytes (Scapini *et al.*, 2000; Schroder *et al.*, 2006).

CCL5 (RANTES) can exist in different truncated variants (Lim *et al.*, 2005). CCL5 production is induced *via* the NF-κB pathway in a Nod-like receptor-dependent manner. CCL5 secretion has

been documented to occur through the standard canonical pathway (Lacy and Stow, 2011). Besides the recruitment of immature dendritic cells and T lymphocytes towards the site of injury (Werts *et al.*, 2007), CCL5 is the only CC chemokine also able to cause chemotaxis and activation of eosinophils (Deshmane *et al.*, 2009).

V Development of chronic inflammatory diseases

The healthiest outcome of acute inflammation is resolution, which is essential in order to maintain homeostatic conditions in the tissues (Henson, 2005). However, long-term inflammation can occur through premature activation of neutrophils during migration, excessive extracellular release of antimicrobial products during killing, inappropriate removal of infected and damaged host cells, and failure to terminate acute inflammatory responses (Smith, 1994). In such a scenario, an imbalance between levels of pro- and anti-inflammatory cytokines evolves (Salvioli *et al.*, 2006). The abnormal host response creates a persistent inflammatory microenvironment with ongoing release of inflammatory mediators (Nathan and Ding, 2010; Serhan, 2011) and thus, predisposes to a series of chronic diseases that include cancer, chronic obstructive pulmonary disease, rheumatoid arthritis, irritable bowel disease, gastric disorders, and autoimmune skin disorders (Henson, 2005; Florean and Diederich, 2012). Some examples of chronic inflammatory diseases are given below.

1. Clinical examples of chronic leukocyte-induced injury

1.1 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory disease mainly characterized by synovial hyperplasia and joint destruction. Neutrophils are the first immune cells to infiltrate the joint at the early disease stage and are activated by soluble immune complexes in the synovial fluid (Smith, 1994; Cascao *et al.*, 2009; Myasoedova *et al.*, 2010). In response, excessive degranulation by neutrophils includes overabundant collagenase and elastase secretion. Also, cytokines have been attributed a role in the pathogenesis of rheumatoid arthritis (Firestein *et al.*, 1990; Brennan *et al.*, 1991; Feldmann, 1996; McInnes and Schett, 2007) and are used for biomarkers of the disease (Burska, 2014).

1.2 Atherosclerosis

Atherosclerosis defines the deposition of fat, blood clots, tissue, and lime in the blood vessels. Literally translated, the term *atherosclerosis* refers to hardening of the connective tissue in the arteries. The origin for the development of atherosclerosis might be damage or dysfunction of the endothelium. As a result, low-density lipoprotein (LDL) molecules can reach subendothelial layers of the tunica intima, and inflammatory reactions can occur: *i)* oxidation of LDLs mediated by oxidants and MPO of neutrophil origin (Smith, 1994; Pattison and Davies, 2006; Loria *et al.*,

2008; Tavora *et al.*, 2009), and *ii)* formation of atherosclerotic patches, the "plaques". Also, cathelicidin (Doring *et al.*, 2012), azurocidin, LL-37, α-defensins, and neutrophil gelatinase-associated lipocalin (NGAL) (Hemdahl *et al.*, 2006) which only can derive from neutrophils, have been identified in atherosclerotic lesions (Soehnlein, 2012). Moreover, cytokines have a modulatory effect on the inflammatory responses in atherosclerosis (Tedgui and Mallat, 2006).

1.3 Inflammatory bowel disease

Inflammatory bowel disease defines a group of inflammatory conditions of the colon and small intestine. Crohn's disease and ulcerative colitis are the principal types of inflammatory bowel disease. In patients with ulcerative colitis, the circulating levels of neutrophils are found to be up to three times the level in healthy controls (Hanai *et al.*, 2004), resulting in an increased production of IL-8 and other cytokines in the inflamed mucosa (Isaacs *et al.*, 1992; Grimm and Doe, 1996; Muzes *et al.*, 2012; Neurath, 2014). Also, ulcerative colitis has been associated with oxidative damage, which could be quantified by MPO-induced oxidation (Winterbourn *et al.*, 2000). Levels of MMP-9, characteristic protein of gelatinase granules, are found elevated in inflammatory bowel disease (Bailey *et al.*, 1994; Baugh *et al.*, 1999).

Since neutrophils are able to recruit, differentiate and activate B and T lymphocytes as well as to program antigen-presenting cells, they represent an important bridge between innate and adaptive immune system. It is therefore essential to maintain a balance in the production of neutrophil-derived mediators in order to prevent excessive release of these products that highly contribute to the development of chronic inflammatory diseases (Akgul *et al.*, 2001). Among the most effective treatment options for chronic inflammatory disorders are blocking agents that specifically target cytokines or their receptors (*e.g.* TNF, IL-1R) (Maini and Taylor, 2000; Bresnihan *et al.*, 2000; Genant, 2001; Taylor, 2003; Furst, 2010). Thus, profound and detailed understanding of regulatory processes in the intracellular vesicle trafficking pathways and exocytosis to the inflammatory environment are essential in order to determine new potential pharmacological targets in chronic inflammatory diseases that could prevent the release of mediators into the pro-inflammatory environment.

VI Pathways of exocytosis

1. Different types of exocytosis

The vesicle transfer of proteins between the cellular compartments is essential for all intra- and intercellular processes (Weber *et al.*, 1998). Proteins that are carried within the vesicles are called cargo proteins. In general, trafficking pathways maintain the inward flux of vesicles from the plasma membrane *via* endocytosis (Maxfield and McGraw, 2004), and in the opposite direction, the liberation of vesicles into the extracellular milieu *via* exocytosis (Cox *et al.*, 1999), (Hackam *et al.*, 1998). Those trafficking pathways are generally known as the endocytic cycle (*Figure 7*). Here, we focus on the exocytotic pathways, for which two general mechanisms exist:

- Constitutive exocytosis, in which no outer signal is needed, defines continuous vesicular trafficking of cargo proteins or dynamic tubulovesicular carriers of newly synthesized proteins deriving directly from mRNA translation, or receptor-mediated *de novo* transcription (Shukla *et al.*, 2000; Lacy and Stow, 2011).
- Regulated exocytosis involves cell-type specific vesicles that are stored in the cytoplasm and undergo fusion with the plasma membrane upon stimulation *via* ligand-receptor signaling (Palade, 1956; Shukla *et al.*, 2000). During this receptor-coupled mechanism that exists in neuroendocrine and exocrine cells, homo- or heterotypic membrane fusion can occur: fusion involving vesicle-intracellular membranes, vesicle-vesicle, or vesicle-plasma membrane (Shukla *et al.*, 2000).

Regulated exocytosis can either occur with a non-secretory or secretory outcome. During non-secretory exocytosis, transfer of membrane patches from specific organelles to the plasma membrane results in no discharge of segregated material. Main purposes are the translocation of membrane components to the cell surface and the compensation for large plasma membrane internalization taking place during phagocytosis. In this sense, exocytosis is highly required for phagocytosis in macrophages (Hackam *et al.*, 1998; Braun and Niedergang, 2006). Regulation of non-secretory exocytosis can occur independently of that of secretory events taking place in the same cell (Chieregatti and Meldolesi, 2005; Falcone *et al.*, 2006). The mechanisms for secretory exocytosis are described in the following sections.

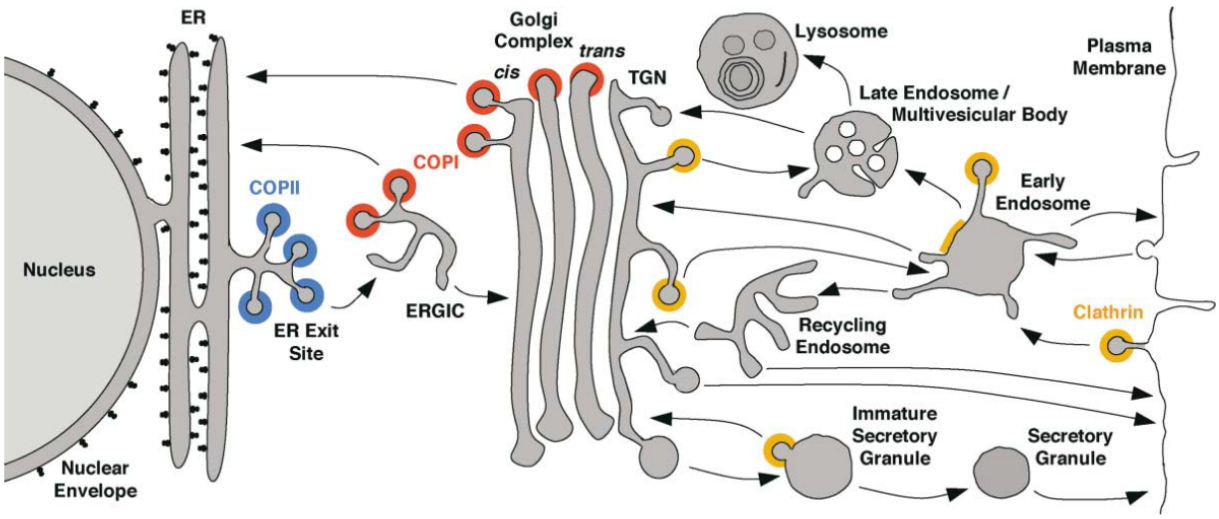

Figure 7 *Intracellular vesicle trafficking between the compartments of the exocytic (secretory, lysosomal/vacuolar) and endocytic pathways (Bonifacino and Glick, 2004). The involvement of coat proteins (COPI in red, COPII in blue and clathrin in orange) in specific transport steps is depicted. Abbreviations: ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; COPI and II, coat proteins I and II; TGN, trans-Golgi network.*

2. Classical secretory exocytosis

Many cells are able to temporarily store proteins in secretory granules until the cell receives an appropriate stimulus. The ability to maintain a regulated secretory pathway is dependent on continued synthesis of proteins that are needed for formation of regulated storage granules (Borregaard and Cowland, 1997). In neutrophils, several cytokines are believed to be stored in granules before extracellular release (Terebuh *et al.*, 1992; Calafat *et al.*, 1997; Bliss *et al.*, 2000; Brandt *et al.*, 2000; Matzer *et al.*, 2001). For example, Denkers *et al.* proposed that IL-6, IL-12 and CXCL2/MIP2 might be stored within secretory vesicles or gelatinase granules (Denkers *et al.*, 2003). Elbim *et al.* discovered an intracellular pool of IL-10 receptors in specific granules of human neutrophils (Elbim *et al.*, 2001). Only about 10% of secretory vesicles are docked to the plasma membrane and immediately available to quickly release their content in response to an extracellular stimulus (Cheviet *et al.*, 2004). These vesicles represent the readily releasable pool (RRP, Martin, 2002) while the majority of the vesicles remains in a reserve pool (RP), which is released at a later time point (Sorensen, 2004). Moreover, the newest vesicles are mainly localized near the plasma membrane, whereas older ones were regrouped deeper in the cytoplasm (Duncan *et al.*, 2003).

Secretory exocytosis was first described by Palade[5] to occur in pancreatic exocrine cells as a stimulation-induced fusion between specialized secretory organelles and the plasma membrane (Palade, 1975). Secretory vesicles travel through many compartments that are interconnected by trafficking routes. An elaborate system of internal membrane-bounded structures is required for the exocytosis of vesicles *via* the classical pathway (Hackam *et al.*, 1998; Cox *et al.*, 1999): the ER, the Golgi apparatus, and the *trans*-Golgi transport vesicles (*Figure 7*). The amount and organization of exocytic organelles varies from organism to organism and cell type to cell type.

Activation of the secretory pathway can be divided into three classes: *i)* Ca^{2+} influx as result of depolarization of excitable membranes or mobilization from intracellular stores; *ii)* G protein activation; and *iii)* receptor tyrosine kinase activation. While proteins are synthesized and properly folded in the ER, they are modified and sorted in the Golgi, in order to be eventually carried by the *trans*-Golgi transport vesicles to the final destination. Here, the pathway involves sequential steps: *i)* wrapping of proteins by membranes deriving from the *trans*-Golgi, *ii)* vesicle budding from the *trans*-Golgi, and *iii)* vesicle biogenesis and maturation (Farquhar and Palade, 1981; Tooze, 1998). This process can occur according to the "sorting by entry" hypothesis, in which proteins are sorted directly from the *trans*-Golgi, the central processing and sorting station of the secretory pathway, into specific nascent vesicles (Arvan and Halban, 2004). For example, lysosome proteins are recognized and sorted by the *trans*-Golgi due to the mannose 6-phosphate residues on the *N*-linked oligosaccharide. Alternatively, according to the "sorting by retention" mechanism, proteins are packaged unsorted into the vesicles and are subsequently sorted throughout vesicle maturation (Blazquez and Shennan, 2000). For example, MPO propeptide undergoes retention in the ER (Bulow *et al.*, 2002).

In mammals, an octameric protein complex (Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, and the hydrophilic proteins, Exo70p and Exo84p) called the exocyst serves the trafficking from *trans*-Golgi in a polarized fashion towards the plasma membrane (TerBush *et al.*, 1996; Kee *et al.*, 1997; Wu *et al.*, 2010; He and Guo, 2009).

During secretory regulatory exocytosis, the cytoskeleton is highly important. While microtubule assembly is responsible for the movement of vesicles over a longer intracellular distance (> 1 μm) and the vectorial exocytosis (Tapper *et al.*, 2002), actin depolymerization is required for releasing vesicles over short distances in the nanometers range (Burgoyne and Morgan, 2003; Varadi *et al.*, 2005; Abu-Hamdah *et al.*, 2006; Malacombe *et al.*, 2006).

[5] George Emil Palade was a Romanian cell biologist whom was awarded the Nobel Prize in Physiology and Medicine in 1974 for his innovations in electron microscopy and cell fractionation leading him to the discovery of ribosomes and the endoplasmic reticulum.

3. Non-classical secretory exocytosis

The most commonly used export strategy for cargo proteins is the classical ER-Golgi pathway, mentioned above. However, also non-classical pathways exist which have been discovered only several years ago. The molecular identity implicated in these export routes has not yet been elucidated in details.

The generation of so-called multivesicular bodies by the internalization of many small vesicles from the membrane of large “mature” endosomes can predispose “prelysosomal exocytosis”, the regulated trafficking from endosomes to lysosomes. The inward budding of vesicles results in the formation of 40 to 100 nm segregated vesicles of endocytotic origins called exosomes (Raposo *et al.*, 1996; Savina *et al.*, 2002; Fevrier and Raposo, 2004; Yu *et al.*, 2006; Schorey and Bhatnagar, 2008; Simons and Raposo, 2009; Thery *et al.*, 2009).

Endosomal-derived vesicles called ectosomes or microvesicles (Hess *et al.*, 1999; Cocucci *et al.*, 2009; Lee *et al.*, 2011) that can bud off by shedding (MacKenzie *et al.*, 2001; Bianco *et al.*, 2005; Pizzirani *et al.*, 2007) are, in general, larger than exosomes (ranging from 100 nm up to 1 µm in diameter), and are characterized by an enrichment of phosphatidylserine on their outer surface.

Additionally, lysosomal exocytosis involving secretory lysosomes tightly linked to endocytic system has been described to occur in leukocytes and macrophages (Griffiths, 1996; Dell'Angelica *et al.*, 2000; Blott and Griffiths, 2002; Luzio *et al.*, 2010). As lysosomes are known to be control centres for clearance of cellular products during autophagy, this mechanism does not represent simple degradation in lysosomes (Wu *et al.*, 2007). Indeed, the soluble contents of the vesicles are released to the outside, and the vesicle membrane becomes part of the plasma membrane.

4. Neutrophil-specific secretory exocytosis

Besides the secretory pathways described above, variations of the export routes have been described in neutrophils.

4.1 Piecemeal degranulation

A variation of regulated secretory exocytosis is called piecemeal degranulation, a type of degranulation that was first identified by electron microscopy in eosinophils (Dvorak *et al.*, 1991), and that leads to the differential and selective transport of cytokines from secretory granules to the cell surface. Originally, this mechanism was hypothesized to provide progressive

losses of granule contents without any evidences for fusion events (Crivellato *et al.*, 2003). However, each transport step in this pathway also requires sets of trafficking machinery molecules to enable vesicular transport and membrane fusion. Piecemeal degranulation has also been observed in mast cells, and basophils. In neutrophils, this specific mechanism of degranulation has been described for azurophil granules (Mahmudi-Azer *et al.*, 2010).

4.2 Compound exocytosis

In another variant of regulated secretory exocytosis, called compound exocytosis, fusion of granules occurs at the plasma membrane, and between granules from deeper inside the cytosol thereby forming degranulation channels (Dvorak and Ishizaka, 1995; Pickett and Edwardson, 2006) (*Figure 8*). This variant of exocytosis has already been described to mobilize more than 50% of granules stored in the cytosol in mast cells (Brochetta *et al.*, 2008, Blank, 2011), basophils, eosinophils (Scepek *et al.*, 1998), neutrophils (Lollike *et al.*, 2002), and platelets (Morgenstern, 1995).

Furthermore, two types of compound exocytosis can be distinguished. During sequential compound exocytosis, a primary fusion occurs between vesicle and plasma membrane. Docking of deeper-lying granules to the first vesicles leads to release of granule content (*Figure 8, left*). During multivesicular compound exocytosis, vesicles can fuse with each other in a homotypic fashion leading to the formation of large intracellular structures, which eventually can fuse with the plasma membrane (Hafez *et al.*, 2003) (*Figure 8, right*).

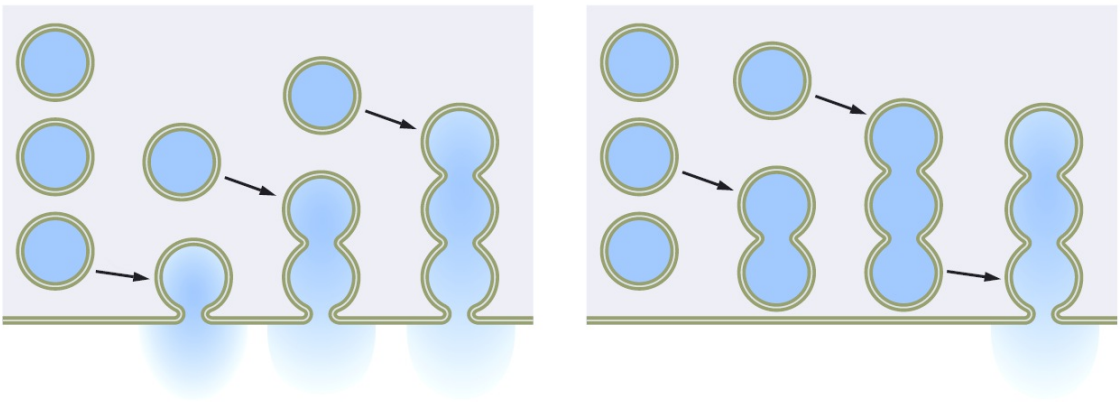

Figure 8 *Compound exocytosis. Scheme depicting the sequential (left) and multivesicular (right) configurations of compound exocytosis (Kasai et al., 2012). Activation stimuli regulate each fusion step between cytosolic vesicles or with the plasma membrane.*

VII Soluble *N*-ethylmaleimide-sensitive-factor attachment receptor proteins

All types of exocytosis mentioned above have been described to depend on regulatory coordinators of membrane fusion events, the evolutionarily conserved soluble *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE) protein superfamily (Sollner *et al.*, 1993; Weimbs *et al.*, 1997; Gerst, 1999; Duman and Forte, 2003; Jeremic *et al.*, 2004). They can be present in multiple tissues in the same organism (Bennett and Scheller, 1993; McMahon *et al.*, 1993; Rossetto *et al.*, 1996). SNAREs are tail-anchored membrane proteins involved in the vesicle fusion process. In mammalian cells, 38 different SNAREs have been identified that lead to the specific trafficking of vesicles (Bennett *et al.*, 1992; Sollner *et al.*, 1993).

1. SNARE classification

Two different classifications of SNARE proteins exist. Firstly, SNAREs can be classified into t-SNAREs localized to the target membrane (Weber *et al.*, 1998; Johannes and Galli, 1998; Pfeffer, 1999; Scales *et al.*, 2001), and v-SNAREs localized to the membrane of the trafficking vesicle. According to the SNARE hypothesis[6] (Rothman and Warren, 1994), one v-SNARE tail-anchored at the transport vesicle, interacts with two t-SNAREs consisting of either two or three polypeptides (Fukuda *et al.*, 2000). In this define stoichiometry, they form *trans*-SNARE complexes (Antonin *et al.*, 2002) and specifically mediate fusion of two opposing membranes (Cho *et al.*, 2002). While t-SNAREs can generally be subdivided into syntaxins (STXs) and synaptosomal associated proteins (SNAP), v-SNAREs consist of vesicle-associated membrane proteins (VAMPs, synaptobrevins). Four of the SNARE members could not be classified, for example, STX6 is the most divergent member of the STX family identified so far (Bock *et al.*, 1997).

Secondly, a more elaborate classification addresses the formation of the *trans*-SNARE complex or SNAREpin (Poirier *et al.*, 1998) and its central region. The so-called “0-layer” is composed of four homologue SNARE motifs, and harbors glutamine (Q) and arginine (R) residues (Katz and Brennwald, 2000) deriving from the relative Q- and R-SNAREs. According to this classification, three Q-SNAREs (subdivided into Q_a, Q_b, and Q_c depending on the position in the SNARE complex) bind to one R-SNARE, thus forming ring complexes of approximately 20 nm in

[6] The SNARE hypothesis, postulated in 1993, (Sollner *et al.*, 1993; Ferro-Novick *et al.*, 1994) is originally based on the discovery of the *trans*-SNARE complex, together with the fusion proteins SNAP (Clary *et al.*, 1990) and NSF (Block *et al.*, 1988).

diameter (Hanson *et al.*, 1997; Cho *et al.*, 2005). Q_a-SNAREs are represented by the STX family, $Q_{b,c}$-SNAREs by SNAP25 homologues and R-SNAREs by the synaptobrevin family. The "Q_{abc}R-rule" indicates that all functional SNARE complexes are thought to contain one member of each subfamily (Jahn and Sudhof, 1999). The Q/R-SNARE nomenclature has been privileged due to its comprehensive classification according to the conserved mechanism and structure of SNARE proteins (Kloepper *et al.*, 2007).

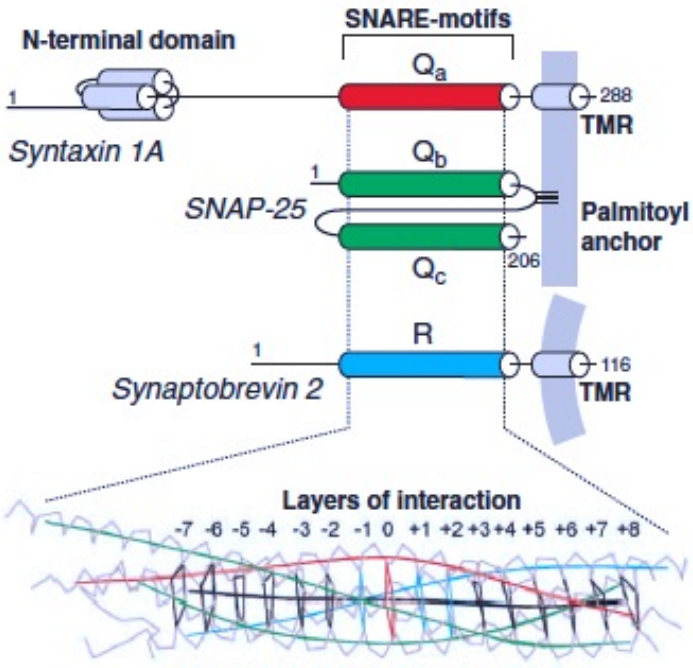

Figure 9 *Structure of the neuronal SNARE proteins (Lang and Jahn, 2008). Syntaxin 1A, SNAP25 and synaptobrevin all contain one to two SNARE motifs. SNARE interaction leads to formation of a stable complex, in which each of the SNARE motifs adopts an α-helical structure. The stability of the four parallel α-helices in the complex is mediated by layers of interaction (−7 to +8). Abbreviations: TMR, transmembrane region.*

1.1 Q_a-SNAREs

The first STX was discovered in 1992 (Bennett *et al.*, 1992). The STX family consists of 16 mammalian members that are integral membrane proteins with a transmembrane domain at the C-terminal that anchors the protein in the membrane (Advani *et al.*, 1998; Teng *et al.*, 2001). The N-terminal cytoplasmic part has coiled-coil regions, which interact with synaptobrevins and SNAP25 homologues (Shukla *et al.*, 2000) (*Figure 9*).

In general, SNAREs are concentrated at glycosphingolipid- and cholesterol-rich sites (Lang *et al.*, 2001; Duman and Forte, 2003; Sieber *et al.*, 2006), namely lipid rafts (Chamberlain *et al.*, 2001), in the plasma membrane of neuronal and non-neuronal cells. The lipid raft might be important to accumulate various sets of molecules (*e.g.* phosphatidylinositol-4,5-bisphosphate

PIP_2; Liu *et al.*, 1998) to facilitate the efficacy of membrane traffic (Martin, 2001; Maxfield, 2002; Salaun *et al.*, 2004). It has been proposed that lipid rafts are primarily important for capturing vesicles from the cytoplasm (Yoshizaki *et al.*, 2008). Additionally, STX1A was found to self-oligomerize and form clusters at concentrations above 2 µM (Murray and Tamm, 2009). Also, STX3 was found enriched in lipid rafts (Pombo *et al.*, 2003) and clusters in the plasma membrane of epithelial cells before establishment of cell polarity (Low *et al.*, 2006). Therefore, it has been proposed that STX clustering is a mechanism of membrane patterning (Sieber *et al.*, 2006) and identifying a compartment. In this sense, homotypic fusion (also referred to as "nucleation") may solely rely on the Q-SNAREs and does not involve R-SNAREs (Weber *et al.*, 1998; Lang and Jahn, 2008). Using stimulated emission depletion (STED) microscopy, the SNARE motif has been described to be essential for the formation of STX clusters in the plasma membrane (170 to 256 nm; Sieber *et al.*, 2006) in order to form vesicular tubular clusters.

1.2 $Q_{b,c}$-SNAREs

$Q_{b,c}$-SNAREs are also called subfamily of SNAP25 homologues. Since its discovery in 1989, SNAP25 has been considered almost exclusively expressed in the brain (Oyler *et al.*, 1989; Bark *et al.*, 1995; Brumell *et al.*, 1995). SNAP23 (23-kDa synaptosome-associated protein) is 59% identical to SNAP25, and has originally been regarded as the SNAP25 homologue in non-neuronal tissues substituting for the lack of SNAP25 in these cells. Although SNAP23 is ubiquitously expressed in all tissues (gastrointestinal and hepatopancreatic epithelial cells, neuroendocrine cells and mast cells (Faigle *et al.*, 2000; Frank *et al.*, 2011) to fibroblasts including brain (Ravichandran *et al.*, 1996; Hepp *et al.*, 1999)), the functional equivalence to SNAP25 remains elusive.

SNAP23 and SNAP25 possess two SNARE motifs, but lack the typical transmembrane domain (TMR) at the C-terminus common to most other SNAREs (Jahn *et al.*, 2003) (*Figure 9*). Instead, SNAP23 has palmitoyl groups for binding to the membrane (Veit, 2000).

SNAP23 has a target sequence for post-translational fatty acid acylation and two potential target sequences of phosphorylation by cAMP/cGMP-dependent protein kinase (Mollinedo and Lazo, 1997). Cell stimulation can lead to the phosphorylation of SNAP23 and thus, to functional activation for mast cell degranulation (Hepp *et al.*, 2005). SNAP23 is subcellularly localized in the apical and basolateral domain of the plasma membrane (Low *et al.*, 1998) but also intracellularly in endosomal and lysosomal compartment of polarized epithelial cells (Rao *et al.*, 2004).

In *in vitro* experiments, SNAP23 bound to glutathione S-transferase (GST)-STX1, STX2, STX3 and STX4 with similar efficiency. Also SNAP23 has been shown to interact to VAMP1 and VAMP2 (Ravichandran *et al.*, 1996). SNAP23 has been reported to interact as well with VAMP3 (Galli *et al.*, 1998). In the exocrine system, SNAP23 and STX4 interact (Wang *et al.*, 2007) and their complex might be involved in histamine secretion in mast cells (Salinas *et al.*, 2008).

Another SNAP25 homologue is SNAP29, which is also ubiquitously located on multiple membranes (Steegmaier *et al.*, 1998). Unlike SNAP23, SNAP29 does not possess a lipid anchor, which suggests that SNAP29 can only associate with membranes by interacting with membrane-bound proteins. SNAP29 protein has been involved in endocytic recycling of transferrin and β_1-integrin (Rapaport *et al.*, 2010) as well as mast cell phagocytosis (Wesolowski *et al.*, 2012). SNAP29 also interacts with several syntaxins (Steegmaier *et al.*, 1998).

1.3 R-SNAREs

Many R-SNAREs have been identified through their specific cleavage by botulinum and tetanus toxins. R-SNAREs are defined by two subfamilies, the “brevin” family, which includes VAMP2 (synaptobrevin 2, *Figure 9*), VAMP3 (cellubrevin) and VAMP8 (endobrevin); and the “longin” family, characterized by an N-terminal extension called the longin domain (*e.g.* TI-VAMP/VAMP7) (Rossi *et al.*, 2004; Wen *et al.*, 2006). Upon their synthesis, VAMPs are post-translationally inserted into the ER membrane. This insertion is dependent on ATP, and sorting is directed by the SNARE motif.

As one of the 8 members of the R-SNARE family (Advani *et al.*, 1998), VAMP3 differs from VAMP1 and VAMP2 by its shorter N-terminus and its lower molecular weight of only 14 kDa (McMahon *et al.*, 1993). Also, while the R-SNARE VAMP1 has been restricted to neuroendocrine and neuronal tissues and was thus not detected in cell extracts of human neutrophils (Trimble, 1993), VAMP3 on the contrary is expressed in a variety of tissues (McMahon *et al.*, 1993), but not in neurons (Chilcote *et al.*, 1995). Subcellularly, VAMP3 is localized mainly in intracellular vesicle and granule membranes and is important for vesicle recycling to endosomes (McMahon *et al.*, 1993; Rossetto *et al.*, 1996). It has been found in the apical as well as in the basolateral endosomal fraction (Steegmaier *et al.*, 2000) but also in the region of the ER and Golgi (Galli *et al.*, 1994; Daro *et al.*, 1996; Bajno *et al.*, 2000; Mallard *et al.*, 2002). In animal experiments, VAMP3 knockout mice do not show any particular biological changes of the intracellular membrane transport processes (Yang *et al.*, 2001). VAMP3 acts in membrane fusion processes with multiple binding partners such as Q_a-SNAREs STX2, 3, 4 and the $Q_{b,c}$-SNAREs SNAP23 and SNAP25 (Galli *et al.*, 1998).

2. SNARE cycle

The defining feature of all Q- and R-SNAREs is an evolutionarily conserved domain of 60 to 70 amino acids that is arranged in heptad repeats (Lu *et al.*, 2010). The SNARE motif (Jahn and Sudhof, 1999) mediates the association of SNAREs into *trans*-SNARE complexes (Antonin *et al.*, 2002) (*Figure 9*). While Q_a-SNAREs (STXs) and R-SNAREs (synaptobrevins) only carry one single SNARE motif, anchored to the plasma membrane by a C-terminal transmembrane region and attached to a large N-terminal domain *via* a linker region, $Q_{b,c}$-SNAREs (SNAP25 homologues) can carry two SNARE motifs (*Figure 9*). Also, $Q_{b,c}$-SNAREs can lack transmembrane domains and are membrane-anchored by post-translationally attached lipids.

During SNARE assembly, each SNARE contributes one helix (Sutton *et al.*, 1998) in the way that one 3-helical bundle from one membrane and one from the opposing membrane assemble according to the *zippering model* (Fasshauer, 2003) towards a twisted parallel 4-helical bundle (Weber *et al.*, 1998) with an overall length of 12 nm (Cho *et al.*, 2005). While single SNAREs can be denatured by heat and are cleaved by botulinum and tetanus neurotoxins (Jahn and Sudhof, 1999), Q- and R-SNAREs form complexes that are resistant to these stresses (Jeong *et al.*, 1998). For example, human STX3 in complex with SNAP23 is insensitive to botulinum toxin C1 (Galli *et al.*, 1998). Upon complex formation, SNARE proteins that are on opposing membrane bilayers bring vesicle and plasma membrane into close proximity, which normally repulse each other due to negatively charged phosphate groups and the hydration barrier. This close proximity of approximately 2.8 Å leads to a destabilization of the normal conformation of the lipid membrane, thus allowing membrane fusion and the subsequent release of the cargo molecules (Lin and Scheller, 1997).

For subsequent rounds of fusion, SNARE complexes are disassembled, recycled and sorted to their appropriate membranes (*Figure 10*). In 2006, Jeremic *et al.* discovered that there is an ATP-dependent disassembly of the highly stable SNARE complexes (Jeremic *et al.*, 2006). In 2007, the chaperone NSF was discovered to be a right-handed molecular motor (Block *et al.*, 1988; Malhotra *et al.*, 1988; Cho *et al.*, 2007) and to act in conjunction with adaptor proteins α- and β-SNAPs (soluble NSF attachment protein) (Clary *et al.*, 1990; Waters *et al.*, 1992; Whiteheart *et al.*, 1993; Jahn *et al.*, 2003) in the uncoiling of each SNARE bundle within the ring complex (Cho *et al.*, 2007). NSF is a hexamer with an N-terminal domain for substrate binding and two nucleotide binding domains D1 and D2 for ATP hydrolysis and hexamer stability. When acting upon STX or SNAP25, its ATPase activity is stimulated more than ten-fold. During SNARE disassembly, Q- and R-SNARE vesicles are dissociated with a rate constant $k=1.1s^{-1}$. The Q-SNAREs are recycled to the starting membrane compartment.

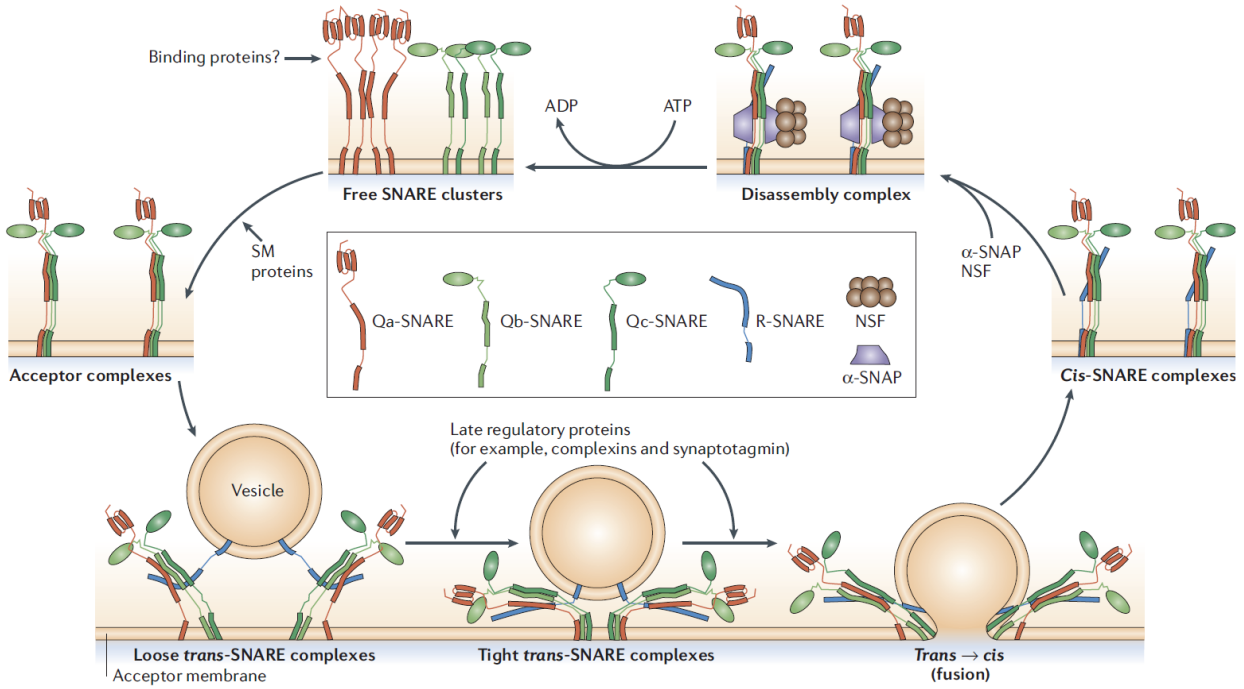

Figure 10 *The SNARE conformational cycle (Jahn and Scheller, 2006). Q_a-, Q_b- and Q_c-SNAREs form clusters at the acceptor membrane prior to assembly with R-SNAREs from a vesicle. This assembly towards a four-helical trans-SNARE complex requires Sec1/Munc18 proteins. The zippering of the SNARE motifs leads to opening of a fusion pore. In regulatory exocytosis, late regulatory proteins control the transition states between loose and tight trans-SNARE complex formation. During membrane fusion, SNAREs undergo cis-configuration and are disassembled by NSF and SNAPs (see text).*

3. Regulation of SNARE complex formation

The SNARE hypothesis proposes that the specificity of vesicle fusion is due to specific combinations of SNARE interactions (Rothman and Warren, 1994; McNew *et al.*, 2000) and that each fusion step of the secretory pathway thus implicates a specific set of SNAREs (Sollner *et al.*, 1993; Bennett and Scheller, 1993; McMahon *et al.*, 1993; Bethani *et al.*, 2007). For example, STX6 mediates vesicle trafficking in the *trans*-Golgi network (Bock *et al.*, 1997). While the mechanisms underlying SNARE complex formation are largely uncovered, the molecular basis of SNARE regulation still remains to be elucidated (Jahn and Scheller, 2006; Bethani *et al.*, 2007). Several studies showed that, *i)* SNARE isoforms that do not fuse *in vivo* can form SNARE complexes *in vitro* (Fasshauer *et al.*, 1999; Yang *et al.*, 1999; Tsui and Banfield, 2000), *ii)* some SNAREs are known to operate in different fusion steps (Bethani *et al.*, 2007), and *iii)* some SNAREs are able to substitute for another *in vivo* (Gotte and Gallwitz, 1997; Liu and Barlowe, 2002; Borisovska *et al.*, 2005). In this sense, it has been demonstrated that SNARE proteins are both necessary and sufficient for specific membrane fusion *in vitro*, but so-called cognate and also non-cognate SNARE complexes with no functional cross-talk can form *in vivo.* Although only cognate SNARE complexes have functional impact, cognate as well as non-cognate configurations are able to form complexes with equally high thermostabilities (Fasshauer *et al.*, 1999; Scales *et al.*, 2000).

Recent findings demonstrate that the formation of acceptor complexes between Q_a- and $Q_{b,c}$-SNAREs is the rate-limiting step of membrane fusion (*Figure 10*). However, only when complementary R-SNAREs engage, a productive fusion event is initiated (McNew *et al.*, 2000) that mediates specificity only in *trans*-configuration and later stage in the SNARE assembly-disassembly cycle (Bethani *et al.*, 2007). Although one SNARE complex is sufficient for membrane fusion (van den Bogaart *et al.*, 2010), the most critical parameter turns out to be the surface density of SNARE proteins, which controls the rate and extent of SNARE-mediated membrane fusion (Ji *et al.*, 2010).

3.1 Regulatory proteins of trafficking specificity

Regulatory proteins that could restrict SNARE activity spatially or temporally, or perform proof-reading for SNARE pairing in *trans*, have been proposed to be essential in the vesicle trafficking requisite for the proper functioning of the cells (Shen *et al.*, 2007). In this sense, kinetics of SNARE assembly *in vivo* (Rothman and Sollner, 1997) can be controlled by several accessory proteins termed SNARE-masters (Gerst, 1999): *i)* Rab proteins (Darchen and Goud, 2000;

Zerial and McBride, 2001; Stenmark, 2009), *ii)* the Ca^{2+} sensor synaptotagmin[7] (SYT) (Brose *et al.*, 1992; Jahn and Niemann, 1994; Chapman, 2002), *iii)* Munc13 proteins and STX-binding Sec1/Munc18-proteins (Jahn and Sudhof, 1999; Guo *et al.*, 2000; Jahn *et al.*, 2003; Gerst, 2003), and *iv)* complexins.

3.1.1 Rab GTPases

The role of Rab (ras genes from rat brain) family members in vesicular trafficking was first discovered in 1988 in yeast. Approximately 70 Rabs and Rab-like proteins are encoded by the human genome (Pfeffer, 2001; Pfeffer, 2005; Stenmark, 2009). Recent evidence suggests that monomeric Rab proteins, which form the largest branch of the Ras superfamily, function in the initial steps of membrane tethering/docking for guidance of vesicular transport towards the acceptor compartment (Balch, 1990; Jahn and Sudhof, 1999; Zerial and McBride, 2001). They are considered to ensure the specificity of vesicle docking by acting upstream of SNARE complex assembly (Zerial and McBride, 2001) but are not involved in the fusion reaction itself. In order to temporally and spatially coordinate membrane transport, Rab GTPases cycle between an active GTP-bound (membrane-associated) and an inactive GDP-bound state (soluble). This conformational cycle highly depends on downstream Rab effectors that control the rates of GDP-GTP exchange and GTP hydrolysis (Takai *et al.*, 2001; Zerial and McBride, 2001; Lee *et al.*, 2009; Stenmark, 2009).

In neutrophils, for example, Rab5a is a key regulator of phagolysosome maturation (Perskvist *et al.*, 2002) while Rab7 determines later fusion events with late endosomes and lysosomes (Vieira *et al.*, 2003; Roberts *et al.*, 2006). Phagosome maturation requires the recycling of certain membrane compounds, a process that involves Rab11 (Leiva *et al.*, 2006). Rab27a controls azurophil granule exocytosis and regulates the activation of the NADPH oxidase at the plasma membrane (Stinchcombe *et al.*, 2001; Izumi *et al.*, 2003; Munafo *et al.*, 2007; Johnson *et al.*, 2010; Johnson *et al.*, 2011). Also, Herrero-Turrión *et al.* identified Rab27a in the exocytosis of gelatinase and specific granules (Herrero-Turrion *et al.*, 2008).

3.1.2 Intracellular Ca^{2+}

Ca^{2+} has been identified as a key point for secretory regulated exocytosis (Blank *et al.*, 2001) due to its effect on the orientation of membrane bilayers. The binding of Ca^{2+} to the phosphate head groups of the opposing bilayers leads to the release of water from hydrated Ca^{2+} ions and

[7] In the 1990s, Thomas Südhof (Nobel Prize in Physiology or Medicine, 2013) discovered the role of synaptotagmins and Munc proteins in the regulation of SNARE-mediated membrane fusion.

bilayer destabilization in order to promote membrane fusion (Jeremic *et al.*, 2006). The low fusion rate of *trans*-SNARE complex formation corresponds in the absence of Ca^{2+} to τ= 16min whereas it changes dramatically to τ~10s in the presence of Ca^{2+}. The phospholipid PIP_2 has long been known as an important activator of Ca^{2+}-dependent regulated exocytosis due to its trigger function of the Ca^{2+} release from the ER, the major reservoir for free Ca^{2+} ions (Montero *et al.*, 1995). Also, Ca^{2+} around the Golgi is contributory to the membrane trafficking in ER-to-Golgi-to-plasma membrane delivery of cargo (Micaroni, 2010; Pizzo *et al.*, 2010). In general, a temporary increase of Ca^{2+} in the cytosol is observed during a pulse of protein traffic (Lew *et al.*, 1986; Micaroni, 2010). However, it is important to note that Ca^{2+} alone cannot regulate the degree of vesicle content release (Snyder *et al.*, 2006). Additional levels of regulation are mediated by Ca^{2+}-binding proteins, such as SYT.

3.1.2.1 Synaptotagmin

SYT was the first regulator discovered in the membrane of synaptic and large dense-core vesicles (Chapman, 2002; Sudhof, 2002). By now, 13 isoforms have been identified in neurons and non-neuronal cells (Sudhof, 2002). SYT has one transmembrane domain, a Q_a-SNARE binding domain, a lysine- and arginine-rich linker and two Ca^{2+} binding domains, C2A and C2B at the C-terminus (Chapman, 2002). SYT is able to interact with STX1 and SNAP25 upon phosphorylation thus bringing them into close proximity (Bennett and Scheller, 1993; Davletov *et al.*, 1993; Popoli, 1993; Verona *et al.*, 2000; Rizo, 2010). Also, SYT was demonstrated to be a substrate for calmodulin-activated kinase II (Popoli, 1993) and casein kinases I and II (Bennett and Scheller, 1993; Davletov *et al.*, 1993). When Ca^{2+} is present, the Ca^{2+} sensor SYT (Geppert *et al.*, 1994; Fernandez-Chacon *et al.*, 2001; Bai and Chapman, 2004) releases the Q_a-SNAREs so they can fully zipper with the R-SNARE, thus affecting kinetics of exocytosis (Nagy *et al.*, 2006; Snyder *et al.*, 2006) and whether full or kiss-and-run fusion will take place (Wang *et al.*, 2003). After activation of exocytosis, the Ca^{2+} binding regions are inserted into the membrane. The exact mechanism by which SYT stimulates membrane fusion before or after Ca^{2+} influx is elusive (Mahal *et al.*, 2002). In neutrophils, SYTII is associated with specific granules, and translocates to the plasma membrane upon stimulation (Lindmark *et al.*, 2002).

3.1.2.2 Munc13 proteins

In the docking step, Sec1/Munc18 (SM) proteins are able to fold into a clasp structure (Misura *et al.*, 2000; Izumi *et al.*, 2007) (*Figure 11*) and specifically bind to the N-terminal regulatory H_{abc} domain of STX, thus preventing other SNARE proteins from binding to it (Fan *et al.*, 2007).

Alternatively, they can accelerate fusion for only the cognate SNAREs for exocytosis, therefore enhancing fusion specificity (Snyder *et al.*, 2006; Shen *et al.*, 2007). Munc18-1 has been shown to interact with STX1, 2, and 3; Munc18-2 with STX1, 3 (Tadokoro *et al.*, 2007; Peng *et al.*, 2010), and slightly with STX2 (Hata and Sudhof, 1995); Munc18-3 interacts with STX2 and 4 (ter Beest *et al.*, 2005; Latham *et al.*, 2006) and to a lesser extent with STX1 (Tellam *et al.*, 1995). Recently, Munc18-2 has also been shown to interact with STX11 (Cote *et al.*, 2009). In neutrophils, Munc18-2 is associated preferentially with azurophil granules but could also be found with secondary and gelatinase granules, while Munc18-3 is majorily associated with secondary and gelatinase granules (Brochetta *et al.*, 2008). Displacement of Munc18 from STX might be regulated by either phosphorylation (Shuang *et al.*, 1998; Fujita *et al.*, 1998; Fletcher *et al.*, 1999; Reed *et al.*, 1999; Liu *et al.*, 2007) or by other proteins such as tomosyn (Fujita *et al.*, 1998) or Munc13 (Betz *et al.*, 1998). Munc13 proteins are Ca^{2+}-binding proteins that are responsive to the stimulation with protein kinase C (Li *et al.*, 2011; Sudhof and Rizo, 2011; Boswell *et al.*, 2012) and play an essential role in vesicle priming. Both releasable vesicle pools, slowly releasable pool (SRP) and readily releasable pool (RRP), are refilled from the unprimed pool (UPP) by a process called vesicle priming (Becherer and Rettig, 2006), a process of different ATP-dependent reactions (Klenchin and Martin, 2000; Stojilkovic, 2005; Becherer and Rettig, 2006). Munc13-1 protein markedly accelerates the transition from the STX-Munc18 complex to the SNARE complex by binding with its C2A domain to the SNARE, and with its C2B to the membrane (Boswell *et al.*, 2012). In neutrophils, Munc13-4 regulates exocytosis of azurophil (Johnson *et al.*, 2011) and gelatinase granules (Pivot-Pajot *et al.*, 2008).

3.1.3 Complexins

Complexins, also called synaphins, are soluble proteins of 15 kDa (Marz and Hanson, 2002) able to bind to the partially assembled *trans*-SNARE complex with nanomolar affinity in order to clamp membrane fusion at later stages of SNARE assembly (McMahon *et al.*, 1995; Tokumaru *et al.*, 2001; Giraudo *et al.*, 2006). Complexin I and II are highly homologous proteins that bind strongly and with a 1:1 stoichiometry to the SNARE complex (McMahon *et al.*, 1995; Marz and Hanson, 2002) at the STX-VAMP interface (Chen *et al.*, 2002; Bracher *et al.*, 2002). Upon an increase in Ca^{2+} concentration, SYT displaces complexin allowing the SNARE complex to fulfill membrane fusion (Tokumaru *et al.*, 2008; Sudhof, 2013).

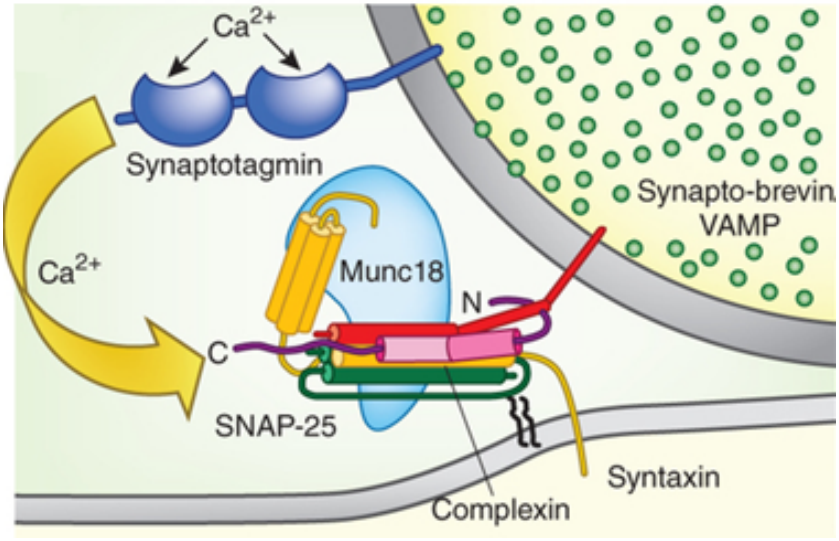

Figure 11 *Primed vesicle fusion complex, involving SNARE proteins, Munc18-1 and complexin with synaptotagmin ready to mediate fusion pore formation upon Ca^{2+} binding (Sudhof, 2013).*

3.2 Phosphorylation of SNAREs and their regulators

Q- and R-SNAREs can be modified post-translationally, mainly by phosphorylation (Turner *et al.*, 1999; Gerst, 2003; Snyder *et al.*, 2006) in order to regulate interactions between SNAREs and their regulators (Craig *et al.*, 2003). SNAREs can be substrates for protein kinase A, C, Ca^{2+}- and calmodulin-activated kinases II as well as casein kinases I and II (Nielander *et al.*, 1995; Lin and Scheller, 2000). Q_a-SNAREs are phosphorylated by casein kinases II and I on two separate phosphorylation sites next to the N-terminal regulatory H_{abc} domains (Bracher and Weissenhorn, 2004) thereby increasing the binding affinities to regulatory proteins Munc18 and SYT (Risinger and Bennett, 1999). On the contrary, phosphorylation of STX4 by protein kinase C has been shown to disrupt their interaction in endothelial cells (Fu *et al.*, 2005). Interestingly, protein tyrosine phosphatase, an ubiquitously expressed non-receptor tyrosine-specific phosphatase localized on the cytoplasmic face of the ER (Frangioni *et al.*, 1992), also regulates the interaction of Munc18-1 with STX1 through dephosphorylation of a specific tyrosine residue of Munc18-1 (Lim *et al.*, 2013).

In vitro phosphorylation of SNAP25 by protein kinase C results in a decrease in affinity for STX (Shimazaki *et al.*, 1996). Phosphorylation of SNAP23 on threonine residue 138 by protein kinase A (Han *et al.*, 2004) can lead to an increased secretion from vesicles. SNAP23 can also be phosphorylated by protein kinase C (Polgar *et al.*, 2003), whereas modification by STX-associated kinase is able to affect SNARE stability and assembly (Cabaniols *et al.*, 1999).

Also SNARE regulatory proteins have been described to have phosphorylation sites. For example, SYT can be phosphorylated by CK I at threonine 128 in rats and threonine 129 in bovine (Davletov *et al.*, 1993).

4. SNAREs in neutrophils

Since the discovery of the first SNARE complex involving SNAP25, STX1A and VAMP2 (Jeremic *et al.*, 2004), the so-called minimal fusion machinery (Weber *et al.*, 1998), SNAREs have intensively been characterized in the regulated exocytosis of neurotransmitters in neurons (Weimer and Jorgensen, 2003). Emerging evidence shows that SNARE proteins also play a critical role in the regulation of immune responses mediated by neutrophils (Stow *et al.*, 2006).

4.1 SNARE expression

Previous evidences have identified a number of SNARE proteins being expressed in neutrophils. In 1997, Nabokina *et al.* reported that SNAP25 is mainly present in peroxidase-negative granules of human neutrophils (Nabokina *et al.*, 1997). Controversial studies questioned the apparently low amount of this protein present in human neutrophils.

In 1999, Martín-Martín *et al.* published the expression profile of SNAREs in DMSO-differentiated HL-60 cells, a cell model for neutrophils. On the mRNA level, the Q_a-SNAREs STX1A, 3, 4, 5, 6, 7, 9, 11, and 16 were expressed in dHL-60 cells. The level of expression of STXs 3, 4, 6, and 11 was increased during neutrophil differentiation of HL-60 cells, whereas the expression of STXs 1A, 5, 9, and 16 was unchanged (Martin-Martin *et al.*, 1999). In 2001, Smolen *et al.* detected VAMP2 and STX4 in neutrophils and in the neutrophil-like HL-60 library to be upregulated upon differentiation (Smolen *et al.*, 2001). In addition, several members of the R-SNARE family, such as VAMP1 to 5, were also found expressed in the dHL-60 cells and primary neutrophils (Mollinedo *et al.*, 2006). The upregulated expression of these SNAREs following differentiation of the HL-60 cells has been argumented to point towards a functional importance.

Many studies have aimed to elucidate the functional importance of these SNARE proteins in neutrophil responses. *Table 1* summarizes the results of studies assessing SNARE implication in neutrophil responses, respectively.

4.2 SNARE proteins in phagocytosis and the respiratory burst

Phagosome maturation during internalization of bacteria in neutrophils requires several interactions mediated by SNARE proteins (Stow *et al.*, 2006). However the precise molecular

mechanisms regulating the formation of the phagolysosome in neutrophils are still elusive (Perskvist *et al.*, 2002). In studies using intracellular and extracellular bacterial pathogens, Perksvist *et al.* found that the Q_a-SNARE STX4 was retained for a longer time period with the Rab GTPase Rab5a in GTP-bound form on the membrane of the intracellular pathogen *Mycobacterium tuberculosis*, compared to the extracellular pathogen *Staphylococcus aureus*.

The expression of the Q_a-SNARE STX7 has been investigated in human peripheral blood neutrophils and neutrophil-differentiated HL-60 cells. Xie *et al.* found that STX7 was located in the membranes of the neutrophil-specific granules, with a major location in azurophil granules (Xie *et al.*, 2010). Due to its primary location, STX7 might be involved in azurophil granule fusion during phagocytosis (Xie *et al.*, 2010).

Regarding the phagocytosis-stimulated H_2O_2 production in neutrophils, treatment with HIV transactivator of transcription (TAT)-SNAP23 fusion protein resulted in reduced respiratory burst activity by 60% (Uriarte *et al.*, 2011). Using a mouse model to investigate sepsis, the same authors also detected that TAT-SNAP23 significantly decreased the neutrophil respiratory burst (Bai *et al.*, 2014). By using the same approach, TAT-STX4 was also introduced into neutrophils and affected the respiratory burst activity (McLeish *et al.*, 2013).

4.3 SNARE proteins in degranulation

Martín-Martín *et al.* show that STX6 and SNAP23 are involved in human neutrophil exocytosis. After localizing STX6 mainly at the plasma membrane and SNAP23 in the gelatinase and specific granules, they found them colocalized upon neutrophil activation. Studies using antibodies against STX6 resulted in inhibited exocytosis of CD67- and CD63-enriched granules, while antibodies against SNAP23 only resulted in inhibited exocytosis of CD63-enriched granules (Martin-Martin *et al.*, 2000).

Using the same approach, specific antibodies against the R-SNARE VAMP2 inhibited the exocytosis of CD66b-enriched specific and gelatinase granules in electropermeabilized neutrophils (Mollinedo *et al.*, 2003). Upon cell activation with phorbol myristate acetate, VAMP2 interacts with STX4 localized to the plasma membrane, and is thus also involved in exocytosis of specific and gelatinase granules (Mollinedo *et al.*, 2003). The localization of VAMP2 is in accordance with the findings of Feng *et al.* who labeled neutrophils with immunonanogold antibodies against VAMP2 (Feng *et al.*, 2001).

While Nabokina found VAMP1, which might be causing decreased capacity to exocytosis in chronic myeloid leukemia (Nuyanzina and Nabokina, 2004), to be associated with membranes of gelatinase and specific secretory granules (Nabokina and Revin, 2002), Mollinedo *et al.*

further investigated the axis with involvement of other SNAREs. Their data indicate that VAMP1 and VAMP7 can complex also with STX4, together with the $Q_{b,c}$-SNARE SNAP23. This SNARE combination has been attributed the regulated secretion of azurophil granules. In addition, the previously discovered VAMP2-STX4 complex potentially interacts with SNAP23 in the exocytosis of specific and gelatinase granules (Mollinedo *et al.*, 2006). Contrary to Martín-Martín *et al.* and in accordance with Mollinedo *et al.*, Logan *et al.* found STX4 but not STX6 interacting with SNAP23 (Logan *et al.*, 2006).

Furthermore, the R-SNAREs VAMP7 and VAMP8 were localized to granule and membrane-enriched fractions in neutrophils. Using anti-VAMP7 antibodies, Logan *et al.* observed impaired secretion of MPO, LTF and MMP-9 (Logan *et al.*, 2006).

Brochetta *et al.* described that the Sec/Munc18 protein Munc18-2 is located in all cytoplasmic granules and interacts with the Q_a-SNARE STX3 in neutrophils (Brochetta *et al.*, 2008).

While Martín-Martín *et al.* and Mollinedo *et al.* both described the $Q_{b,c}$-SNARE SNAP23 to be localized on gelatinase and specific granules, Uriarte *et al.* observed significant inhibition of stimulated exocytosis of secretory vesicles and gelatinase and specific granules by introducing TAT-SNAP23 fusion protein into neutrophils (Uriarte *et al.*, 2011). Also studies by Uriarte *et al.* confirmed the SNAP23 interplay with VAMP2 and STX4.

In a mouse model, D'Orlando *et al.* investigated the knockout effect of the Q_a-SNARE STX11. While STX11 controls the cytotoxic release from NK cells and CD8 T lymphocytes, it also regulates neutrophil degranulation (D'Orlando *et al.*, 2013).

As described above, several SNAREs have already been investigated to play an important role in neutrophil functions. However, to date, none of them has been implicated in neutrophil-mediated cytokine secretion.

Table 1 *SNARE proteins involved in neutrophil functions.*

SNARE	Species	Biological function(s)	Reference
Q_a-SNAREs			
STX3	Human	Degranulation	(Brochetta *et al.*, 2008)
STX4	Human	Phagocytosis	(Perskvist *et al.*, 2002)
STX4	Human	Degranulation	(Mollinedo *et al.*, 2003)
STX4	Human	Respiratory burst	(McLeish *et al.*, 2013)
STX7	Human	Phagocytosis	(Xie *et al.*, 2010)
STX11	Mouse	Degranulation	(D'Orlando *et al.*, 2013)
$Q_{b,c}$-SNAREs			
SNAP23, STX6 (Q_c)	Human	Degranulation	(Martin-Martin *et al.*, 2000)
SNAP23	Human	Degranulation	(Mollinedo *et al.*, 2006)
SNAP23	Human	Respiratory burst, Degranulation	(Uriarte *et al.*, 2011)
SNAP23	Mouse	Respiratory burst	(Bai *et al.*, 2014)
SNAP25	Human	Degranulation	(Nabokina *et al.*, 1997)
R-SNAREs			
VAMP1	Human	Degranulation	(Nabokina and Revin, 2002) (Nuyanzina and Nabokina, 2004) (Mollinedo *et al.*, 2006)
VAMP2	Human	Degranulation	(Feng *et al.*, 2001) (Mollinedo *et al.*, 2003) (Mollinedo *et al.*, 2006)
VAMP7	Human	Degranulation	(Logan *et al.*, 2006)

Scope and Aim of the Thesis

Scope and Aim of the Thesis

Neutrophil granulocytes are crucial effector cells in the innate and adaptive immunity. As the first cells to be recruited to sites of infection, they are able to rapidly release a broad variety of granule proteins and pro-inflammatory cytokines to efficiently kill intruding pathogens and recruit other immune cells to the inflammatory environment, respectively. In eukaryotic cells, specific interactions of so-called soluble *N*-ethylmaleimide-sensitive-factor attachment receptor (SNARE) proteins lead to the formation of SNARE complexes in order to guide intracellular vesicle trafficking and exocytosis. Due to the important contributory impact of neutrophil-derived products to the development of chronic inflammatory diseases, a profound and detailed understanding of the regulatory release mechanisms of neutrophil-derived products is essential for the development of anti-inflammatory therapies in chronic inflammatory diseases.

The overall aim of the present study was to investigate the regulatory processes of mediator release in neutrophils during inflammation.

So far, not a single study has focused on the expression and the role of the SNARE exocytic machinery in the release of neutrophil-derived cytokines and granules during inflammation. Therefore, our objectives are the following:

1. **Characterization of the pro-inflammatory response in regard to exocytosis.**

Primary human neutrophils were incubated with bacterial LPS to mimic pro-inflammatory conditions. The effect of LPS on degranulation and secretion of pro-inflammatory cytokines, described to have a prominent role in the development of chronic inflammatory diseases, was characterized *via* flow cytometry. A novel linear fitting approach was built to perform correlative investigations on the kinetic profiles derived from cytokine secretion and degranulation. This approach gives a first basis for deeper understanding of the relationship between these two processes.

2. **Profiling the expression of Q_a-, $Q_{b,c}$-, and R-SNAREs**.

Expression levels of Q_a-, $Q_{b,c}$-, and R-SNAREs in native and differentiated HL-60 cells (dHL-60 cells), a neutrophil-like cell model, as well as primary neutrophils were determined *via* whole-transcript analysis, qPCR and immunoblotting. Since *i)* syntaxin 3 (STX3) expression was upregulated upon HL-60 differentiation, *ii)* no study has considered its role in neutrophils, we investigated whether STX3 is required for neutrophil functions.

3. **Determination of the functional role of the Q_a-SNARE STX3 in cytokine release and degranulation during inflammation**.

Using an RNAi strategy and cytometric bead array approach, the Q_a-SNARE STX3 was identified as an essential actor in the release of IL-1α, IL-1β, IL-12b, and CCL4 without alteration of other cytokine secretion in dHL-60 cells. Additionally, ELISA results demonstrated that STX3 was involved in MMP-9 exocytosis from gelatinase granules.

4. **Determination of STX3 intracellular localization**.

Neutrophils and dHL-60 cells were labeled against STX3 in order to determine its intracellular localization by immunofluorescence analysis. STX3 was localized to gelatinase granules as well as secretory vesicles.

5. **Identification of $Q_{b,c}$- and R-SNARE interaction partners of STX3.**

To further investigate the precise mechanism by which STX3 regulates the trafficking pathway, we aimed to find potential $Q_{b,c}$- and R-SNARE partners interacting with STX3 that specifically direct the pro-inflammatory mediator release.

On one hand, immunofluorescence analysis presented that STX3 colocalizes with the $Q_{b,c}$-SNAREs SNAP23 and SNAP29. However, knockdown experiments showed that SNAP23 only affected IL-8 release while SNAP29 regulated the release of IL-12b, CCL2 and IL-8 as well as MMP-9.

On the other hand, dHL-60 cells treated with siRNA against the R-SNARE VAMP3 resulted in deregulated secretion of CCL4, IL-8 as well as MMP-9. Proximity-ligation studies although revealed that VAMP3 did not interact with the $Q_{b,c}$-SNARE SNAP29. Hence, VAMP3 is hypothesized to interfere with the secretory pathway at the level of the plasma membrane.

Taken together, our findings provide first evidence that the Q_a-SNARE STX3 controls secretion of IL-1α, IL-1β, IL-12b, and CCL4 during gelatinase degranulation in a neutrophil-like cell model. Also, STX3 together with SNAP29 might form so-called “cognate” SNARE complexes that specifically mediate IL-12b and MMP-9 trafficking under pro-inflammatory conditions. The role of the R-SNARE VAMP3 is not entirely determined due to its localization at the plasma membrane.

Results

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I Regulation of neutrophil degranulation and cytokine secretion: a novel model approach based on linear fitting

Neutrophil granulocytes represent the first immune cells recruited to the site of inflammation. For many decades, they have been solely regarded as storage cells of preformed granules. Over the last years, the discovery that neutrophils are also capable of *de novo* synthesis and secretion of pro-inflammatory cytokines reveals that their role is not only limited to the direct killing of pathogens but also includes immunomodulation of the adaptive immune system. Here, we aimed to **characterize the concurrent behavior of neutrophil-mediated cytokine release and degranulation upon pro-inflammatory conditions.**

Purified human neutrophils were stimulated for different time points with bacterial lipopolysaccharide (LPS). Cells as well as supernatants were analyzed by flow cytometry techniques and used to establish kinetic secretion profiles of granules and cytokines, which have been described to play essential roles in the development of inflammatory disorders. The capacity for degranulation was investigated by the upregulation of granule-specific membrane molecules as a consequence of membrane fusion from granules with the plasma membrane (Borregaard and Cowland, 1997). The secreted amount of several cytokines was detected simultaneously in supernatants *via* antibody-labeled beads with discrete fluorescence intensities in a cytometric bead array approach.

To analyze the link between cytokine release and degranulation time series, we propose an original strategy based on linear fitting, which may be used as a guideline, to investigate in more details the relationship of granule proteins and cytokines secreted to the inflammatory site.

Regulation of neutrophil degranulation and cytokine secretion: a novel model approach based on linear fitting

Isabelle Naegelen *), Nicolas Beaume *), Sébastien Plançon, Eric J. Tschirhart, and Sabrina Bréchard

*) contributed equally

University of Luxembourg, Life Sciences Research Unit, Calcium Signaling and Inflammation, 162A avenue de la Faïencerie, L-1511 Luxembourg

Corresponding author:

Sabrina Bréchard, PhD

University of Luxembourg, Life Sciences Research Unit, Calcium Signaling and Inflammation

162A avenue de la Faïencerie

L-1511 Luxembourg, Luxembourg

Phone number: +352/466644-6434

Fax number: +352/466644-6435

E-mail address: sabrina.brechard@uni.lu

Running title: Kinetic modelling of neutrophil functions during inflammation

Abbreviations: CBA, cytometric bead array; LPS, bacterial lipopolysaccharide

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Abstract

Neutrophils participate in the maintenance of host integrity by releasing various antimicrobial and cytotoxic proteins during degranulation. Due to recent advances, a major role has been attributed to neutrophil-derived cytokine secretion in the initiation, exacerbation and resolution of inflammatory responses. Because the release of neutrophil-derived products orchestrates the action of other immune cells at the infection site and thus, can contribute to the development and chronicity of inflammatory diseases, we aimed to investigate in more details the spatiotemporal regulation of neutrophil-mediated release mechanisms of pro-inflammatory mediators.

Purified human neutrophils were stimulated for different time points with bacterial lipopolysaccharide. Cells and supernatants were analyzed by flow cytometry techniques and used to establish secretion profiles of granules and cytokines, which have been described to play crucial roles in the development of inflammatory disorders. To analyze the link between cytokine release and degranulation time series, we propose an original strategy based on linear fitting, which may be used as a guideline, to *i)* define the relationship of granule proteins and cytokines secreted to the inflammatory site, and to *ii)* investigate the spatial regulation of neutrophil cytokine release.

The model approach presented here aims to predict the correlation between neutrophil-derived cytokine secretion and degranulation and may easily be extrapolated to investigate the relationship between other types of short-time series of functional processes.

Keywords: inflammation, neutrophils, cytokine secretion, degranulation, linear fitting

Introduction

Historically, neutrophils were described as simple professional killers of invading pathogens to the human organism (Nathan, 2006). In this regard, it was considered that only the release of various antimicrobial and cytotoxic proteins, synthesized and distributed into different types of granules, participated in the innate immune response mediated by neutrophils. Granule types have been characterized to be readily mobilized upon an inflammatory stimulus towards the plasma membrane in reverse order to their formation according to the *formed-first-released-last model* (Pellme *et al.*, 2006). Indeed, in the different stages of neutrophil development, azurophil granules are formed first followed by specific granules, gelatinase granules and lastly by secretory vesicles, which are the most easily mobilized organelles in the mature neutrophils.

Due to recent progress, this classical view has been expanded by the acknowledgment that appropriately activated neutrophils constitute a substantial source of a variety of secreted cytokines supporting a direct contribution of these cells in the regulation framework of the adaptive immune response (Cassatella *et al.*, 1995; Scapini *et al.*, 2000; Kasama *et al.*, 2005). Neutrophils are not only a source of *de novo* synthesis of cytokines dependent on gene induction but have also the capacity to express cytokines at a basal level from preformed stores (Pellme *et al.*, 2006). However, precise intracellular localization of these packaged cytokines and mechanisms underlining their secretion remain largely elusive. The assumption widely accepted is that multiple secretory pathways coexist in neutrophils allowing the regulated release of diverse pro-inflammatory mediators (Stanley and Lacy, 2010). Preformed cytokines are instantly released upon ligand-receptor signaling during the so-called "regulated exocytosis" process (Duitman *et al.*, 2011) whereas *de novo* synthesized cytokines may be released after trafficking *via* recycling endosomes during the mechanism referred to as "constitutive exocytosis" (Crivellato *et al.*, 2003; Moqbel and Coughlin, 2006). Additionally, variations of these two main classical secretion pathways have also been reported (Pickett and Edwardson, 2006). These distinct processes selectively control the combination of granule proteins and cytokines released into the local microenvironment from neutrophils over a temporal and spatial range, and are thus regulatory mechanisms important for the onset and resolution of inflammation enabling the development of an appropriate inflammatory response (Matsukawa and Yoshinaga, 1998).

It is now largely recognized that neutrophil-derived granule proteins and cytokines contribute to the maintenance of the inflammatory response and the ongoing process of tissue damage leading to the development of many chronic inflammatory disorders such as inflammatory bowel diseases (Radford-Smith and Jewell, 1996), rheumatoid arthritis (Edwards and Hallett, 1997),

and atherosclerosis (Soehnlein, 2012). Determination of the regulatory mechanisms mediating the different patterns of cytokine trafficking and release may create opportunities to define new targets or strategies to selectively reduce cytokine secretion in clinical diseases.

Therefore, we selected relevant cytokines, secreted by neutrophils and described to contribute to the development of chronic inflammation diseases, in order to investigate their release in combination to degranulation upon stimulation with bacterial lipopolysaccharide (LPS).

Here, we propose an appealing model based on a linear fitting approach of cytokine secretion and degranulation giving a first basis for deeper understanding of the relationship between these two processes. It also provides a predictive view on the distribution of cytokines in neutrophils and offers an outstanding starting point to target future research on release mechanisms involved in inflammatory processes.

Materials and Methods

Purification of human neutrophils

Peripheral blood of healthy volunteers was collected in EDTA-containing tubes (BD Vacutainer®, BD Biosciences, Erembodegem, Belgium). Samples were collected in accordance with the good clinical practices and following the national and international ethical recommendations. Neutrophils were isolated from blood samples by Polymorphprep™ separation procedure (Axis-Shield, Dundee, Scotland) according to manufacturer's instructions. Remaining erythrocytes in the neutrophil cell suspension were lysed for 10 min with red blood cell lysis buffer (pH 7.4). Neutrophils were washed and resuspended in PBS 1X (pH 7.4). Purity of isolated neutrophils was analyzed by the BD FACSCanto™ II flow cytometer (BD Biosciences) using two mixtures of selection markers CD66b-FITC/CD11b-PE/CD14-APC and CD15-FITC/CD16-PE/CD45-APC (Immunotools, Friesoythe, Germany) on 10,000 events in the gated population of homogenous (FSC-A *vs.* SSC-A), single (SSC-A *vs.* SSC-H) and living cells (negative cells for Sytox Blue staining (Invitrogen, Gent, Belgium)). Purified neutrophils are positive for all the selection markers used by flow cytometry. Eosinophils can be distinguished from the neutrophil population since they are known to be $CD16^-$ (van de Geijn *et al.*, 2011). Human neutrophils were cultured in X-VIVO™ 15 medium with L-glutamine, and gentamicin (Lonza, Verviers, Belgium) at 37°C and 5% CO_2 up to 24 h after purification.

Cell stimulation

Purified neutrophils were stimulated with bacterial LPS from *E. coli* serotype O111:B4 (Sigma, Bornem, Belgium) for simulating pro-inflammatory conditions. For kinetic studies of cytokine secretion and degranulation, neutrophils were stimulated with 100 ng/mL LPS for 2, 4, 6, 12,

and 24 h under serum-free conditions to avoid any serum component contamination, which could interfere with specific LPS-induced cell responses.

Cell analysis by flow cytometry

The most relevant markers have been selected for degranulation analysis, in accordance with the literature (Borregaard and Cowland, 1997). Degranulation was determined by measuring the expression of CD markers characteristic for azurophil granules (CD63-PE), specific granules (CD15-FITC, CD66b-FITC), gelatinase granules (CD11b-PE), and secretory vesicles (CD13-APC, CD14-APC, CD18-FITC, CD35-FITC, CD45-APC) at the plasma membrane by flow cytometry (all antibodies are from BD Biosciences except CD14-APC from Immunotools).

IgG1-FITC, IgG2a-PE (BD Biosciences) and IgG1-APC antibodies (Immunotools) were used as negative isotype controls, whereas CD45-FITC, CD45-PE or CD45-APC (BD Biosciences) single dye staining was used to set compensations. Data analysis was performed by measuring the mean fluorescence intensity (MFI) for each CD marker with BD FACSDiva software (BD Biosciences) on the gated population of granulocytes (FSC-A *vs.* SSC-A), single (SSC-A *vs.* SSC-H) and living cells (negative cells for Sytox Blue staining (Invitrogen)). In total, 10,000 events were recorded *per* staining. The relative translocation of CD markers to the plasma membrane for each granule was determined by calculating the ratio between MFI of LPS-stimulated cells and non-stimulated control from the same time point.

Measurement of cytokine secretion by Cytometric Bead Array (CBA)

Density of human neutrophils was adjusted to $10x10^6$ cells *per* condition for subsequent quantitative measurement of cytokine secretion by LPS-stimulated cells, respectively. Fresh supernatants were collected and used directly for cytometric bead array (CBA, BD Biosciences) analysis. The multiplex standard curve composed of mixed cytokine standards was set up by serial dilutions according to the manufacturer's instructions. Selected capture beads were prepared and added to supernatants. The following beads were used: CCL2 (MCP1, bead D8), CCL3 (MIP1α, bead B9), CCL4 (MIP1ß, bead E4), CCL5 (RANTES, bead D4), IL-1α (bead D6), IL-1β (bead B4), IL-6 (bead A7), IL-8 (CXCL8, bead A9), IL-12b (bead E5), and TNFα (bead C4). After 1 h of incubation, detection reagent was added to each sample. After 2 h of incubation, samples were rinsed with wash buffer and centrifuged. Samples were washed again prior to flow cytometry analysis (BD FACSCanto™ II, BD Biosciences). Results were quantified using the standard curves and the Flow Cytometric Analysis Program (FCAP) Array software (Soft Flow, Minneapolis, USA).

Linear fitting approach via R statistical software

Kinetic profiles of cytokine secretion and degranulation were imported into R statistical software (www.r-project.org) and a linear regression approach was applied. This approach was used to find the optimal proportionality factor, namely the slope of the model, and provide methods to evaluate the significance of our models. All ratio values between LPS- and non-stimulated control conditions (stimulation points 0, 2, 4, 6, 12, and 24 h) from the time series of cytokine secretion and degranulation were $\log_{10}$ normalized to minimize scale effect. For each granule-specific CD marker, a linear model has been fitted with the secreted cytokines. For each model, ANOVA analysis has been performed and the adjusted R-squared (RSQ) value and the slope of the model were retained. Models with a significant difference to the null model (p-value ≤ 0.05) and with a high adjusted RSQ value between degranulation marker *vs.* secreted cytokine (RSQ ≥ r, r being determined by simulations, see *Simulations to determine the optimal RSQ threshold*) were considered as underlying a strong similarity of pattern between a cytokine secretion and a degranulation marker. Then, cytokines linked to the same degranulation marker were clustered. To visualize these clusters, time series of the degranulation marker and its relative cytokines of the cluster were plotted. To permit comparison between time series from different scales, values from the secreted cytokines were divided by the slope of their linear model.

Simulations to determine the optimal RSQ threshold

To define the optimal RSQ threshold r, we simulated the fitting between pairs of granule marker and cytokine with controlled perturbations between them and choose the threshold r by determining at which level of perturbation the RSQ was drastically dropping. The simulations were designed as follows: Time series of each granule-specific marker and cytokines were taken individually (19 time series in total) and used to simulate matching time series with more or less perturbations. For the six points of each time series (0, 2, 4, 6, 12, and 24 h), a random number between V_i - e and V_i + e was drawn. V_i is the i^{th} element of the time series and e is a pre-defined constant. e controls the intensity of the perturbation: the highest e is, the more different both profiles are expected to be. All values from 0 to 1 with a step of 0.1 were tested for e (0, 0.1, 0.2, *etc.* to 1). Then the RSQ of the original profile *vs.* simulated profile was computed as mentioned in *Linear fitting approach via R statistical software*. This process was repeated 100 times and, for each of the 19 original profiles, the average of the RSQs was computed and plotted against the e value. To define the optimal RSQ threshold that defines a cut-off between linear fitting models with “high RSQ” and “low RSQ”, we clustered the distribution of averaged RSQ into two groups, using a k-means approach (a silhouette analysis of all clustering solution

with 2 to 10 clusters confirmed that 2 clusters was the best solution, *data not shown*). The last element of the cluster with the highest center, namely 0.82, was taken as RSQ threshold for our analysis.

Statistical analysis

Statistical analysis was performed by using the 2-tailed unpaired Student's t-test in the Prism5 software (Graph Pad Software, La Jolla, CA, USA). The difference between the means are considered statistically significant when the p-value is * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Results

Behavior of cytokine levels secreted by human neutrophils upon LPS stimulation

Since recent reports have implicated neutrophils in the development of chronic inflammatory disorders, we wanted to characterize the regulatory mechanisms in the release of neutrophil-derived products. In a first step, cytokine candidates found to be secreted by highly purified (≥ 98%) neutrophils upon LPS stimulation (see *Manuscript II*), were selected for integration into our mathematical model. These cytokines have a particular relevance since they have been reported to contribute to the development of different chronic inflammatory diseases through the recruitment of diverse immune cells to the inflammatory site (**Table I**).

To develop a reliable model that investigates the relationship between degranulation and cytokine secretion, different sets of experimental data were generated. Time series of cytokine secretion were determined to serve as input to our model. Neutrophils were treated with 100 ng/mL LPS for 2, 4, 6, 12, 24 h and subsequent quantitative measurement of cytokine secretion was performed by the CBA technique. A basal secretion level of all cytokines was detected in neutrophils under non-stimulated conditions. Secretion of most of the cytokines released into the extracellular medium was augmented with increasing time stimulation by LPS, except for CCL5, IL-1α and IL-12b, whose release was not significantly affected by LPS treatment (**Fig. 1, Table IIA**).

The secretion pattern was different for each cytokine in the way that different profiles could be identified. Except for IL-1α, secretion levels of cytokines increased up to 6 h or 12 h after treatment with LPS and decreased after 24 h. Maximal cytokine secretion was observed at *i)* 6 h LPS for IL-1β, CCL3, and TNFα; *ii)* 12 h LPS for IL-6, IL-8, CCL2, CCL4, and IL-12b. Moreover, quantities of released cytokines were highly variable. TNFα, IL-1α, IL-1β, CCL3, CCL5, and IL-12b were only discretely secreted (≤ 150 pg/mL) whereas IL-6, CCL2, and CCL4 were secreted at an intermediate level (~ 250-600 pg/mL) and IL-8 was highly secreted (≥ 30000 pg/mL).

Effect of LPS on degranulation in neutrophils

To collect data for the implementation of the model, the second series of experiments consisted in identifying the kinetic profiles of the different granule types upon time-dependent LPS stimulation. Degranulation can be determined by the upregulation of granule membrane molecules as a consequence of membrane fusion from granules with the plasma membrane (Borregaard and Cowland, 1997). Therefore, LPS-treated cells were analyzed for cell surface expression of several CD molecules known as degranulation markers, as described in *Materials and Methods*.

Results showed that LPS stimulation clearly affected the ease of mobilization of intracellular granule types in neutrophils in a time-dependent manner, as reflected by increased translocation of CD markers to the cell surface (**Fig. 2, Table IIB**). In a temporal pattern, LPS stimulation increased the presence of CD15 and CD66b, CD11b as well as CD13, CD18, CD35 and CD45 reflecting an increased release of specific granules, gelatinase granules and secretory vesicles, respectively.

Maximal increase for the majority of these CD markers was detected after 12 h of LPS stimulation, except for *i)* CD35 which showed maximal translocation towards the plasma membrane already after 6 h of LPS, and *ii)* CD13 whose expression was highest upon 24 h LPS at the plasma membrane (related to the non-stimulated control at the same time point). On the other hand, LPS was unable to trigger the mobilization of azurophil granules since CD63 expression was not changed at the plasma membrane.

Linear fitting of cytokine secretion and degranulation kinetics

Many approaches exist for the examination of time series of expression data (*e.g.* Ernst and Bar-Joseph, 2006), but none of them could be applied to analyze short-time series of secretion. For this reason, we used a novel model approach to explore the relationship between cytokine secretion and degranulation by their kinetic profiles (**Fig. 3**).

We hypothesized that time series of secreted cytokines with similar pattern to time series of degranulation markers present at the plasma membrane should have proportional values at each time point of LPS stimulation, so that a proportionality factor between the two profile curves can be defined. To address this question, we choose to use the linear regression approach, which fits best to our needs: *i)* it captures proportionality well, *ii)* it can be used with only one pair of profiles (in our case cytokine *vs.* granule-specific marker), and *iii)* it includes measures to evaluate the results (significance of the model and R-squared value, see *Materials and Methods*). All ratio values between LPS- and non-stimulated control conditions from the time series of cytokine secretion and degranulation (**Table II**) were $\log_{10}$ normalized.

For each model, ANOVA analysis has been performed and the adjusted R-squared (RSQ) value and the slope of the model were retained. While the ANOVA analyses the efficiency of this model (*i.e.* proportional kinetic profile curves are significantly different from the null model), the adjusted RSQ value measures the correlation between the kinetic profiles (*i.e.* proximity to the linear fitting). Models with a significant difference to the null model (p-value ≤ 0.05) and with a high adjusted RSQ value between degranulation marker *vs.* secreted cytokine were considered as underlying a strong similarity of pattern between a degranulation marker and a secreted cytokine. The optimal RSQ value was determined by simulations, in which pre-defined perturbations were introduced to our kinetic profiles (see *Materials and Methods*). **Fig. 4A** shows how augmenting perturbations (e from 0 to 1) influenced the linear fitting between two time series, in our example the linear fitting between the granule marker CD15 and the cytokine CCL4. The original kinetic profile is depicted by the black line, whereas the one with perturbations is represented by the red line. By plotting the average RSQ values (derived from 100 repetitions of simulations) against e values, k-means clustering can differentiate between "high" and "low" RSQ (**Fig. 4B**). By setting these parameters non-significant outcomes with "low" RSQ were eliminated, and the threshold for RSQ was set to RSQ ≥ 0.82. In **Fig. 4C**, an example of a significant linear fitting model is shown, in which the behaviour of CCL4 is correlated to CD15. Due to its p-value of 0 and RSQ of 0.91, the correlation between CCL4 and CD15 fits to the model.

Relationship between degranulation and cytokine secretion

After filtering only highly significant correlation data, results from human neutrophils were plotted. The linear fitting approach applied to the time series data showed that the secretion of 6 out of our 10 selected pro-inflammatory cytokines strongly correlated with the release of secretory vesicles, gelatinase granules, and specific granules. Four cytokines have not been correlated to any degranulation markers: TNFα, IL-12b, CCL2, and CCL5.

Secretory vesicles represented by the markers CD13, CD14, CD18, CD35 and CD45, were fitted to 4 cytokines, namely IL-1α, IL-1β, IL-8 and CCL3 (**Fig. 5A**). Moreover, time series of IL-1α and IL-8 release were strongly correlated to the degranulation marker CD11b, showing a relationship between gelatinase granules and these cytokines (**Fig. 5B**). Furthermore, the release of the cytokines IL-6, and CCL4 fitted to CD15 suggesting that secretion of these cytokines correlated to specific granules. In line with our results for IL-1α and IL-8, the two cytokines were also associated with CD66b. Interestingly there was a high overlap of cytokines that was fitted to the given markers of specific and gelatinase granules. Since no significant

cytokine correlation has been observed for CD63, azurophil granules are probably not associated with cytokine secretion.

Discussion and Conclusion

For many years, the contributory impact of neutrophils to the development of chronic inflammation was not extensively taken into account since they have been considered as terminally differentiated cells synthesizing low amount of RNA and protein (Witko-Sarsat *et al.*, 2000). Though, the vast number of neutrophils found at the site of infection cannot be neglected due to the fact that their secreted amounts of granule proteins and cytokines exert a synergistic and cumulative effect on the inflammatory tissue environment (Soehnlein, 2012). These pro-inflammatory soluble mediators are highly decisive for the onset of inflammatory processes, the activation and the recruitment of various immune cells to the infection site (Nathan, 2006). However, little is known about the combination in which cytokines and granule proteins are secreted by neutrophils. In the present report, we therefore aimed to predict the spatiotemporal regulation of pro-inflammatory mediator release by neutrophils.

Our study proposes an original approach allowing the establishment of a relationship between cytokine secretion and degranulation in neutrophils. We used the linear fitting approach to integrate data generated from own experiments and obtained from LPS-mediated short-time series of degranulation and cytokine secretion. Other approaches, such as Pearson correlation, are based only on the average of all correlated values. In contrast, our model is able to reliably establish time-specific associations between the two dynamic functions in neutrophils, respecting each time point of stimulation.

According to our results, a number of cytokines could be fitted to the different types of neutrophil granules. The cytokines IL-1α, IL-1β, IL-8 and CCL3 correlated to secretory vesicles (**Fig. 6A**), which are the most easily mobilized organelles in mature neutrophils (Borregaard and Cowland, 1997). Interestingly, IL-1α and IL-8 secretion was correlated to gelatinase and specific granules. The release of IL-6 and CCL4 was also fitted to specific degranulation. In contrast, degranulation of azurophil granules seems unchanged by LPS suggesting that either *i)* this granule type may require further cell activation to induce its mobilization towards the plasma membrane (Metzler *et al.*, 2011), or *ii)* the upregulation of CD63 at the plasma membrane might not be significant enough to be detected since azurophil granules are rather poor in receptors in contrast to secretory vesicles (Borregaard and Cowland, 1997). For this reason, no cytokine could probably be fitted to CD63 in human neutrophils.

Given our results, the linear fitting approach characterizes the concurrent behavior of a large range of neutrophil-derived cytokines and granules upon inflammation. While granules have already been characterized to be mobilized towards the plasma membrane in reverse order to their formation according to the *formed-first-released-last model* (Pellme *et al.*, 2006), our linear fitting approach underlines the key role of granules and cytokines released in a hierarchical sequence in accordance to their roles during the microbial elimination processes and inflammatory response. In addition, we would like to postulate the hypothesis that neutrophil cytokines are localized in or mobilized to the different granule types (**Fig. 6B**).

Indeed, we found a significant correlation between kinetics of IL-8 secretion and the release of specific and gelatinase granules as well as secretory vesicles in human neutrophils. Given that cytokines can exert pleiotropic functions, some of them are probably localized in different types of granules as suggested by our model. In line with this observation, Pellmé *et al.* reported that IL-8 is stored in cytoplasmic granules in resting peripheral blood neutrophils and thus, can be rapidly mobilized and released by the cells (Pellme *et al.*, 2006). Further, Terebuh *et al.* showed by immunohistochemical staining of neutrophils that IL-6 might be localized in gelatinase granules and secretory vesicles (Terebuh *et al.*, 1992). The fact that their results derived from murine neutrophils might explain the discrepancy to our observations in which human neutrophil-derived IL-6 could be fitted to specific granules only. Moreover, the role of azurophil granules during cytokine secretion appears very restricted. In line with our results showing that this granule type is not significantly translocated to the plasma membrane upon LPS stimulation in neutrophils, the release of these granule contents might be rather concentrated to their involvement in neutrophil extracellular trap formation (Metzler *et al.*, 2011).

Additional studies are required to investigate the intracellular localization of cytokines in neutrophils, but this determination remains largely elusive by the fact that reliable staining for electron microscopy is facing challenges due to the low intracellular amount of cytokines. Further, other techniques used to document subcellular organelle location of cytokines have limited resolution (*e.g.* subcellular fractionation) (Pasquali *et al.*, 1999).

In comparison to the modelling approach proposed by Rørvig *et al.* (Rorvig *et al.*, 2013), which is based on proteomic and mRNA array data to predict localization of proteins in granules, our approach is complementary by including functional data analysis. Hence, the linear fitting between degranulation and cytokine release in LPS-treated neutrophils represents an attractive method to investigate the potential localization of cytokines in the different types of granules even if additional experiments are required to confirm the intracellular localization of cytokines. Also, since our linear fitting approach has been adapted to investigate secretion kinetics, it can

easily be extrapolated for the analysis of other short-time series deriving from other cell-types, disease or developmental states, *e.g.* protein arrays or proteomics data.

Acknowledgements

We thank the healthy volunteers for their blood donation, the Croix-Rouge Luxembourgeoise and the Centre Hospitalier du Luxembourg for their collaboration.

Table I List of selected pro-inflammatory cytokines contributing to the development of chronic inflammatory disorders.

Cytokine	Cell recruitment	Chronic inflammatory disorders
TNFα	Monocytes, neutrophils	RA (Edwards and Hallett, 1997), IBD (Billiet *et al.*, 2014), A (Zhang *et al.*, 2014)
IL-1α	Neutrophils, monocytes, T cells	RA (Hueber *et al.*, 2007), IBD (Ludwiczek *et al.*, 2004)
IL-1β	Neutrophils, monocytes, T cells	RA (Edwards and Hallett, 1997)
IL-6	Monocytes, B cells	RA (Edwards and Hallett, 1997), IBD (Carey *et al.*, 2008), A (Hartman and Frishman, 2014)
IL-12b	T cells	RA (Vandenbroeck *et al.*, 2004), IBD (Schmidt *et al.*, 2002), A (Yong *et al.*, 2013)
CCL2	Monocytes, T cells	RA (Hueber *et al.*, 2007), A (Gerszten *et al.*, 1999)
CCL3	Monocytes, T cells	RA (Chintalacharuvu *et al.*, 2005), A (Kuziel *et al.*, 2003)
CCL4	Monocytes, T cells	RA (Robinson *et al.*, 1995), IBD (Banks *et al.*, 2003), A (Kuziel *et al.*, 2003)
CCL5	Eosinophils, dendritic cells and T cells	RA (Yao *et al.*, 2006), A (Kuziel *et al.*, 2003)
IL-8	Neutrophils, macrophages, and lymphocytes	RA (Edwards and Hallett, 1997), A (Gerszten *et al.*, 1999), IBD (Mitsuyama *et al.*, 1994)

RA *rheumatoid arthritis*, IBD *inflammatory bowel disease*, A *atherosclerosis*

Table II Time-dependent secretion of cytokines and granules expressed in ratio between LPS- and non-stimulated cells ± SEM of at least three independent experiments. Significantly different from non-stimulated control at the same time point: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(A)

Relative secretion	0h LPS	2h LPS	4h LPS	6h LPS	12h LPS	24h LPS
TNFα	0.85 ± 0.15	5.00 ± 1.86	19.91 ± 7.59	43.44 ± 18.23 *	44.23 ± 15.54 *	8.38 ± 2.90
IL-1α	0.49 ±0.49	1.12 ± 0.24	3.06 ± 1.39	6.46 ± 4.53	6.43 ± 2.90	9.79 ± 1.80
IL-1β	0.79 ±0.41	1.69 ± 0.32 *	4.53 ± 0.40	6.78 ± 2.99	8.05 ± 1.20 *	2.99 ± 0.40
IL-6	0.92 ±0.09	1.22 ± 0.21	27.79 ± 20.60	55.48 ± 38.34	162.39 ± 44.37 *	13.40 ± 4.68
IL-12b	1.05 ±0.11	0.86 ± 0.08	1.10 ± 0.02	1.21 ± 0.21	9.49 ± 4.87	1.39 ± 0.37
CCL2	0.89 ±0.09	0.84 ± 0.11	1.17 ± 0.20	1.18 ± 0.25	73.21 ± 28.97 *	3.25 ± 1.32
CCL3	1.00 ±0.00	14.55 ± 5.92 *	50.66 ± 20.85 *	97.10 ± 54.82 *	60.90 ± 32.28 *	6.55 ± 5.20
CCL4	1.01 ±0.07	1.97 ± 0.58	7.03 ± 2.93	10.32 ± 6.72	100.54 ± 52.95 *	6.27 ± 5.42
CCL5	0.93 ±0.02	1.61 ± 0.70	1.00 ± 0.08	0.90 ± 0.17	0.93 ± 0.06	1.03 ± 0.07
IL-8	1.00 ±0.10	4.89 ± 1.76 *	7.80 ± 0.47 *	13.18 ± 1.13 *	25.40 ± 3.31 *	25.87 ± 2.54

(B)

Relative degranulation	0h LPS	2h LPS	4h LPS	6h LPS	12h LPS	24h LPS
CD63	1.47 ± 0.63	1.09 ± 0.07	0.97 ± 0.07	1.02 ± 0.11	0.88 ± 0.16	0.92 ± 0.07
CD15	1.02 ± 0.08	1.08 ± 0.11	1.26 ± 0.08 **	1.23 ± 0.19	1.56 ± 0.24 **	1.34 ± 0.12
CD66b	1.02 ± 0.08	1.14 ± 0.09	1.37 ± 0.11 *	1.72 ± 0.05 ***	2.41 ± 1.77 ***	1.87 ± 0.26 ***
CD11b	1.05 ± 0.14	1.29 ± 0.17	1.62 ± 0.18 **	2.11 ± 0.13 ***	2.57 ± 0.23 ***	2.03 ± 0.34 *
CD13	1.07 ± 0.18	1.08 ± 0.07	1.18 ± 0.09	1.38 ± 0.07	1.94 ± 2.33 *	1.76 ± 0.19 *
CD14	1.00 ± 0.04	1.13 ± 0.12	1.31 ± 0.08	1.58 ± 0.31	1.23 ± 0.55	1.00 ± 0.23
CD18	1.05 ± 0.14	1.26 ± 0.06	1.23 ± 0.08	1.61 ± 0.08	2.22 ± 0.88 **	1.68 ± 0.15 *
CD35	1.11 ± 0.23	1.26 ± 0.15	1.37 ± 0.18	1.60 ± 0.27	1.01 ± 0.33	1.05 ± 0.19
CD45	1.03 ± 0.12	1.19 ± 0.09	1.39 ± 0.07 *	1.58 ± 0.12 **	2.18 ± 0.39 ***	1.84 ± 0.29 **

Figure Legends

Figure 1 Time-dependent effect of LPS on cytokine secretion in human neutrophils. Cytokine secretion was measured by CBA upon stimulation with 100 ng/mL LPS for 0, 2, 4, 6, 12 and 24 h. Results are mean secretion (pg/mL) ± SEM of at least three independent experiments. Significantly different from cytokine secretion in non-stimulated control at the corresponding time: * $p < 0.05$.

Figure 2 Temporal pattern of degranulation upon LPS stimulation in human neutrophils. Translocation of degranulation markers to the plasma membrane was assessed using flow cytometry after cell treatment with 100 ng/mL LPS for 0, 2, 4, 6, 12 and 24 h. Results are expressed in mean fluorescence intensity (MFI) of LPS-stimulated cells and non-stimulated control ± SEM of at least three independent experiments. Significantly different from non-stimulated control at the same time point: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3 Workflow of linear fitting of LPS-mediated cytokine secretion and degranulation. A novel approach based on linear fitting was used to find linear relationship between short-time series of secreted cytokines with similar pattern to degranulation markers present at the plasma membrane. After importing data into R statistical software, all ratio values between LPS-stimulated and non-stimulated control conditions were $\log_{10}$ normalized and treated as mentioned in *Materials and Methods*.

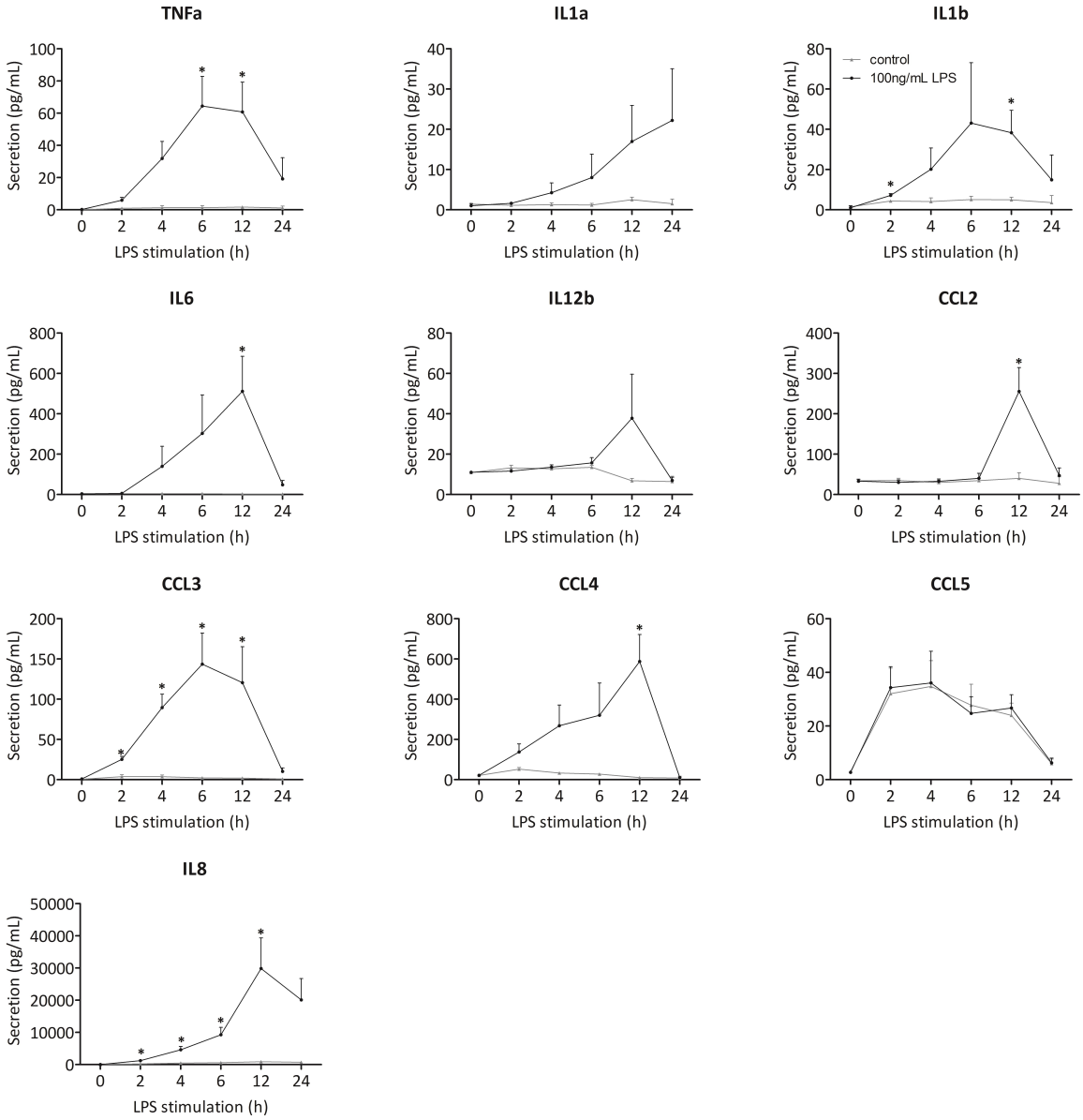
Figure 4 Linear fitting approach for the kinetic analysis of degranulation and cytokine secretion. (A) Simulations were performed to find the optimal threshold for RSQ value, as described in *Materials and Methods*. After introducing perturbations (e value) to the kinetic profiles, the linear fitting decreases in significance. Here, the example depicts how the linear fitting between the granule marker CD15 and the cytokine CCL4 evaluates by introducing perturbations to the kinetic profiles. (B) Plot of average RSQ deriving from simulations (n=100) against augmenting e values. (C) Linear fitting model in which the behaviour of CCL4 is correlated to CD15. Due to its p-value of 0 and high RSQ of 0.91, the correlation between CCL4 and CD15 corresponds to a significant linear fitting model.

Figure 5 Linear fitting of LPS-mediated kinetics of cytokine release and degranulation in human neutrophils. Enlisted cytokines on the histograms fitted to the kinetics of the degranulation markers characteristic for (A) secretory vesicles (CD13, CD14, CD18, CD35, CD45), (B) azurophil granules (CD63), specific granules (CD15, CD66b) and gelatinase granules (CD11b), according to the selection of p-value ≤ 0.05, and RSQ ≥ 0.82.

Figure 6 Hypothetical model of fitted cytokines and granules in human neutrophils. (A) Neutrophil-derived cytokines are released in a hierarchical sequence coincident with the roles of granules (IV secretory vesicles, III gelatinase granules, II specific granules,

I azurophil granules) during the microbial elimination processes and inflammatory response. Cytokines and granules are released in a concurrent fashion but (B) could additionally be localized in or mobilized to the different granule types. Classical secretory pathways are mediated through the endoplasmic reticulum (ER) and Golgi complex (*e.g.* IL-6, IL-8, CCL3, and CCL4). IL-1α and IL-1β are secreted on a non-conventional pathway. Possible routes for cytokine trafficking (after ER-Golgi or after cleavage) and mobilization to the plasma membrane relative to degranulation are shown in *red*. Different types of granules are characterized by their CD markers and proteolytic enzymes (triangles): CD63, myeloperoxidase and elastase for azurophil granules; CD66b as well as lactoferrin for specific granules; CD11b and gelatinase for gelatinase granules; and CD13, CD14, CD18, CD35 and CD45 for secretory vesicles. Upon mobilization of the granules to the plasma membrane, granule docking and fusion leads to the translocation of the CD markers to the plasma membrane as well as the release of proteolytic enzymes (myeloperoxidase, elastase, lactoferrin, and gelatinase) into the extracellular environment.

Figure 1


TNFa
IL1a
IL1b
control
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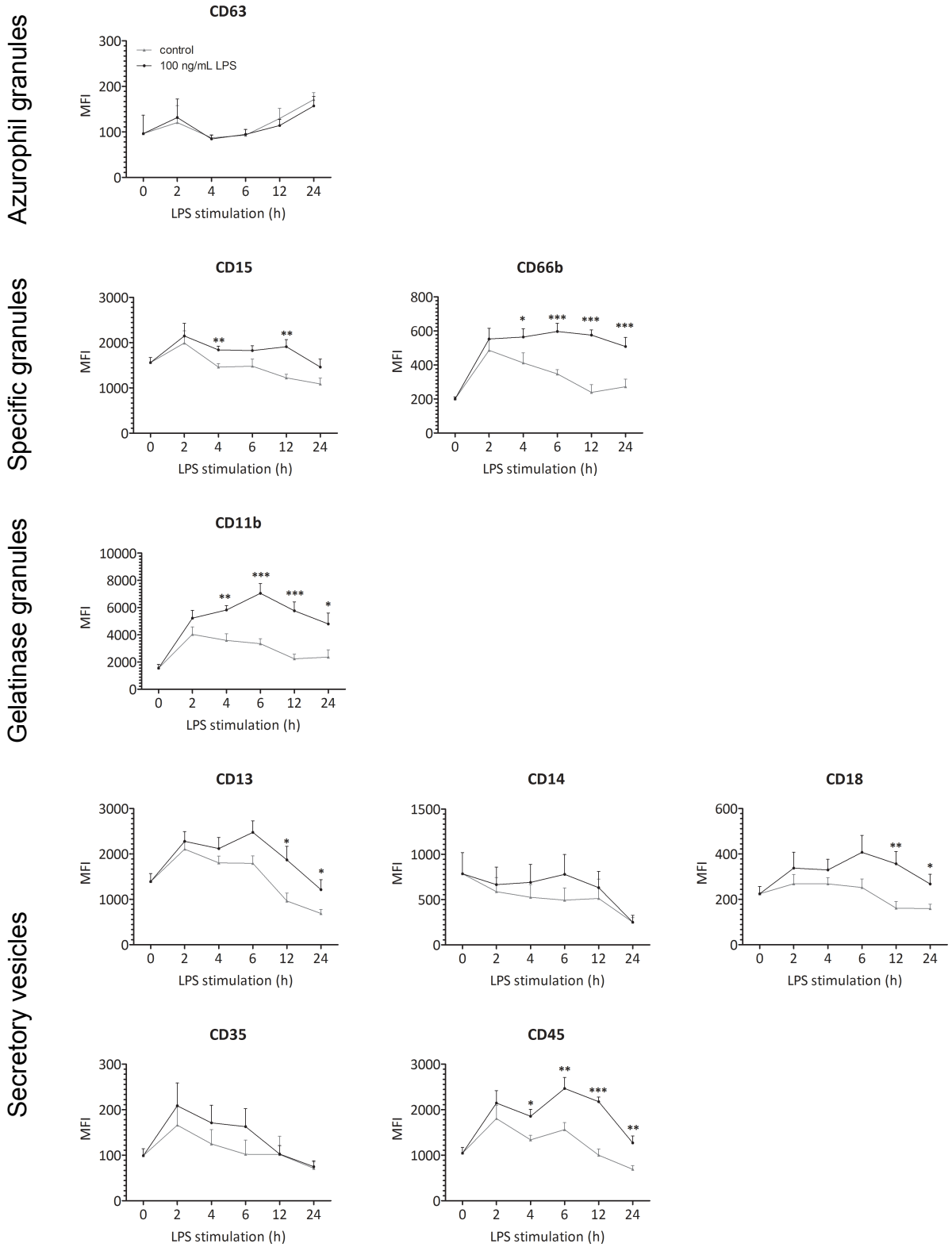
IL12b
CCL2
CCL3
CCL4
CCL5
IL8
Secretion (pg/mL)
LPS stimulation (h)

Figure 2


Azurophil granules
CD63
control
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MFI
LPS stimulation (h)
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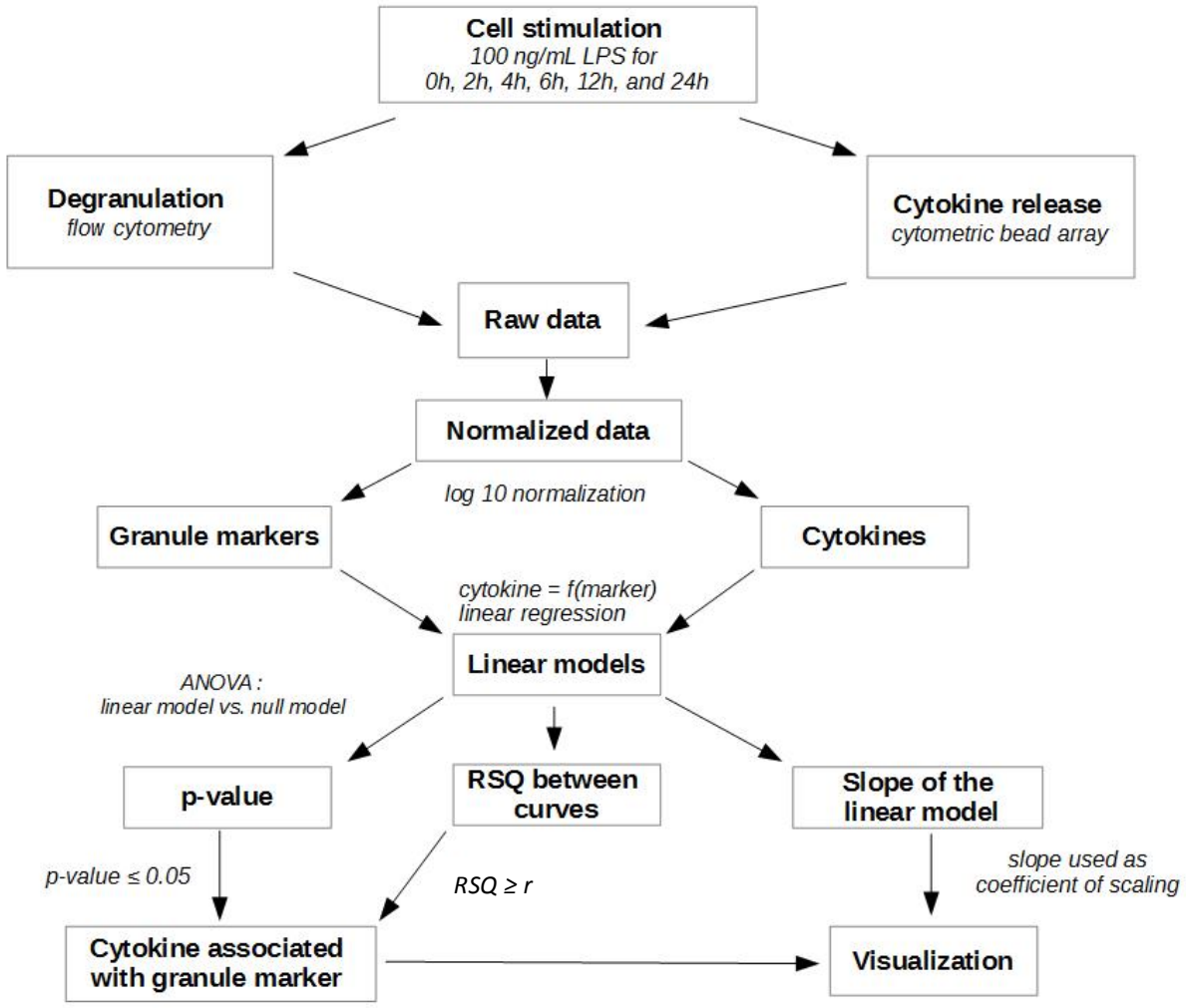
Gelatinase granules
CD11b
Secretory vesicles
CD13
CD14
CD18
CD35
CD45

Figure 3


Cell stimulation
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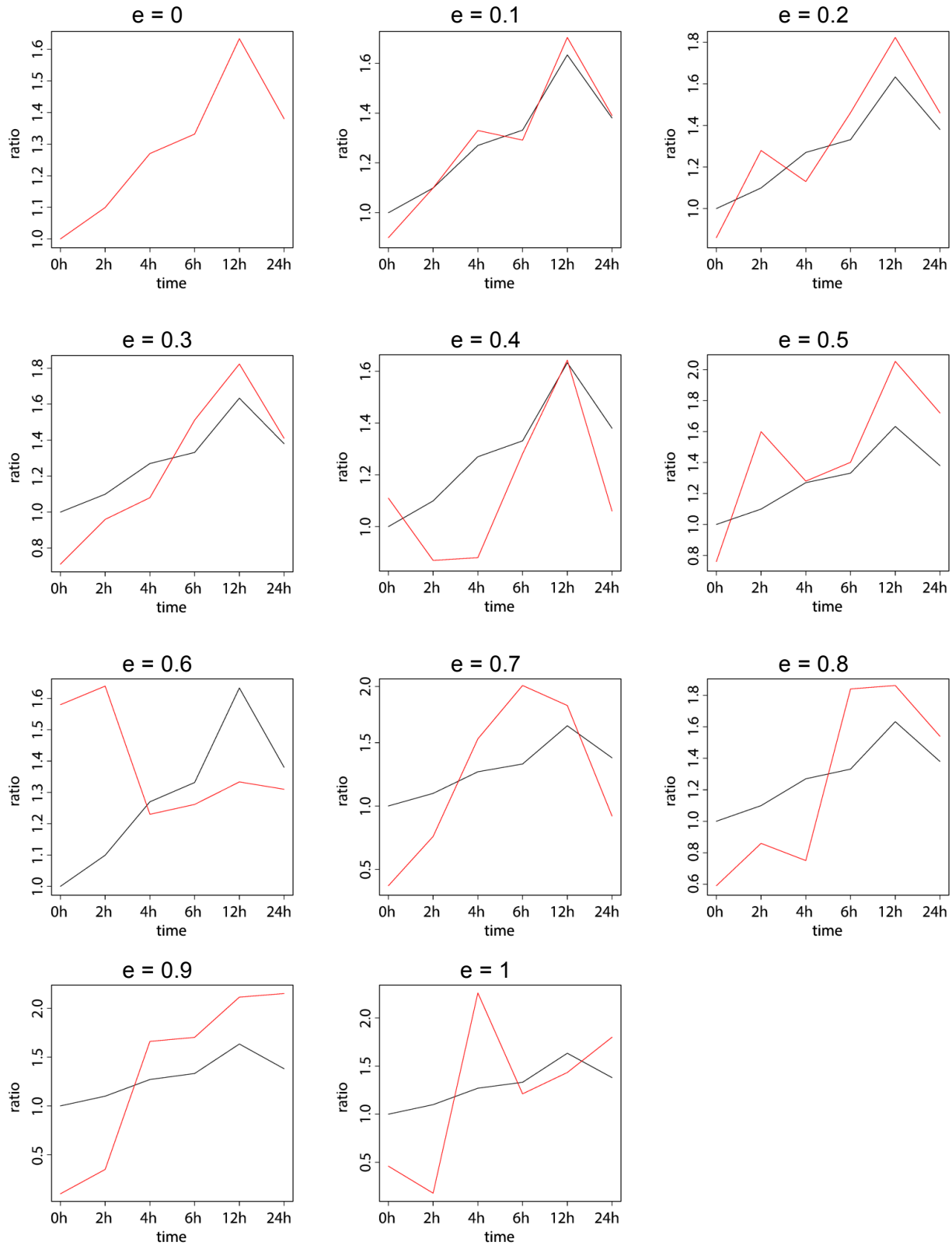
0h, 2h, 4h, 6h, 12h, and 24h
Degranulation
flow cytometry
Cytokine release
cytometric bead array
Raw data
Normalized data
log 10 normalization
Granule markers
Cytokines
cytokine = f(marker)
linear regression
Linear models
ANOVA :
linear model vs. null model
p-value
RSQ between curves
Slope of the linear model
p-value ≤ 0.05
RSQ ≥ r
slope used as coefficient of scaling
Cytokine associated with granule marker
Visualization

Figure 4

(A)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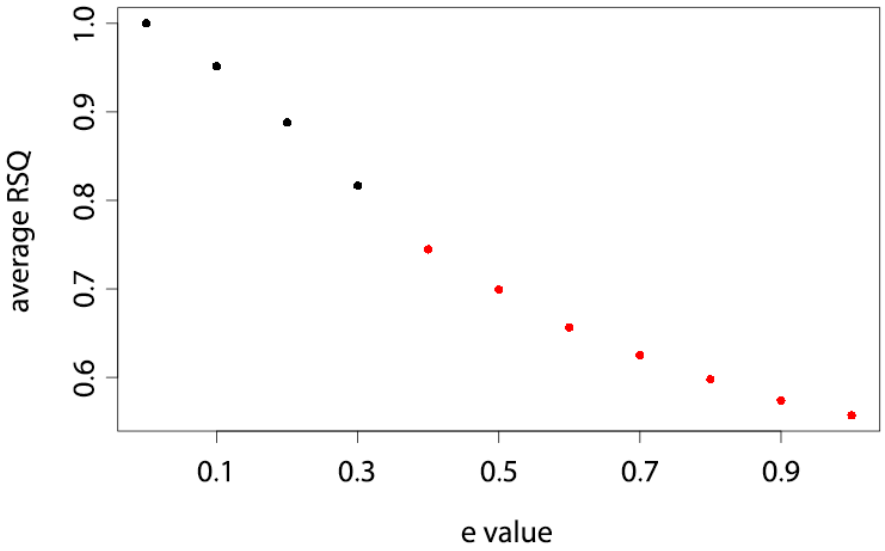
e = 1
ratio
time
0h
2h
4h
6h
12h
24h

(B)


average RSQ
1.0
0.9
0.8
0.7
0.6
0.1
0.3
0.5
0.7
0.9
e value

(C)

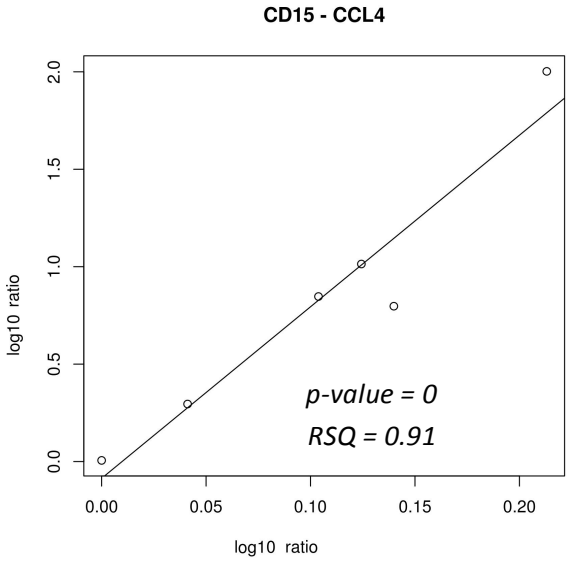
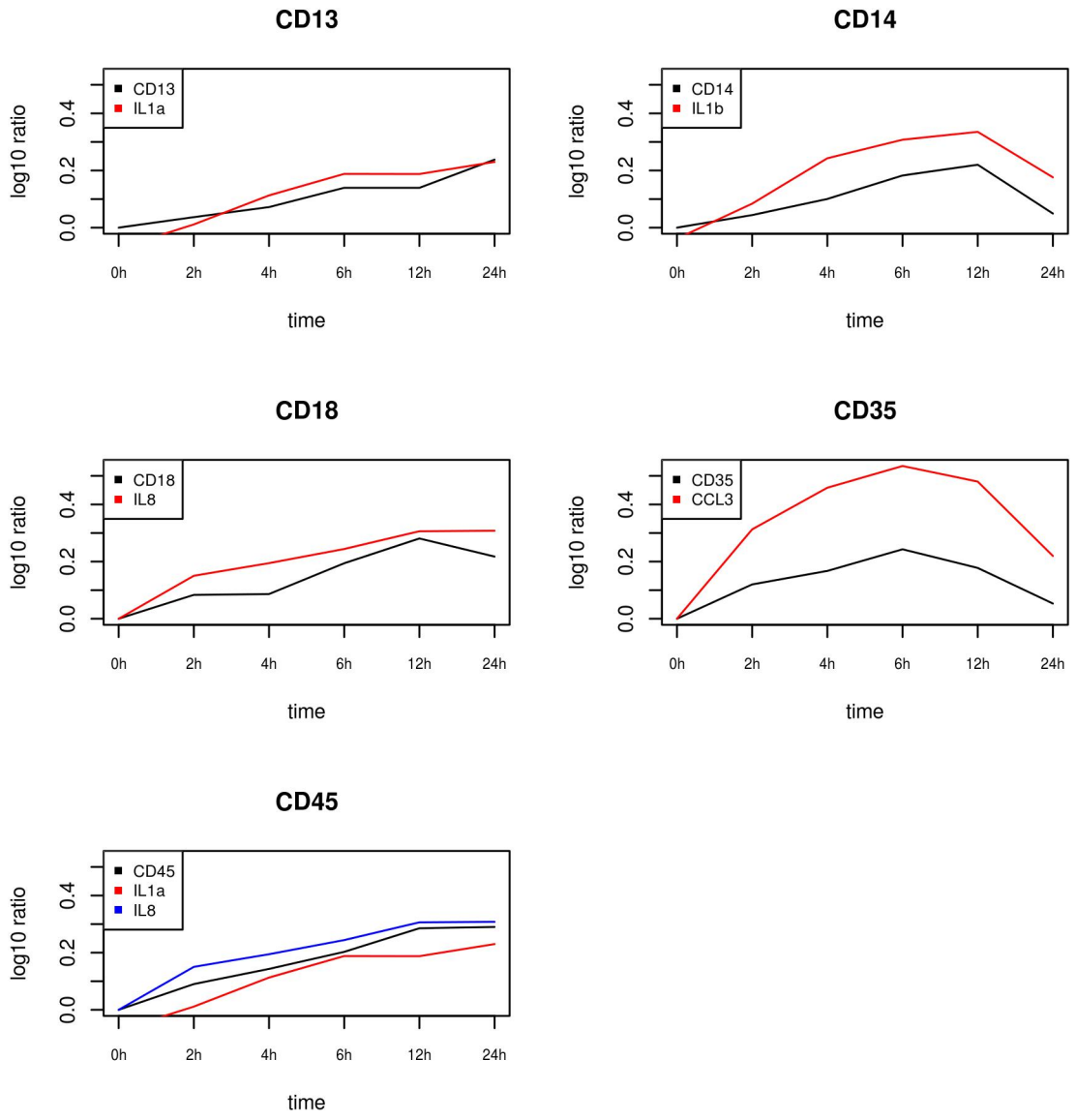

CD15 - CCL4
log10 ratio
2.0
1.5
1.0
0.5
0.0
0.00
0.05
0.10
0.15
0.20
log10 ratio
p-value = 0
RSQ = 0.91

Figure 5

(A)


CD13
CD13
IL1a
log10 ratio
0.0
0.2
0.4
0h
2h
4h
6h
12h
24h
time
CD14
CD14
IL1b
log10 ratio
0.0
0.2
0.4
0h
2h
4h
6h
12h
24h
time
CD18
CD18
IL8
log10 ratio
0.0
0.2
0.4
0h
2h
4h
6h
12h
24h
time
CD35
CD35
CCL3
log10 ratio
0.0
0.2
0.4
0h
2h
4h
6h
12h
24h
time
CD45
CD45
IL1a
IL8
log10 ratio
0.0
0.2
0.4
0h
2h
4h
6h
12h
24h
time

(B)

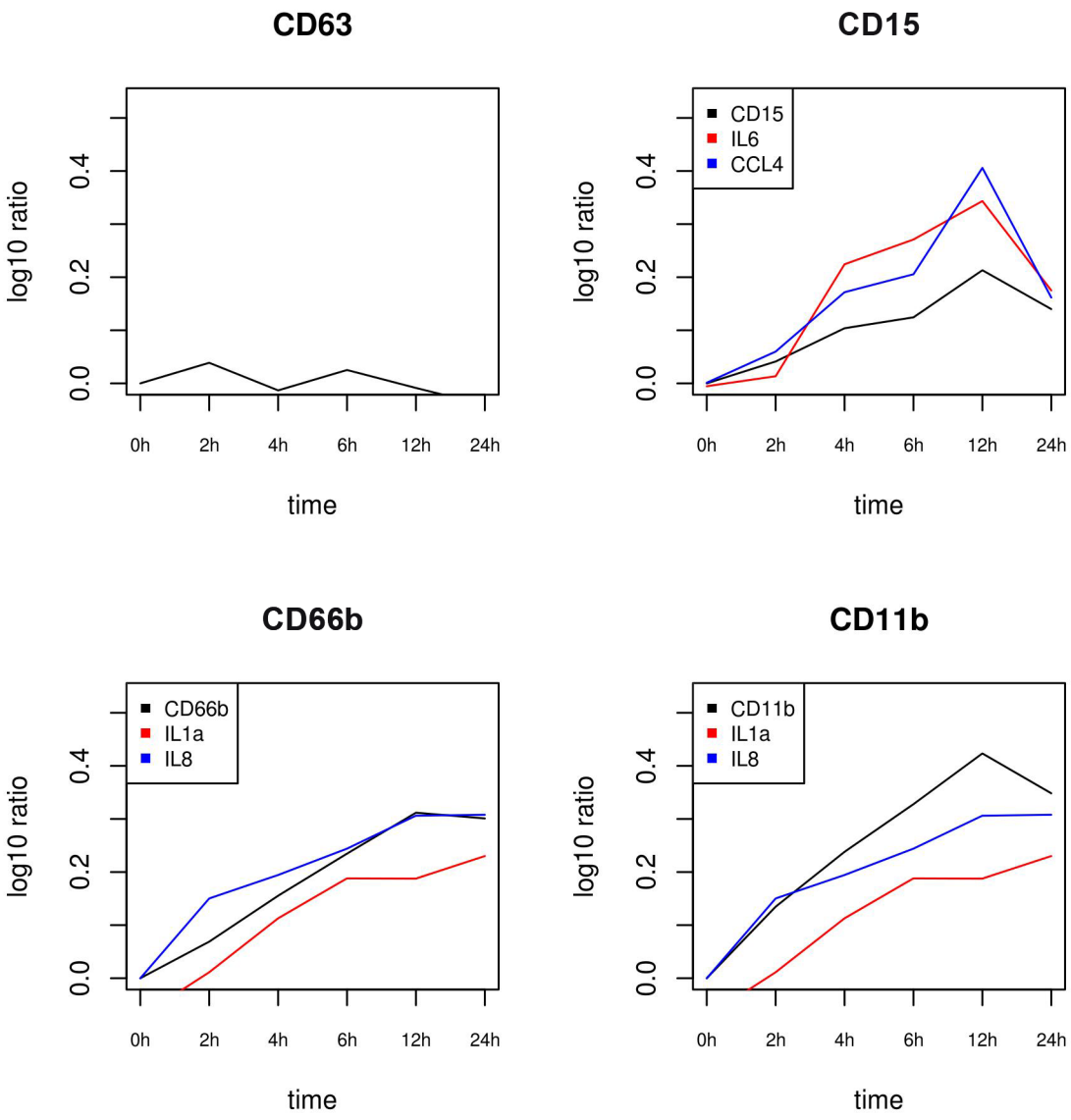
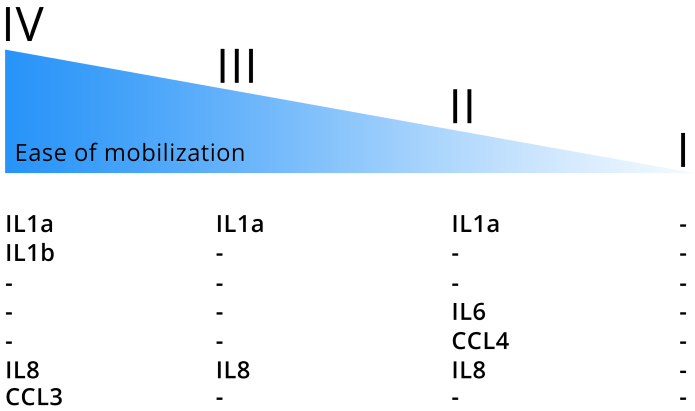
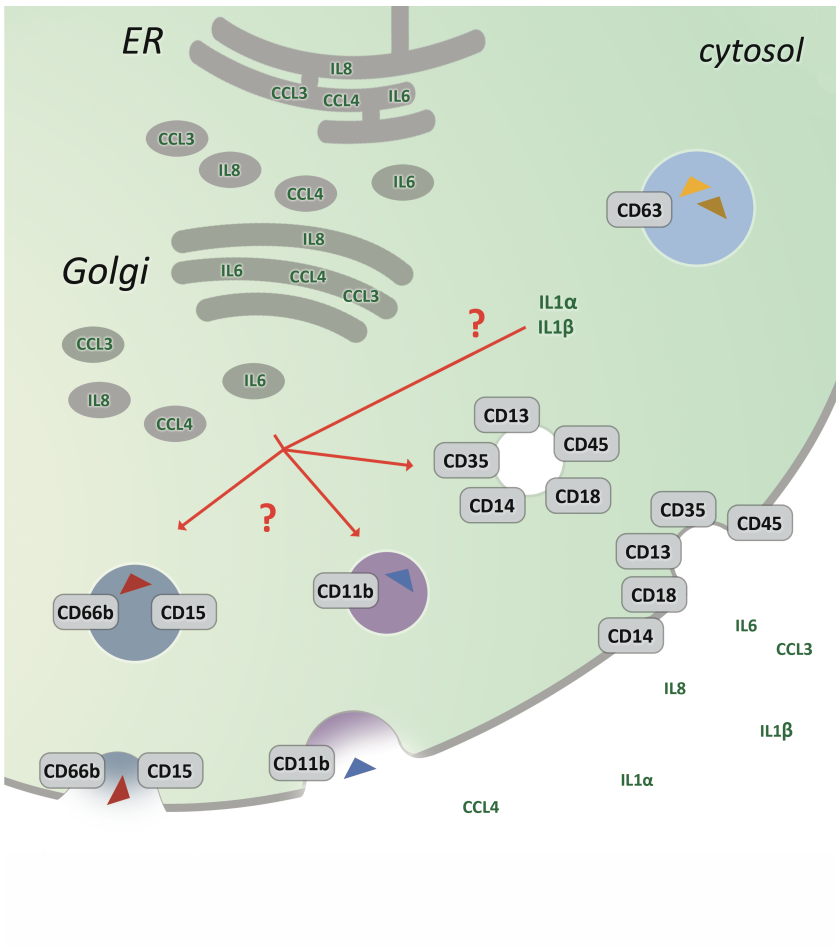

CD63
log10 ratio
0.0
0.2
0.4
0h
2h
4h
6h
12h
24h
time
CD15
CD15
IL6
CCL4
CD66b
CD66b
IL1a
IL8
CD11b
CD11b
IL1a
IL8

Figure 6

(A)


IV
III
II
I
Ease of mobilization
IL1a
IL1b
-
-
-
IL8
CCL3
IL1a
-
-
-
-
IL8
-
IL1a
-
-
IL6
CCL4
IL8
-
-
-
-
-
-
-
-
-

(B)


ER
cytosol
Golgi
IL8
CCL3
CCL4
IL6
CD63
IL1α
IL1β
?
CD13
CD45
CD35
CD14
CD18
CD66b
CD15
CD11b
IL6
CCL3
IL8
IL1β
IL1α
CCL4

Manuscript II *currently under major revision, Journal of Leukocyte Biology*

II An essential role of syntaxin 3 protein for granule exocytosis and secretion of IL-1α, IL-1β, IL-12b, and CCL4 from differentiated HL-60 cells

Emerging evidence shows that neutrophils are able to package a variety of cytokines into cytoplasmic granules. In a prior study, we determined the concurrent behavior of LPS-mediated cytokine release and degranulation. In this part of the study, the aim is **to characterize the intracellular SNARE machinery regulating neutrophil-derived cytokine secretion and degranulation**.

To investigate the functional role of SNAREs in neutrophil functions, we downregulated SNARE expression *via* RNAi interference. Since primary neutrophils are merely transfectable, we used the HL-60 cell line, which originates from malignant cells in the peripheral blood of a 36-year old female patient with acute promyelocytic leukemia (Lubbert and Koeffler, 1988). HL-60 cells can be differentiated into neutrophil-like cells (Koeffler and Golde, 1980; Collins, 1987) upon addition of dimethyl sulfoxide for 4.5 days (Collins *et al.*, 1978; Mollinedo *et al.*, 2008).

In a first step, we identified the expression profiles of key regulators in exocytosis, members of the SNARE protein superfamily. We focused on the Q_a-SNARE syntaxin 3 (STX3) whose expression is upregulated upon cell differentiation. Functional analysis revealed that STX3 knockdown affects the secretion of the cytokines IL-1α, IL-1β, IL-12b, and CCL4, as well as gelatinase degranulation upon LPS stimulation. Using immunofluorescence microscopy, STX3 could be partially localized in gelatinase B-positive compartments.

In this part of the study we provide first evidence that STX3 interferes with cytokine secretion and degranulation while being localized in gelatinase granules in neutrophil-like HL-60 cells under pro-inflammatory conditions.

An essential role of syntaxin 3 protein for granule exocytosis and secretion of IL-1α, IL-1β, IL-12b, and CCL4 from differentiated HL-60 cells

Isabelle Naegelen [*], Sébastien Plançon [*], Nathalie Nicot [†], Tony Kaoma [†], Arnaud Muller [†], Laurent Vallar [†], Eric J. Tschirhart [*], and Sabrina Bréchard [*]

[*] University of Luxembourg, Life Sciences Research Unit, Calcium Signaling and Inflammation, 162A avenue de la Faïencerie, L-1511 Luxembourg

[†] Centre de Recherche Public Santé (CRP-Santé), 84 Val Fleuri, L-1526 Luxembourg

E-mail addresses: isabelle.naegelen@uni.lu; sebastien.plancon@uni.lu; nathalie.nicot@crp-sante.lu; tony.kaoma@crp-sante.lu; arnaud.muller@crp-sante.lu; laurent.vallar@crp-sante.lu; eric.tschirhart@uni.lu; sabrina.brechard@uni.lu

One-sentence summary: This study provides first evidence that STX3 regulates the secretion of IL-1α, IL-1β, IL-12b, and CCL4 during gelatinase degranulation in differentiated HL-60 cells.

Running title: STX3 regulates degranulation and cytokine secretion.

Corresponding author: Sabrina Bréchard, Calcium Signaling and Inflammation group, Life Sciences Research Unit, University of Luxembourg, 162A avenue de la Faïencerie, L-1511 Luxembourg, Luxembourg, phone number: +352/466644-6434, fax number: +352/466644-6435, e-mail address: sabrina.brechard@uni.lu

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Abbreviations

CBA	cytometric bead array
dHL-60	DMSO-differentiated HL-60 cells
LPS	bacterial lipopolysaccharide from *E.coli*
LTF	lactoferrin
MMP-9	gelatinase B, matrix metallopeptidase-9
MPO	myeloperoxidase
SNARE	soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
STX3	syntaxin 3

Abstract

Besides their roles in the killing of pathogens, neutrophils have the capacity to package a variety of cytokines into cytoplasmic granules for subsequent release upon inflammatory conditions. Because the rapid secretion of cytokines orchestrates the action of other immune cells at the infection site and thus, can contribute to the development and chronicity of inflammatory diseases, we aimed to determine the intracellular soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) machinery responsible for the regulation of cytokine secretion and degranulation.

From a constructed gene expression network, we first selected relevant cytokines for functional validation by the cytometric bead array approach. We established a cytokine secretion profile for human neutrophils and differentiated HL-60 cells (dHL-60 cells) underlining their similar ability to secrete a broad variety of cytokines within pro-inflammatory conditions mimicked by LPS stimulation.

Secondly, after screening of SNARE genes by microarray experiments, we selected syntaxin 3 (STX3) for further functional studies. Using a small interfering RNA strategy, we show that STX3 is clearly required for the maximal release of IL-1α, IL-1β, IL-12b, and CCL4 without alteration of other cytokine secretion in dHL-60 cells. In addition, we demonstrate that STX3 is involved in MMP-9 exocytosis from gelatinase granules where STX3 is partly localized.

Our results suggest that the secretion of IL-1α, IL-1β, IL-12b, and CCL4 occurs during gelatinase degranulation, a process controlled by STX3. In summary, these findings provide first evidence that STX3 has an essential role in trafficking pathways of cytokines in neutrophil granulocytes.

Introduction

Neutrophil granulocytes are involved in the early recognition of microbial pathogens and subsequent triggering of an extensive pro-inflammatory response. To protect hosts from invading pathogens and ensure an efficient killing of infecting microorganisms, neutrophils possess, among other properties, the ability to liberate a plethora of potent cytotoxic molecules during the degranulation process (Borregaard and Cowland, 1997).

During neutrophil maturation, over 300 antimicrobial and proteolytic proteins are distributed in a hierarchical order within four different types of granules: azurophil (primary), specific (secondary), gelatinase (tertiary) granules, as well as secretory vesicles (Borregaard and Cowland, 1997). These distinct intracellular granules are readily mobilized under pro-inflammatory conditions and their contents are released in a reverse chronological order compared to their synthesis (Le Cabec *et al.*, 1996; Cowland and Borregaard, 1999; Almkvist *et al.*, 2001).

Neutrophils are also a source of cytokines, which have a pivotal position in innate and adaptive immune regulatory networks. Initially, it was assumed that cytokines were stored by the neutrophil as preformed mediators within granules and released upon appropriate stimulation. Despite their terminal differentiation, it is now obvious that neutrophils display the capacity to rapidly *de novo* synthesize cytokines in response to a variety of inflammatory stimuli (Stanley and Lacy, 2010). Although subcellular fractionation of human neutrophils demonstrates that VEGF is stored within specific granules (Gaudry *et al.*, 1997), the precise intracellular localization of other neutrophil-derived cytokines has not yet been clearly characterized.

Pro-inflammatory mediators orchestrate the action of other immune cells at the site of inflammation and also many functions of neutrophils themselves (Scapini *et al.*, 2000), but they may also contribute to the persistence of inflammatory processes and hence the development of chronic inflammatory diseases, such as sepsis, rheumatoid arthritis, atherosclerosis or inflammation-related cancer (Kasama *et al.*, 2005). On this basis, it is therefore evident that cytokine secretion needs to be tightly controlled to prevent deregulated or inappropriate release and subsequent pathological conditions.

The signaling event required for rapid delivery of cytokines to the cell surface for extracellular secretion is still poorly characterized. In the past years, evidence has accumulated, which shows that soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNAREs) proteins (Sollner *et al.*, 1993) are required for the regulation of granule fusion in neutrophils and release of their contents (Martin-Martin *et al.*, 2000; Mollinedo *et al.*, 2003; Uriarte *et al.*, 2011). Although it is now unequivocal that many SNARE proteins are expressed and involved in

intracellular vesicular trafficking pathways in neutrophils, specific mechanisms underlying the release of cytokines in neutrophils have not yet been clearly reported and data about involvement of SNAREs in this process appear poor in these cells.

The aim of our work was to determine the role of the SNARE syntaxin 3 (STX3) in cytokine secretion and degranulation following activation by bacterial lipopolysaccharide (LPS) through cell surface receptors. Because neutrophils are short-lived and terminally differentiated, these cells are not amenable to genetic manipulation, such as transfection. For this reason, we used DMSO-differentiated HL-60 (dHL-60) cells, which exhibit neutrophil-like phenotypes (Harris and Ralph, 1985), to investigate molecular mechanisms related to exocytosis and SNARE proteins. Complementary experiments were performed in human primary neutrophils to ensure the relevance of our results.

After determination of cytokine and SNARE expression profiles in dHL-60 cells and validation in human neutrophils, we showed that STX3 is involved in the secretion of IL-1α, IL-1β, IL-12b, and CCL4. We pointed out that STX3 is partly localized in gelatinase granules and is also required for exocytosis of their contents suggesting a common secretory pathway involving cytokine release and degranulation.

Materials and Methods

Cell culture

The promyelocytic HL-60 cell line (Collins *et al.*, 1977) was purchased at ATCC-American Type Culture Collection (Manassas, Virginia, USA) (CCL-240) and was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin (Lonza, Verviers, Belgium). HL-60 cells were differentiated into neutrophil-like cells by addition of 1.3% DMSO to the culture medium for 4.5 days. The differentiation status of HL-60 cells upon DMSO treatment is evidenced by the monitored upregulation of CD11b (Carrigan *et al.*, 2005), and downregulation of transferrin receptor (CD71) (Mora-Jensen *et al.*, 2011) (*data not shown*).

Purification of human neutrophils

Peripheral blood of healthy volunteers was collected in EDTA-containing Vacutainer® tubes (BD Biosciences, Erembodegem, Belgium). Samples were collected in accordance with the good clinical practices and following the national and international ethical recommendations. Neutrophils were isolated from blood samples by Polymorphprep™ separation procedure (Axis-Shield, Dundee, Scotland) according to manufacturer's instructions. The efficacy of the

Polymorphprep method for neutrophil isolation from peripheral blood has been validated (Degel and Shokrani, 2010). Remaining erythrocytes in the neutrophil cell suspension were lysed for 10 min with red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4). Neutrophils were washed and resuspended in PBS 1X (pH 7.4). Purity of the isolated neutrophil population was ≥ 98% as analyzed by the BD FACSCanto™ II flow cytometer (BD Biosciences) using two mixtures of selection markers CD66b-FITC (clone B13.9)/ CD11b-PE (clone MEM-174)/ CD14-APC (clone MEM-18) and CD15-FITC (clone MEM-158)/ CD16-PE (clone LNK16)/CD45-APC (clone MEM-28) (Immunotools, Friesoythe, Germany) on 10,000 events in the gated population of homogenous (FSC-A *vs.* SSC-A), single (SSC-A *vs.* SSC-H) and living cells (negative cells for Sytox Blue staining (Life Technologies, Gent, Belgium)). Purified neutrophils were positive for all the selection markers used by flow cytometry. Monocytes could be distinguished from the neutrophil population by $CD11b^+$ $CD66b^-$ $CD14^{high}$, lymphocytes by $CD11b^-$ $CD66b^-$ $CD14^-$, and eosinophils by $CD45^+$ $CD16^-$ and SSC^{high} (**Suppl. Fig. 5**). Human neutrophil samples were cultured in X-VIVO™ 15 medium with L-glutamine, and gentamicin (Lonza) at 37°C and 5% CO_2 up to 24 h after purification.

Cell stimulation

dHL-60 cells and highly purified neutrophils were stimulated with bacterial LPS from *E. coli* serotype O111:B4 (Sigma, Bornem, Belgium) for simulating pro-inflammatory conditions. Because LPS at concentrations of 100ng/mL also acts on neutrophils independently of serum lipopolysaccharide-binding protein (Nakatomi *et al.*, 1998), and to avoid any serum component contamination, which could interfere with specific LPS-induced cell responses, stimulations were carried out under serum-free conditions. For kinetic studies of cytokine mRNA expression, $1x10^7$ cells were treated with 100 ng/mL LPS for 2, 6 and 12 h. To analyze cytokine secretion and degranulation, dHL-60 cells or neutrophils were stimulated with different LPS concentrations (10, 30 and 100 ng/mL) for 6 or 12 h, respectively. Time points of 6 and 12 h of LPS stimulation were chosen according to the maximal cytokine mRNA expression levels.

Total RNA extraction and quality control

RNA from dHL-60 cells was extracted using innuPrep RNA mini kit (Analytik, Jena, Germany) according to manufacturer's instructions. The quantity and quality of total RNA were evaluated by a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific, Erembodegem, Belgium). To determine the integrity of total RNA, samples were examined on Agilent 2100 Bioanalyzer (Paolo Alto, USA) using an RNA 6000 Nano LabChip kit following supplier's instructions.

Affymetrix microarray and data analysis

Total RNA samples were reverse-transcribed to double-stranded cDNA using specific primers, which reduce the priming of rRNA. Then, cRNA was generated by *in vitro* transcription and reverse transcribed into a sense single-stranded cDNA. Afterwards, the cDNA was fragmented, labeled and hybridized on Affymetrix GeneChip® Human Gene 1.0 ST Arrays (Santa Clara, USA) according to the Ambion® WT Expression kit for Affymetrix® GeneChip® Whole Transcript (WT) Expression Array Protocol (P/N 4425209 Rev.B 05/2009) and GeneChip® WT Terminal Labeling and Hybridization User Manual for use with the Ambion® WT Expression kit (P/N 702808 Rev.6) (Santa Clara, California, USA). Microarrays were then washed, stained and scanned according to manufacturer's instructions. CEL files containing hybridization raw signal intensities were imported into Partek GS software (St. Louis, Missouri, USA) for further statistical analysis. First, probe intensities were summarized to gene expression signals using Partek default options (GC-content adjustment, RMA background correction, quantile normalization, $\log_2$ transformation and summarization by means). Variance in the dataset was then analyzed by using the Principal Component Analysis (PCA) approach. As PCA separated samples into four groups according to treatment and time, a two-way ANOVA followed by false discovery rate (FDR) correction were applied to detect differential expressed genes. Two cut-offs were considered to identify those genes: FDR < 0.05 and absolute fold change ≥ 2. The networks were generated through the use of IPA (Ingenuity® Systems, Redwood City, CA, USA, www.ingenuity.com). Microarray expression data from LPS-stimulated dHL-60 cells are available at ArrayExpress (The EMBL-European Bioinformatics Institute, Cambridge, UK, www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-1393. Microarray expression data for SNARE expression from undifferentiated HL-60 cells, dHL-60 cells and human neutrophils are available under the accession number E-MTAB-2107.

Quantitative real-time PCR (qPCR)

cDNA was synthesized from 2 µg total RNA by using the ThermoScript™ Reverse Transcriptase System (Life Technologies). Forward and reverse primers (**Table 1** and **Table 2**) were designed with the aid of the GenScript Online PCR Primer Design Tool (www.genscript.com). Primer efficiency and amplicon integrity were controlled by analysis of melting-curves, agarose gel and sequencing. Real-time qPCR was performed with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Nazareth, Belgium) for 40 cycles on a MyIQ single color real-time qPCR detection system. All data were analyzed in triplicates. Quantification of mRNA levels was carried out with Bio-Rad iQ5 software and normalization of mRNA levels was

calculated with respect to the reference gene β-actin, according to the $2^{-\Delta\Delta CT}$ Livak method (Livak and Schmittgen, 2001).

Short interfering RNA assays

Double-stranded short interfering RNA (siRNA) of 19 nucleotides targeting human STX3 (as well as a non-silencing scramble siRNA) were custom-ordered from Eurogentec (Seraing, Belgium). Three synthesized siRNA candidate duplexes were tested for silencing efficiency. The most efficient siRNA to mediate knockdown expression of STX3 was chosen leading to ~80% expression inhibition and used for functional analysis. All experiments were performed with a siRNA duplex (sense: 5'-GGCACGAGAUGAAACGAAA-3', antisense: 5'-UUUCGUUUCAUCUCGUGCC-3'). The non-silencing control siRNA (sense: 5'-UGCGCUACGAUCGACGAUG-3', antisense: 5'-CAUCGUCGAUCGUAGCGCA-3') was used as a negative control. siRNA target sequences chosen in this study were screened by National Center for Biotechnology Information BLAST searches to avoid mismatches. dHL-60 cells ($2x10^6$) were transiently transfected with 2 μg of STX3 siRNA or non-silencing control sequence at day 3 of differentiation using the Nucleofector apparatus (Amaxa Biosystems, Cologne, Germany) and the Nucleofector V kit with program T-019 according to the manufacturer's protocol. Terminally differentiated cells were processed for further experiments upon 48 h post-transfection.

Western Blot analysis

Cells were lysed in a buffer containing 50 mM Hepes, pH 7.5; 150 mM NaCl; 10% glycerol; 1 mM EDTA; 10 mM NaF; 0.5 mM Na_3VO_4; 1% Triton X-100; 1.5 mM $MgCl_2$ supplemented with a mix of serine and cysteine protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets, Roche Molecular Biochemicals, Basel, Switzerland). For human neutrophil lysates, 30 µL of 0.05 M diisopropylfluorophosphate (D0879, Sigma) was added to the $40x10^6$ cell pellet prior to the addition of 500 µL lysis buffer. Lysates were centrifuged at 10 000 g at 4°C for 10 min and an equal volume of Laemmli 2X buffer was added to supernatants. After resolving heat-denatured samples on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, proteins were transferred onto nitrocellulose (Hybond™-ECL, Amersham, GE Healthcare, Belgium) membranes, which were saturated with blocking buffer containing 3% w/v non-fat milk. Immunodetection was realized by using rabbit polyclonal anti-STX3 (1:2000 dilution, clone A31534, Sigma) and mouse monoclonal anti-β-actin (1:500 dilution, clone C4, Millipore, Bruxelles, Belgium) as primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies directed against rabbit IgGs (1:10000 dilution, AP132P, Millipore) or mouse IgGs (1:20000 dilution, AP124P, Millipore). Revelation of bands was visualized by ECL

detection. Band intensities were quantified by densitometry using Image J densitometry software (National Institutes of Health, Bethesda, Maryland, USA). The integrated intensity of the target protein band was normalized to the reference protein (β-actin).

Enzyme-linked immunosorbent assay (ELISA)

To quantify the release of granule matrix proteins upon degranulation, supernatants from LPS-stimulated dHL-60 cells were analyzed *via* human myeloperoxidase (MPO) ELISA kit (BMS2038INST, eBioscience, Vienna, Austria), human matrix metallopeptidase 9 (MMP-9) ELISA kit (ELH-MMP9-001, RayBio, Boechout, Belgium) and bovine albumin ELISA kit (E11-113, Bethyl Laboratories) according to the manufacturer's instructions. Cell cytotoxicity upon treatments was examined using lactate dehydrogenase assay (LDH, CytoTox 96® Non-Radioactive Cytotoxicity Assay, G1780, Promega, Leiden, The Netherlands) according to manufacturer's instructions. For normalization, ELISA absorbance results were divided by LDH absorbance values.

Cell analysis by flow cytometry

To analyze the rate of cell death, apoptotic cells were assessed by the use of annexin V-FITC apoptosis detection kit (556570, BD Biosciences) according to the manufacturer's instructions. The percentage of apoptotic cells was determined as cells positive for annexin V-FITC labeling on the entire cell population.

The most relevant markers have been selected for degranulation analysis, in accordance with the literature (Borregaard and Cowland, 1997). Degranulation was determined by measuring the expression of CD markers specific for azurophil granules (CD63-PE), gelatinase granules (CD11b-PE), and secretory vesicles (CD13-APC, CD14-APC, CD18-FITC, CD35-FITC, CD45-APC) at the plasma membrane by flow cytometry (all antibodies are from BD Biosciences except CD14-APC from Immunotools). IgG1-FITC, IgG2a-PE (BD Biosciences) and IgG1-APC antibodies (Immunotools) were used as negative isotype controls, whereas CD45-FITC, CD45-PE or CD45-APC (BD Biosciences) single dye staining was used to set compensations. Data analysis was performed by measuring the mean fluorescence intensity (MFI) for each CD marker with BD FACSDiva software (BD Biosciences) on the gated population of granulocytes (FSC-A *vs.* SSC-A), single (SSC-A *vs.* SSC-H) and living cells (negative cells for Sytox Blue staining (Life Technologies)). In total, 10,000 events were recorded *per* staining. The relative translocation at the plasma membrane of CD markers for each granule was determined by calculating the ratio between MFI of (+LPS/-LPS) of STX3 siRNA-treated dHL-60 cells and

Measurement of cytokine secretion by cytometric bead array (CBA)

Cell density was adjusted to $2x10^6$ cells *per* condition for subsequent quantitative measurement of cytokine secretion by LPS-stimulated cells. Fresh supernatants were collected and used directly for cytometric bead array (CBA, BD Biosciences) analysis. The multiplex standard curve composed of mixed cytokine standards was set up by serial dilutions according to the manufacturer's instructions. Selected capture beads were prepared and added to supernatants. The following beads were used: CCL2 (MCP1, bead D8), CCL3 (MIP1α, bead B9), CCL4 (MIP1ß, bead E4), CCL5 (RANTES, bead D4), IL-1α (bead D6), IL-1ß (bead B4), IL-6 (bead A7), IL-8 (CXCL8, bead A9), IL-12b (bead E5), and TNFα (bead C4). After 1 h of incubation, detection reagent was added to each sample. After 2 h of incubation, samples were rinsed with wash buffer and centrifuged. Samples were washed again prior to flow cytometry analysis (BD FACSCanto™ II, BD Biosciences). Results were quantified using the standard curves and the Flow Cytometric Analysis Program (FCAP) Array software (Soft Flow, Minneapolis, USA).

Immunofluorescence analysis of STX3 localization

Cells were centrifuged for 5 min at 300 g to adhere to glass coverslips. After fixation with 3% w/v paraformaldehyde/2% w/v sucrose in 1xPBS, cells were permeabilized in a 1xPBS buffer containing 0.1% w/v saponine and 0.5% w/v BSA, then blocked with 5% human IgG (Sigma). Cells were immunostained with the following primary antibodies: rabbit anti-human STX3 (1:400 dilution, clone A31534, Sigma), mouse anti-human CD43 for plasma membrane (1:100 dilution, clone 290111, R&D Systems, Abingdon Oxon, United Kingdom), mouse anti-human calnexin (CNX) for endoplasmic reticulum (1:50 dilution, clone 546828, Millipore), mouse anti-human GM130 for Golgi (1:25 dilution, clone 35, BD Biosciences), mouse anti-human EEA1 for early endosomes (1:25 dilution, clone 14, BD Biosciences), mouse anti-human LAMP-1 (1:50 dilution, clone H4A3, BD Biosciences) and LAMP-2 (1:200 dilution, clone H4B4, BD Biosciences) for late endosomes, mouse anti-human myeloperoxidase (MPO) for azurophil granules (1:2500 dilution, clone 2C7, Thermo Scientific), mouse anti-human lactoferrin (LTF) for specific granules (1:1000 dilution, clone 2B8, AbD Serotec, Düsseldorf, Germany), mouse anti-human MMP-9 for gelatinase granules (1:600 dilution, clone 5G3, Thermo Scientific), mouse anti-human tetranectin for secretory vesicles (1:40 dilution, clone 5B7, Thermo Scientific). To assure the specificity of the rabbit anti-human STX3 antibody, STX3 was immunostained in dHL-60 cells treated with non-silencing control siRNA and STX3 siRNA-treated dHL-60 cells. STX3 pixel intensities were quantified by densitometry using Image J densitometry software. The intensity of STX3 in STX3 siRNA-treated dHL-60 cells was normalized to the intensity in control dHL-60 cells. These results show that STX3 fluorescent signal was significantly reduced in STX3

siRNA-treated dHL-60 cells, compared to the control siRNA-treated cells (**Suppl. Fig. 3**) and confirm the efficiently silenced STX3 protein level detected by Western blot in the entire siSTX3-transfected cell population. In addition, immunostaining with each marker alone as positive controls served to detect and avoid any fluorescent signal bleed-through to other channels. The use of donkey serum (1:500 dilution, Jackson ImmunoResearch, Newmarket Suffolk, UK) served as negative control. After washing with blocking buffer, cells were incubated with cyanine 3-conjugated donkey anti-mouse (1:150 dilution, 715-165-150, Jackson ImmunoResearch), AlexaFluor488-conjugated donkey anti-rabbit (1:200 dilution, A21206, Life Technologies) antibodies as well as DAPI for nuclear staining (1μM, D1306, Life Technologies). After washing, samples were mounted on Moviol/DABCO and monitored by confocal microscopy using a 63×/1.4 oil objective with a 405 nm laser and a band pass BP 420-480 nm to excite and detect DAPI, a 488 nm laser and a BP 505-550 nm to excite and detect AlexaFluor488, and a 561 nm laser and a BP 575-615 nm to excite and detect cyanine 3, respectively (LSM 510 META; Zeiss, Zaventem, Belgium). Confocal 2D and z-stack images were deconvolved using AutoQuant X3 (Media Cybernetics, Rockville, USA). A minimum of 100 cells *per* condition from z-stack images only was evaluated for Pearson correlation (PCC). The PCC was calculated since it is a rapid, simple and most importantly, robust tool to investigate co-localization of two proteins (Dunn *et al.*, 2011). The PCC was evaluated for each entire image according to the equation mentioned by Barlow *et al.* (Barlow *et al.*, 2010). In our case, we put the threshold of the PCC ≥ 0.7 in order to select only for highly positive correlation between green and red pixels.

Statistical analysis

Statistical analysis was performed by using the two-tailed unpaired Student's t-test in the Prism5 software (Graph Pad Software, La Jolla, CA, USA). The difference between the means are considered statistically significant when the p-value is * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Cytokine expression network in differentiated HL-60 cells upon LPS stimulation

So far, no complete pattern of cytokine secretion has yet been defined in dHL-60 cells upon LPS treatment. Further, data from the literature concerning the expression of many cytokines in human neutrophils (*e.g.* CCL2, CCL5, CCL7, CCL8, IL-10, IL-1B, IL-6 and TNF) are contradictory (Cassatella, 1995) and there are still many open questions about the exact cytokines actually released by human neutrophils upon pro-inflammatory conditions. To

examine the cytokine regulatory network, we firstly established the cytokine expression profile in LPS-treated dHL-60 cells and then validated our data in highly purified human neutrophils.

We performed microarray gene expression analysis to define transcriptional alterations in dHL-60 cells upon LPS stimulation. dHL-60 cells were left untreated (non-stimulated control) or incubated in the presence of 100 ng/mL LPS for 2 or 6 h, and total RNA extracted from triplicate assays was analyzed. Hierarchical clustering of array data with average linkage and Pearson correlation as similarity metrics were used to examine the variability within the expression dataset. Correlation coefficients for replicates were > 0.995 indicating high assay reproducibility. Interestingly, among the 472 genes differentially regulated upon 6 h of LPS stimulation (only 70 genes upregulated upon 2 h of LPS stimulation), 44 genes encoding cytokine molecules or receptors were found to be upregulated (microarray expression data are available under E-MTAB-1393 at www.ebi.ac.uk/arrayexpress) emphasizing the importance of cytokine activity in the response to LPS. These include *e.g.* IL-6, TNF, IL-1B, CCL2, CCL5, CCL7, and CCL8. No downregulation of any of these 44 genes was observed at any time point of the study.

To further explore the behavior of key players involved in the inflammatory response mediated by cytokines following LPS stimulation, our experimental data were analyzed using the Ingenuity Pathway Analysis platform (Ingenuity Systems®, www.ingenuity.com). Based on information available in the Ingenuity knowledge base describing the cytokines implicated in the biological response to LPS, a regulatory network was built by considering all genes that were overexpressed in our experimental dataset (treated *vs.* untreated cells after 6 h of stimulation). The resulting network represented in **Fig. 1A** shows that IL-8, IL-1A, IL-1B and TNF constitute four important nodes in the cytokine response to LPS. A direct impact of LPS treatment on CCL2, IL-12B and IL-6 gene expression was apparent from this analysis. In addition to these genes, three other relevant chemokines (CCL3, CCL4 and CCL5), described to have prominent roles in neutrophil-mediated inflammatory response (Lord *et al.*, 1991; Kasama *et al.*, 1993; VanOtteren *et al.*, 1995; Kasama *et al.*, 2005), have been chosen for qPCR validation.

Cytokine mRNA expression in dHL-60 cells upon LPS stimulation

Gene expression analysis of the selected set of cytokines by qPCR (**Fig. 1B**) was highly similar to the microarray results. The correlation of cytokine expression between microarray and qPCR data, derived from LPS-treated dHL-60 cells for 6 h, has been validated for statistical analysis using Pearson's correlation coefficient ($R=0.79$, $p=4.45\times10^{-5}$, *data not shown*) underlining the robustness of the analyses. Real-time qPCR time-series confirmed significant expression changes for 7 out of 10 genes examined. Transcript expression of selected cytokines was upregulated upon LPS stimulation, except for TNF, CCL3 and IL-8, for which no significant

change upon 6 h LPS treatment or downregulation was observed. Most of the cytokines presented a peak expression after 6 h, which decreased after 12 h of LPS stimulation. In addition, cytokine expression varied in magnitude. On this basis, cytokines can be classified into two groups: one grouping TNF, IL-1A, IL-1B, CCL2, CCL3, CCL5 and IL-8 showing moderate differential expression (inferior to 50-fold after LPS stimulation) and another including IL-6, IL-12B and CCL4, exhibiting a high differential expression, superior to 50-fold after LPS stimulation.

LPS-dependent cytokine release by dHL-60 cells

Because mRNA levels do not necessarily reflect the relative amount of proteins, quantification of cytokine mRNA by qPCR needs to be interpreted carefully. To corroborate mRNA expression results at the protein functional level, we studied the ability of LPS to induce the release of cytokines.

Differentiated HL-60 cells were treated with increasing concentrations of LPS and secreted cytokines were quantified using the CBA technology. Under non-stimulated conditions, CCL4 and IL-8 were secreted to a basal level by dHL-60 cells. Stimulation of cells with low concentrations of LPS (0.1 ng/mL, 0.3 ng/mL, 1 ng/mL and 3 ng/mL) triggered a secretion of only IL-8 and CCL4 when compared to non-stimulated conditions (*data not shown*). Therefore, IL-8 and CCL4 can constitute a pool of pro-inflammatory mediators rapidly mobilized.

In the presence of higher LPS concentrations (10 ng/mL, 30 ng/mL, and 100 ng/mL), all selected cytokines, whose transcripts were expressed, were also released into the extracellular medium upon LPS stimulation in a concentration-dependent manner (**Fig. 2A**). IL-8 and CCL4 were found to be highly secreted whereas IL-1α, IL-1β, TNFα and CCL5 were only discretely detected.

No variation of apoptosis rate has been observed in our experimental conditions (*data not shown*). In dHL-60 cells, the previously reported anti-apoptotic effect of LPS in neutrophils (Lee *et al.*, 1993), is not pronounced, probably due the fact that they are derived from a patient with acute promyelocytic leukemia.

Validation of cytokine secretion in highly purified human neutrophils

Secretion of selected cytokines was additionally determined in primary human neutrophils isolated from peripheral blood. A preparation of neutrophils highly purified (≥ 98%) has been used for CBA experiments, avoiding as much as possible the presence of monocytes which have been reported to profoundly affect the data due to their high ability to secrete cytokines (Cassatella, 1999). To underline the fact that neutrophils are the source of the detected

cytokines, we analyzed cytokine secretion levels between neutrophils highly purified ≥ 98% and those purified to ≥ 90% (**Suppl. Fig. 6**) and observed that they are similar. Our purification data shows that the amount of monocytes is as low as 0.1%. According to our experimental conditions, we can conclude that the detected level of secreted cytokines such as IL-6 and CCL2 might not derive from monocytes. Additionally, monocytes are known to produce not only IL-6 in great quantities, but also TNFα, and IL-1. However, the latter two cytokines are secreted to a very low level (< 100 pg/mL).

The maximal peak of secretion for the majority of the cytokines was reached at 12 h for a concentration of 100 ng/mL LPS. **Fig. 2B** shows that all the cytokines secreted by dHL-60 cells were also released by neutrophils. TNFα, IL-1α, IL-1β, CCL3, CCL5, and IL-12b were only discretely secreted (< 150 pg/mL) whereas IL-6, CCL4, and CCL2 were secreted at an intermediate level (~ 250-600 pg/mL) and IL-8 highly secreted (~ 10 000 pg/mL). The response is qualitatively similar in dHL-60 cells and neutrophils, despite quantitative difference in the cytokine secretion profile upon LPS stimulation. The similar cytokine secretion response from highly purified neutrophils as well as the neutrophil-like cell model additionally underlines the fact that neutrophils are the actual source of the detected cytokines.

In accordance with the literature (Lee *et al.*, 1993), cell apoptosis in neutrophils was shown to be significantly diminished upon 12 h of LPS treatment in our experimental conditions underlining the fact that LPS is able to delay apoptosis in freshly isolated human neutrophils (*data not shown*).

Endogenous expression of v- and t-SNAREs in myeloid cells

In a second step, we examined the expression of SNARE genes in promyelocytic non-differentiated and DMSO-differentiated HL-60 cells to induce terminal differentiation along granulocyte lineage (Collins *et al.*, 1977). For that, microarray experiments were conducted to detect the expression profile of SNARE genes (microarray expression data are available at www.ebi.ac.uk/arrayexpress). The docking and fusion of transport vesicles with target membranes are mediated by the direct interaction of vesicle SNAREs (v-SNAREs) including synaptobrevin/VAMP family members with target SNAREs (t-SNAREs) including syntaxins and SNAP25 homologues (Sollner *et al.*, 1993). Data analysis revealed that 22 v- and t-SNAREs were expressed in HL-60 cells (**Fig. 3A**).

To examine possible divergence of SNARE gene expression in native and differentiated HL-60 cells, we checked if SNARE mRNA patterns were modified during the process of DMSO differentiation. Considering a false discovery rate (FDR) < 5% and absolute fold changes (FC) ≥ 2, **Fig. 3A** and **Table 3** show that upon differentiation, the expression of two t-SNAREs (STX3

and STX11) and one v-SNARE (VAMP1) expression is upregulated at a level approximately 2.5- to 3- fold higher than in undifferentiated HL-60 cells. To the contrary, mRNA expression levels of other SNAREs were not dramatically affected during the differentiation process.

Then, microarray data were validated by qPCR using primers specifically designed and validated. Gene expression analysis was highly similar to the microarray results as highlighted by the upregulated expression of the same genes: STX3, STX11, and VAMP1 (**Fig. 3B**). For statistical analysis, correlation between the microarray and qPCR results has been validated using Pearson's correlation coefficient ($R = 0.98$, $p < 0.0001$, **Fig. 3B insert**). A comparable profile of SNARE mRNA expression has been found in highly purified human neutrophils except for STX2, STX18 and VAMP5, which were not expressed in primary cells (**Table 3**).

Given that *i)* STX3, STX11 and VAMP1 are upregulated during the differentiation process in HL-60 cells, *ii)* STX11 and VAMP1 had already been implicated in granule exocytosis in neutrophils (Nabokina, 2001; Mollinedo *et al.*, 2006; Xie *et al.*, 2009; D'Orlando *et al.*, 2013), and *iii)* no study has ever considered the functional role of STX3 in neutrophils, we investigated whether STX3 is required for cytokine release in dHL-60 cells.

Western blot analysis was carried out to correlate STX3 mRNA level to protein level and confirmed that this protein is expressed in mature peripheral blood human neutrophils and upregulated during neutrophil differentiation of HL-60 cells with DMSO (**Fig. 4A**). As expected, STX3 was found to be expressed in human neutrophils and dHL-60 cells using a specific antibody that recognized a band of 29 kDa. The level of STX3 protein was ~4.5-fold higher in differentiated HL-60 cells than in non-differentiated HL-60 cells.

STX3 is required for LPS-induced release of IL-1α, IL-1β, IL-12b, and CCL4 from dHL-60 cells

To address the issue whether STX3 is functionally involved in cytokine secretion, we used specific siRNA sequences to target and selectively suppress endogenous STX3 protein. STX3 siRNA led to an effective inhibition of the relevant target molecule. The mRNA level of targeted STX3 was reduced with a percentage of roughly 80% (**Fig. 4B**). Specificity of this knockdown was confirmed by the absence of any significant changes in the levels of other syntaxin isoforms (STX6, STX7 and STX11, *data not shown*). At the protein level, western blotting revealed that the knockdown resulted in a decrease of more than 50% of STX3 (**Fig. 4C**).

In accordance with our results above, dHL-60 cells were stimulated for 6 h with 10 ng/mL LPS to analyze the role of STX3 on cytokine release. We found that downregulation of STX3 reduced the secretion of IL-1α, IL-1β, IL-12b, and CCL4 elicited by LPS (**Fig. 5**). While STX3

deficiency affected the release of ~33% for IL-1α and IL-12b, ~23% for IL-1β, and ~16% for CCL4, STX3 siRNA did not affect the capacity of dHL-60 cells to release other cytokines.

Downregulation of STX3 affects the LPS-mediated release of gelatinase granules in dHL-60 cells

Since *i)* STX3 affects the LPS-induced release of IL-1α, IL-1β, IL-12b, and CCL4, *ii)* it has been described that cytokines may be located in intracellular granules and vesicles (Gaudry *et al.*, 1997), and that *iii)* SNARE proteins are also involved in neutrophil granule exocytosis (Martin-Martin *et al.*, 2000; Mollinedo *et al.*, 2003; Uriarte *et al.*, 2011), we also investigated the requirement of STX3 for degranulation in dHL-60 cells.

To this purpose, supernatants of dHL-60 cells stimulated by 10 ng/mL LPS for 6 h were tested by ELISA for secretion of soluble mediators that have been described characteristic for the different granule types: myeloperoxidase (MPO) for azurophil granules, matrix metalloproteinase-9 (MMP-9) for gelatinase granules, and serum albumin for secretory vesicles (Borregaard and Cowland, 1997). No marker of specific granules has been used since dHL-60 cells are known to lack this type of granules (Harris and Ralph, 1985).

As shown in **Fig. 6A**, dHL-60 cells treated with STX3 siRNA displayed about 50% reduction in their capacity to release MMP-9, compared to the control siRNA. On the contrary, MPO and albumin secretion was unchanged by the downregulation of STX3 expression. In parallel to the ELISA data, we performed additional experiments to investigate the effect of STX3 siRNA on the LPS-mediated degranulation in dHL-60 cells (**Fig. 6B**). Degranulation can be determined by the upregulation of granule membrane molecules as a consequence of membrane fusion from granules with the plasma membrane (Borregaard and Cowland, 1997; Cowland and Borregaard, 1999). Consequently, LPS-treated cells were analyzed for cell surface expression of several CD molecules known as degranulation markers, *e.g.* CD63 for azurophil granules. Consistent with our ELISA results, STX3 siRNA was able to inhibit the presence of CD11b at the plasma membrane about 65% upon 6 h of LPS stimulation and thus, the release of gelatinase granules in dHL-60 cells. Interestingly, knockdown of STX3 also altered the release of secretory vesicles, represented by the degranulation markers CD13, CD18, and CD45.

Localization of STX3 in dHL-60 cells and human neutrophils

Since STX3 knockdown affected the release of IL-1α, IL-1β, IL-12b, and CCL4, we investigated whether STX3 co-localized with organelles from the secretory pathways in dHL-60 cells and human neutrophils. In order to visualize intracellular proteins, immunofluorescence staining was performed: dHL-60 cells and human neutrophils were double-labeled with antibodies against

STX3 and markers for the nucleus (DAPI), plasma membrane (CD43), endoplasmic reticulum (calnexin), Golgi apparatus (GM130), early endosomes (EEA1), as well as the late endosomes (LAMP-1 and LAMP-2). Localization of STX3 was not observed at any of these subcellular levels in dHL-60 cells (**Fig. 7A**) and human neutrophils (**Fig. 8A**).

Additional experiments were performed to address the question whether STX3 was located in the different types of granules. In this view, cells were co-labeled with STX3 antibodies and markers for the cytoplasmic granules, namely myeloperoxidase (MPO, azurophil granules), lactoferrin (LTF, specific granules), matrix metalloproteinase-9 (MMP-9, gelatinase granules), and tetranectin (TN, secretory vesicles). Results show that STX3 was partly co-localized with MMP-9 and TN in the membranes of gelatinase granules and secretory vesicles in dHL-60 cells (**Fig. 7B**), respectively. In human neutrophils, STX3 was also present in specific granules (**Fig. 8B**).

Same labeling procedures were performed in dHL-60 cells stimulated for 6 h with 100 ng/mL LPS and neutrophils stimulated for 12 h with 100 ng/mL LPS; STX3 did not relocalize in different organelles upon LPS stimulation (*data not shown*).

Discussion

Intensive research efforts have focused on the mechanisms leading to the production of pro-inflammatory soluble mediators intimately involved in host immunity and the development of inflammatory disorders. Initially, macrophages were described as the major source of cytokines and neutrophils were considered unable to produce such molecules. Gradually, it became evident that cytokines are also released by neutrophils, which rapidly migrate to the inflammatory sites and play a preponderant role in the activation and recruitment of other cells of the immune system to sites of inflammation (Nathan, 2006). The first cytokine, and certainly the most studied, shown to be produced by human neutrophils is IL-8 (Altstaedt *et al.*, 1996). Later, several studies have indicated that other pro-inflammatory cytokines including IL-1β, IL-6 and TNFα as well as CCL3, CCL4, and IL-12 are also produced by neutrophils (Cassatella, 1999), which may display some diversity in terms of expression.

It is primordial to undoubtedly determine the cytokine profile in neutrophils and dHL-60 cells since controversies subsist on the nature of cytokines secreted by these cells (Cassatella, 1999). After LPS stimulation, our data from microarray and qPCR experiments indicated that neutrophils and dHL-60 cells display a similar profile of cytokine mRNA expression (**Suppl. Fig. 4**). Multiplex bead-based immunoassays afford a powerful strategy to sensitively detect

cytokines in the extracellular milieu, thereby allowing validation of cytokine expression at the functional level. Using the CBA technique, we found that TNFα, IL-1α, IL-1β, IL-6, IL-12b, CCL2, CCL3, CCL4, CCL5 and IL-8 are released by human neutrophils highly purified from peripheral blood and differentiated HL-60 cells confirming the capacity of these two cell types to similarly produce and secrete inflammatory mediators. For the first time, we were able to establish the strikingly similar transcriptome and cytokine secretion profiles in dHL-60 cells and human neutrophils. These results not only validated the attribute of the dHL-60 cell model, but also helped us to select significant pro-inflammatory mediators for further functional investigations in human cells.

The mechanisms underlining cytokine release have been largely elusive but a few recent studies provided compelling evidence for an essential role of SNARE proteins in the exocytic pathway of myeloid cells. Initially, SNARE proteins have been involved in the process of cytosolic secretory granule fusion and exocytosis of their contents (Herrero-Turrion *et al.*, 2008; Pivot-Pajot *et al.*, 2008). Thereafter, several studies emphasized the critical role of SNAREs in many kinds of regulated secretory pathways in diverse cell types. In activated immune cells, an abundant increase of SNAREs at the plasma membrane allows the upregulation of vesicular traffic and secretion of cytokines (Pagan *et al.*, 2003). Recently, a subset of t-SNAREs (STX4, SNAP23) has been reported to be substantially increased by LPS in a temporal pattern coinciding with TNFα peak secretion in activated macrophages. The increased trafficking of TNFα by overexpression of STX4 provided evidence that upregulation of STX4 is a necessary event for the TNFα secretion process (Pagan *et al.*, 2003). A similar study using overexpression of intracellular SNARE proteins showed that STX6 has a rate-limiting role in TNFα trafficking and secretion (Murray *et al.*, 2005). Therefore, we speculated on the contribution of distinct SNAREs in the release of cytokines in human neutrophils.

Microarrays were used in a wider screen to search for all SNARE genes expressed in dHL-60 cells and human neutrophils. In accordance with previous studies, we identified a range of SNAREs in both cell types among them diverse t-SNAREs: STX1, 3-7, 9, 11, 16 (Martin-Martin *et al.*, 1999; Mollinedo *et al.*, 2006). In particular, the expression of STX3 at mRNA and protein levels was upregulated upon differentiation of HL-60 cells towards neutrophil-like cells suggesting a functional role of this protein in mature neutrophils. Thus, STX3 has been selected from this screen as a potential regulator of cytokine release. By analyzing the participation of STX3 to cell-specific functional activities, we provide the first evidence of a role of STX3 in the release of four particular cytokines (IL-1α, IL-1β, IL-12b, and CCL4) in a model of neutrophils. Even though STX3 had been implicated in the release of *de novo* synthesized cytokines IL-8,

CCL2, CCL3 and CCL4 by mature human mast cells based on the use of neutralizing antibodies (Frank *et al.*, 2011), its comparable role may not be obvious in neutrophils. In accordance with the SNARE hypothesis, different SNARE complexes constituted of variable SNARE proteins could be associated with specific functions in different cell types. For example, STX4 acts with VAMP3 in TNF secretion and phagocytosis in macrophages (Pagan *et al.*, 2003; Murray *et al.*, 2005) whereas these two proteins are involved in degranulation in mast cells (Paumet *et al.*, 2000; Vaidyanathan *et al.*, 2001; Pombo *et al.*, 2003; Puri and Roche, 2006). It is also conceivable that STX3 interacts with different partners according cell types to control similar or different functions (Stow *et al.*, 2009). To support this hypothesis, data established that STX4-SNAP23 form a complex with VAMP1 or VAMP2 according to the different granule types for directing secretion (Martin-Martin *et al.*, 2000; Mollinedo *et al.*, 2006).

The knockdown of STX3 protein led to different inhibition levels on secretion of IL-1α, IL-1β, IL-12b, and CCL4 as well as MMP-9. In fact, the maximal amount of IL-1α and IL-1β was present in the supernatants of dHL-60 cells upon stimulation with 10 ng/mL LPS. IL-12b and CCL4 were maximally secreted upon stimulation with 100 ng/mL LPS. However, the different release pattern of these four cytokines does not preclude a role of STX3 in the regulation of the secretion pathway of these cytokines. It remains elusive in which way STX3 is specifically affecting cytokine release, therefore as a perspective, SNARE partners that form complexes with STX3 and lead to the specific release of these cytokines need to be revealed. Effectively, STX3 can have different SNARE partners in order to define the specific transport of the cytokines to the extracellular milieu. Alternatively, in neutrophils a release mechanism called compound exocytosis has been described in which granules fuse in a multivesicular manner with one another in the cytosol prior to fusion with the plasma membrane, or sequentially with the plasma membrane (Lollike *et al.*, 2002). Thus, STX3 could be involved in the secretion of cytokines with different release pattern by regulating the fusion between individual vesicles before exocytosis.

Traditionally, neutrophils have been seen as stores of preformed granules, and degranulation has been believed to mainly occur in a short-time range, meaning within maximally 2 h upon stimulation (Bentwood and Henson, 1980). However, this assumption needs to be balanced since granule mobilization differs on the agonist used (*e.g.* fMLF, PMA) and its applied concentration. Under our experimental conditions, the maximal degranulation level upon LPS stimulation occurs far later than upon 2 h. In accordance with our ELISA results, flow cytometry analysis confirms the time-dependent degranulation upon LPS stimulation (see *Manuscript I*). Also, it is generally admitted that neutrophils have the capacity to store and release loaded

cytokines into secretory granules (Gaudry *et al.*, 1997). In dHL-60 cells, our data underline the evidence that STX3 is partly localized in gelatinase granules and secretory vesicles and involved in the regulation of the exocytosis of their components such as MMP-9. In neutrophils, STX3 co-localizes also with MMP-9 that tends to prove that similar mechanisms involving STX3 for exocytosis may also occur in primary cells. Confirmation of this assumption is necessary but remains challenging since neutrophils are not amenable for genetic manipulations. Our results show that the knockdown of STX3 affects only MMP-9 release upon 6 h of LPS stimulation. Although STX3 siRNA was not able to trigger inhibition of albumin release, the mobilization of degranulation markers characteristic for secretory vesicles to the plasma membrane was disturbed upon STX3 deficiency (**Fig. 6B**). Because, the preponderant role of secretory vesicles is to fuse with the plasma membrane to deliver receptors to the cell surface (Borregaard and Cowland, 1997), we can hypothesize that STX3 is nevertheless involved in this phenomenon.

Controversial reports have been published about the expression of MMP-9 in dHL-60 cells (Le Cabec *et al.*, 1996). Our data provide clear evidence that *i)* MMP-9 mRNA is expressed (**Suppl. Fig. 1**), *ii)* MMP-9 protein is expressed in non-stimulated and LPS-stimulated dHL-60 cells (6h, 100ng/mL LPS), and *iii)* MMP-9 protein is time-dependently secreted from dHL-60 cells upon stimulation with 100 ng/mL LPS. Additionally, we observed that MMP-9 mRNA expression was significantly upregulated upon LPS stimulation of dHL-60 cells pointing out that neutrophil-specific granule proteins are not only preformed but also *de novo* synthesized (**Suppl. Fig. 1**). To assess whether dHL-60 cells and neutrophils release MMP-9 similarly, we observed that MMP-9 release levels were not as high in human neutrophils as in dHL-60 cells but were time-dependently increased upon LPS stimulation (*data not shown*).

According to our results, we can hypothesize on the location of IL-1α, IL-1β, IL-12b, and CCL4 in gelatinase granules before exocytosis. These types of granules may be readily mobilized at the early stages of neutrophil activation inferring a major role of these four cytokines in the initial stage of inflammation. Though, confirmation of the subcellular organelle location of IL-1α, IL-1β, IL-12b, and CCL4 still remains a challenge since no convincing methods to determine the intracellular location of cytokines in neutrophils have been described to our knowledge. Actually, the low amount of cytokines often leads to non-reliable staining by electron microscopy and other techniques used to document subcellular organelle localization of cytokines have limited resolution (*e.g.* subcellular fractionation experiments) (Pasquali *et al.*, 1999). Currently, some research groups dedicate efforts to develop more sensitive techniques that would be a significant achievement for the understanding of mechanisms underlining cytokine secretion (Lacy and Stow, 2011). Also, since STX3 knockdown induced a ~50% inhibition in the release

of MMP-9 but lower inhibition of IL-1α, IL-1β, IL-12b, and especially CCL4, it remains elusive to which amount the given cytokines could be stored in granules and to which extent the STX3 interaction partners can lead to the specificity of this transport.

We provide evidence that STX3, which has typically been described as a t-SNARE, is mainly localized in granules and secretory vesicles in neutrophils. In line with this result, STX7 has been previously reported to be present in azurophil granule membranes and relocalized to the plasma membrane after cell activation (Xie *et al.*, 2010). Although in our experimental conditions, we did not observe any relocalization of STX3 upon LPS stimulation. These findings suggest that STX3 participates in fusion events between a compartment of the secretory pathway and gelatinase granules/secretory vesicles but not with the plasma membrane. As mentioned, at this level other SNARE partners could be implicated in the STX3-mediated release of IL-1α, IL-1β, IL-12b, and CCL4 and direct vesicle trafficking in a specific manner. Further interaction studies are required to identify partners of STX3 at the compartment of the secretory pathway and characterize potential other SNARE complexes involved in the release of these cytokines.

Although mechanisms for cytokine secretion in neutrophils are still elusive, lines of evidence demonstrated that multiple pathways co-exist, in which SNAREs appear to play a fundamental role (Lollike *et al.*, 2002; Stanley and Lacy, 2010). These distinct pathways may ensure a spatiotemporal regulation of cytokine release in line with their intended functions during the inflammatory response. The next interesting step is the identification of SNARE interactions and their distribution in order to determine relevant SNARE complexes dedicated to specific functions of neutrophils in a physiological state as well as in pathological conditions. Disruption of SNARE complexes may also offer a window for the development of novel pharmacological molecules for the treatment of chronic inflammatory diseases.

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Table I Primer sequences used for cytokine gene expression by qPCR.

Gene	GenBank Ref	Forward primer (5' – 3')	Reverse primer (5' – 3')
β-actin	NM_001101	TGA-CCC-AGA-TCA-TGT-TTG-AGA	AGT-CCA-TCA-CGA-TGC-CAG-T
TNF	NM_000594.2	GGA-CAC-CAT-GAG-CAC-TGA-AA	AAG-AGG-CTG-AGG-AAC-AAG-CA
IL1A	NM_000575.3	GGA-GAT-GCC-TGA-GAT-ACC-CA	CCG-TGA-GTT-TCC-CAG-AAG-AA
IL1B	NM_000576.2	CAC-ATG-GGA-TAA-CGA-GGC-TT	TCC-ATA-TCC-TGT-CCC-TGG-AG
IL6	NM_000600.3	CAC-ACA-GAC-AGC-CAC-TCA-CC	AGT-GCC-TCT-TTG-CTG-CTT-TC
IL12B	NM_002187.2	AAG-GAG-GCG-AGG-TTC-TAA-GC	GAA-TAA-TTC-TTG-GCC-TCG-CA
CCL2	NM_002982.3	AGG-AAC-CGA-GAG-GCT-GAG-AC	GCA-GAG-ACT-TTC-ATG-CTG-GA
CCL3	NM_002983.2	TGC-TCA-GAA-TCA-TGC-AGG-TC	TGA-TGC-AGA-GAA-CTG-GTT-GC
CCL4	NM_002984.2	GGA-CAC-AGC-TGG-GTT-CTG-A	TGG-TAT-TGG-TGG-CAA-AGA-GG
CCL5	NM_002985.2	GGT-TCT-GAG-CTC-TGG-CTT-TG	GCC-AGT-AAG-CTC-CTG-TGA-GG
IL8	NM_000584.2	CTC-CAT-AAG-GCA-CAA-ACT-TTC-A	GTT-CCT-TCC-GGT-GGT-TTC-TT

Table II Primer sequences used for SNARE gene expression by qPCR.

Gene	GenBank Ref	Forward primer (5' – 3')	Reverse primer (5' – 3')
STX3	NM_000594.2	GAG-TGG-CCT-AAG-AGG-CTG-CTG-CA	GAA-CGG-GTC-GCA-CTC-GCT-CTC
STX6	NM_005819.4	TGA-TCC-TGT-GTC-TGA-TCC-CA	ACA-TTT-GGA-GCC-TGA-GTG-GA
STX7	NM_003569.2	CTT-AGT-GCT-GTG-CTG-CCT-GA	ACC-TGG-AGT-GAA-CTG-GTG-CT
STX8	NM_004853.2	TGA-AGA-GGC-TAA-GCG-AGG-AG	AAA-CCC-AAG-CCT-CTG-GTC-TC
STX11	NM_003764.3	TTC-GCA-CTC-TCG-CTC-CCA-GTC-C	GGA-TGT-GGT-CCG-TCT-CGA-ACA-CG
STX16	NM_001001433.1	CCG-ACG-CTT-TCT-TGT-TGT-T	GCA-ATG-CTA-CGT-GAA-TGC-AG
SNAP23	NM_003825.2	AAG-AAC-TTT-GAG-TCT-GGC-AAG-G	TTG-CTG-AAG-CTG-ACC-ATT-TG
SNAP29	NM_004782.3	CAA-AGC-AAA-GGA-AAC-CTC-CA	GCC-TAT-GGA-GGC-TGT-GGA-TA
VAMP3	NM_004781	CAG-CTG-GCA-GTG-TTA-GGA-CA	CAG-GAG-CCA-GTG-GGT-TAC-AT
VAMP4	NM_003762.4	TCA-CTT-TGG-TAA-GTG-CCT-TTG-A	TGA-GGA-ATT-GAG-AAG-CTA-GGG-A
VAMP7	NM_005638.5	GCT-CGA-GCC-ATG-TGT-ATG-AA	GCT-TGG-CCA-TGT-AAA-TCC-AC
VAMP8	NM_003761.4	AAA-GTT-GGA-GCA-AGC-AGG-AA	CAC-GAT-CAT-TTC-CTC-CAC-CT

Table III Genomic expression of SNAREs in HL-60 cells and neutrophils.

Official Symbol	Gene Name	HL-60 differentiation	Neutrophils
STX2	syntaxin 2	→	X
STX3	syntaxin 3	↗	ν
STX4	syntaxin 4	→	ν
STX5	syntaxin 5	→	ν
STX6	syntaxin 6	→	ν
STX7	syntaxin 7	→	ν
STX8	syntaxin 8	→	ν
STX10	syntaxin 10	→	ν
STX11	syntaxin 11	↗	ν
STX12	syntaxin 12	→	ν
STX16	syntaxin 16	→	ν
STX17	syntaxin 17	→	ν
STX18	syntaxin 18	→	X
SNAP23	synaptosomal-associated protein (23 kDa)	→	ν
SNAP29	synaptosomal-associated protein (29 kDa)	→	ν
VAMP1	vesicle-associated membrane protein 1	↗	ν
VAMP2	vesicle-associated membrane protein 2	→	ν
VAMP3	vesicle-associated membrane protein 3	→	ν
VAMP4	vesicle-associated membrane protein 4	→	ν
VAMP5	vesicle-associated membrane protein 5	→	X
VAMP7	vesicle-associated membrane protein 7	→	ν
VAMP8	vesicle-associated membrane protein 8	→	ν

→ = no significant variation of genomic expression after differentiation of HL-60 cells
↗ = increase of genomic expression after differentiation of HL-60 cells
X = SNARE mRNA is not expressed in neutrophils
ν = SNARE mRNA is expressed in neutrophils

Figure Legends

Figure 1 Cytokine transcript analysis of dHL-60 cells under pro-inflammatory conditions. (A) Microarray analysis was used to generate a literature-based cytokine expression network in LPS-stimulated dHL-60 cells using Ingenuity Pathway designer. The representation includes the biological interaction network of significant differentially expressed cytokine genes (cut-off: FDR < 0.05 and FC ≥ 2) upon stimulation of dHL-60 cells for 6 h with 100 ng/mL LPS from *E. coli* O111:B4. LPS is represented as the single agonist for induction of cytokine expression network. Arrows represent interactions that promote expression. (B) Time-dependent effect of LPS on cytokine mRNA expression. Validation of cytokine mRNA expression was performed by qPCR after treatment with 100 ng/mL LPS for 2, 6, and 12 h. Data were normalized to β-actin and expressed relative to the calibrator (non-stimulated control) at the corresponding time ± SEM of at least five independent experiments. Significantly different from cytokine expression in the non-stimulated control: * $p < 0.05$, ** $p < 0.01$.

Figure 2 LPS-induced cytokine secretion in dHL-60 cells. (A) Concentration-dependent effect of LPS on cytokine secretion in dHL-60 cells. Cytokine secretion was measured by CBA upon 6 h stimulation with 10, 30, and 100 ng/mL LPS. Results are mean ± SEM of at least three independent experiments. Significantly different from cytokine secretion in 6 h non-stimulated control: ** $p < 0.01$. (B) LPS-induced cytokine secretion in highly purified peripheral blood neutrophils. Cytokine secretion was measured by CBA in ≥ 98% pure neutrophils upon 12 h stimulation with 100 ng/mL LPS. Results are mean ± SEM of at least three independent experiments. Significantly different from cytokine secretion in non-stimulated cells (control): ** $p < 0.01$.

Figure 3 Endogenous SNARE mRNA expression in dHL-60 cells compared to undifferentiated cells. (A) Screening of 22 SNARE genes was performed by microarray analysis. Data are given as ratio between DMSO-differentiated and undifferentiated HL-60 cells of three independent experiments (ANOVA, FDR < 0.05 and absolute FC ≥ 2). (B) SNARE expression of 14 genes was validated by qPCR in undifferentiated and differentiated HL-60 cells. Data were normalized to β-actin and expressed relative to the SNARE expression in undifferentiated HL-60 cells ± SEM of at least three independent experiments. Significantly different from the SNARE expression in undifferentiated HL-60 cells: * $p < 0.05$. Correlation between microarray and qPCR SNARE expression log data is shown in insert.

Figure 4 STX3 protein expression and efficiency of siRNA-mediated knockdown of STX3. (A) STX3 protein expression in undifferentiated HL-60 cells (ND), differentiated HL-60 cells (D) and neutrophils was determined by western blotting. Proteins were detected with specific rabbit

polyclonal anti-STX3. A western blot representative of nine independent experiments is shown. Histogram representing the densitometry evaluation of western blots was performed by Image J software. Protein level was normalized to β-actin. Results are given in ratio between differentiated and undifferentiated HL-60 cells ± SEM of at least nine independent experiments. Significantly different from undifferentiated HL-60 cells (control): * $p < 0.05$. (B) Efficiency of STX3 siRNA was determined by qPCR analysis. Differentiated HL-60 cells were transiently transfected with STX3 siRNA. The amount of STX3 mRNA in cells was normalized to β-actin and expressed as % of STX3 mRNA obtained from cells transfected with a non-silencing sequence used as control. Results are expressed as % ± SEM of at least five independent experiments. Significantly different from control: * $p < 0.05$. (C) Specificity of STX3 antibody was determined by western blot analysis. A western blot representative of three independent experiments is shown. Histogram shows efficiency of STX3 siRNA on protein expression. The integrated intensity of the target protein band was normalized to β-actin and expressed as % of control (non-silencing siRNA sequence). Results are expressed as % ± SEM of at least three independent experiments. Significantly different from control: * $p < 0.05$.

Figure 5 Effect of STX3 knockdown in dHL-60 cells on cytokine secretion. Differentiated HL-60 cells were transiently transfected with STX3 siRNA. Transfected cells were assessed for cytokine secretion using CBA after 6 h stimulation by LPS (10 ng/mL). Results are expressed in relative cytokine secretion meaning the ratio between siRNA STX3 (+LPS/-LPS) and control-transfected cells (+LPS/-LPS) ± SEM of at least three independent experiments. Significantly different from non-silencing siRNA: * $p < 0.05$.

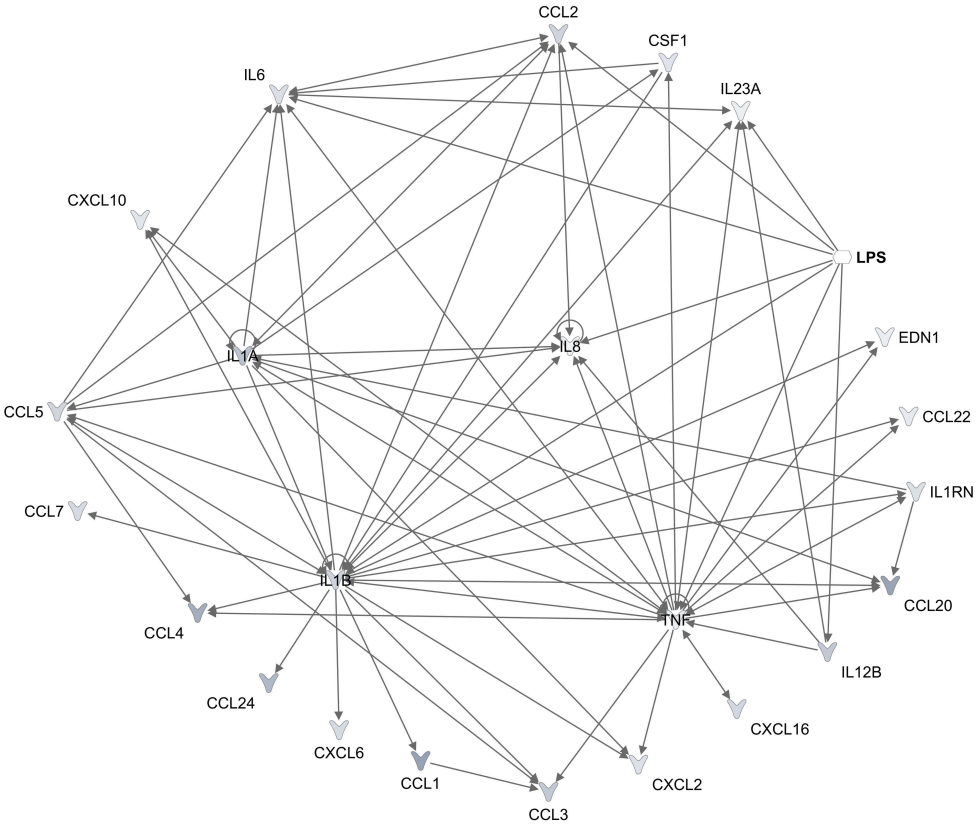
Figure 6 Effect of STX3 knockdown in dHL-60 cells on granule exocytosis. (A) Transfected cells were assessed for degranulation using myeloperoxidase (MPO), MMP-9, and albumin ELISA kits after 6 h stimulation by LPS (10 ng/mL). Cells transfected with a non-silencing sequence were used as control. Results are expressed in relative degranulation meaning the ratio between siRNA STX3 (+LPS/-LPS) and control-transfected cells (+LPS/-LPS) ± SEM of at least three independent experiments. Significantly different from non-silencing siRNA: ** $p < 0.01$. (B) Degranulation was determined by measuring the expression of CD markers specific for different types of granules at the plasma membrane by flow cytometry. The relative translocation at the plasma membrane of CD markers for each granule was determined by calculating the ratio between MFI of (+LPS/-LPS) of STX3 siRNA-treated dHL-60 cells and (+LPS/-LPS) of non-silencing siRNA-treated dHL-60 cells. Data are given as fold control ± SEM of at least three independent experiments. Significant differences are indicated as follows: **$p < 0.01$, ***$p < 0.001$.

Figure 7 Intracellular STX3 localization in dHL-60 cells. Colocalization experiments by immunofluorescence staining were performed with STX3 (green), (A) CD43 for plasma membrane, calnexin (CNX) for endoplasmic reticulum, GM130 for Golgi, EEA1 for early endosomes, LAMP-1 and LAMP-2 for late endosomes/lysosomes, (B) MPO for azurophil granules, lactoferrin (LTF) for specific granules, MMP-9 for gelatinase granules, tetranectin (TN) for secretory vesicles (red), and DAPI for cell nucleus (blue). Scale bar, 5 μm. Merge confocal images only represent green and red channels in order to conclude for co-localization. Corresponding scatter plots of the paired intensities of the green (y-axis for STX3 pixels) and red (x-axis for CD43, CNX, GM130, EEA1, LAMP-1, LAMP-2, MPO, LTF, MMP-9, or TN pixels) channels are depicted on the right column for the entire overlay image. Pearson correlation coefficients were calculated for both channels in deconvolved z-stack confocal images by AutoQuant X3. Values ≥ 0.7 were considered strongly positive for co-localization. I, II, III and IV represent the azurophil, specific and gelatinase granules as well as secretory vesicles, respectively.

Figure 8 Intracellular STX3 localization in human neutrophils. Colocalization experiments by immunofluorescence staining were performed with STX3 (green), (A) CD43 for plasma membrane, calnexin (CNX) for endoplasmic reticulum, GM130 for Golgi, EEA1 for early endosomes, LAMP-1 and LAMP-2 for late endosomes/lysosomes, (B) MPO for azurophil granules, lactoferrin (LTF) for specific granules, MMP-9 for gelatinase granules, tetranectin (TN) for secretory vesicles (red), and DAPI for cell nucleus (blue). Scale bar, 5 μm. Merge confocal images only represent green and red channels in order to conclude for co-localization. Corresponding scatter plots of the paired intensities of the green (y-axis for STX3 pixels) and red (x-axis for CD43, CNX, GM130, EEA1, LAMP-1, LAMP-2, MPO, LTF, MMP-9, or TN pixels) channels are depicted on the right column for the entire overlay image. Pearson correlation coefficients were calculated for both channels in deconvolved z-stack confocal images by AutoQuant X3. Values ≥ 0.7 were considered strongly positive for co-localization. I, II, III and IV represent the azurophil, specific and gelatinase granules as well as secretory vesicles, respectively.

Figure 1

(A)


CCL2
CSF1
IL6
IL23A
CXCL10
LPS
EDN1
IL1A
IL8
CCL5
CCL22
IL1RN
CCL7
IL1B
CCL20
TNF
CCL4
IL12B
CCL24
CXCL16
CXCL6
CCL1
CXCL2
CCL3

(B)

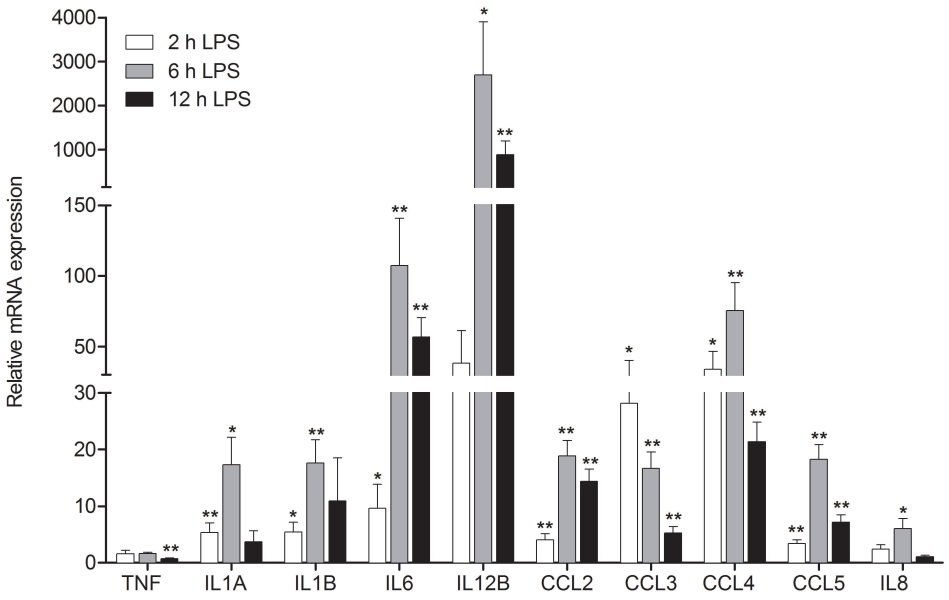
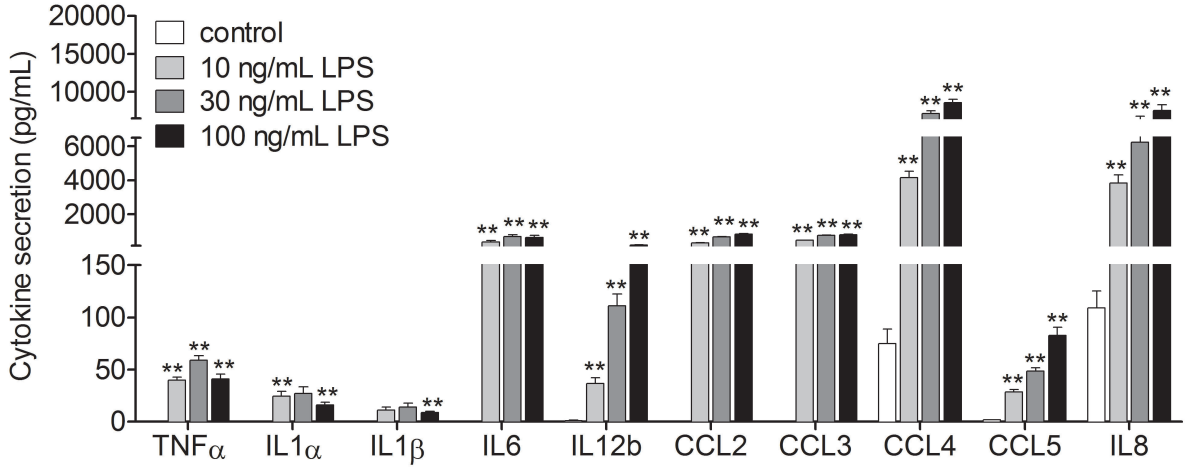

2 h LPS
6 h LPS
12 h LPS
Relative mRNA expression
4000
3000
2000
1000
150
100
50
30
20
10
0
TNF
IL1A
IL1B
IL6
IL12B
CCL2
CCL3
CCL4
CCL5
IL8

Figure 2

(A)


Cytokine secretion (pg/mL)
control
10 ng/mL LPS
30 ng/mL LPS
100 ng/mL LPS
TNFα
IL1α
IL1β
IL6
IL12b
CCL2
CCL3
CCL4
CCL5
IL8

(B)

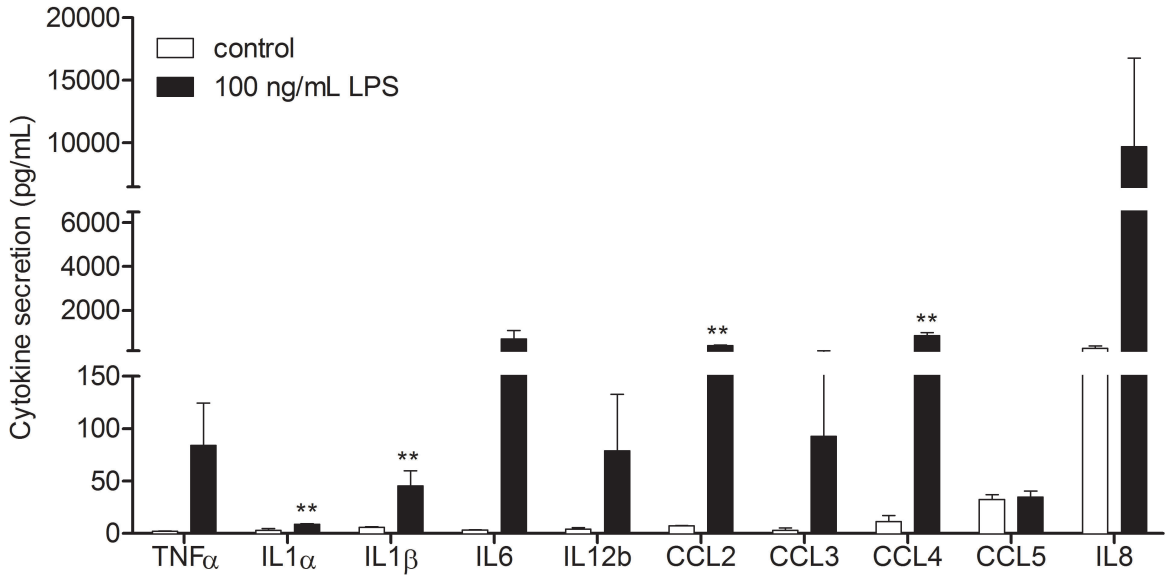

Cytokine secretion (pg/mL)
control
100 ng/mL LPS
TNFα
IL1α
IL1β
IL6
IL12b
CCL2
CCL3
CCL4
CCL5
IL8

Figure 3

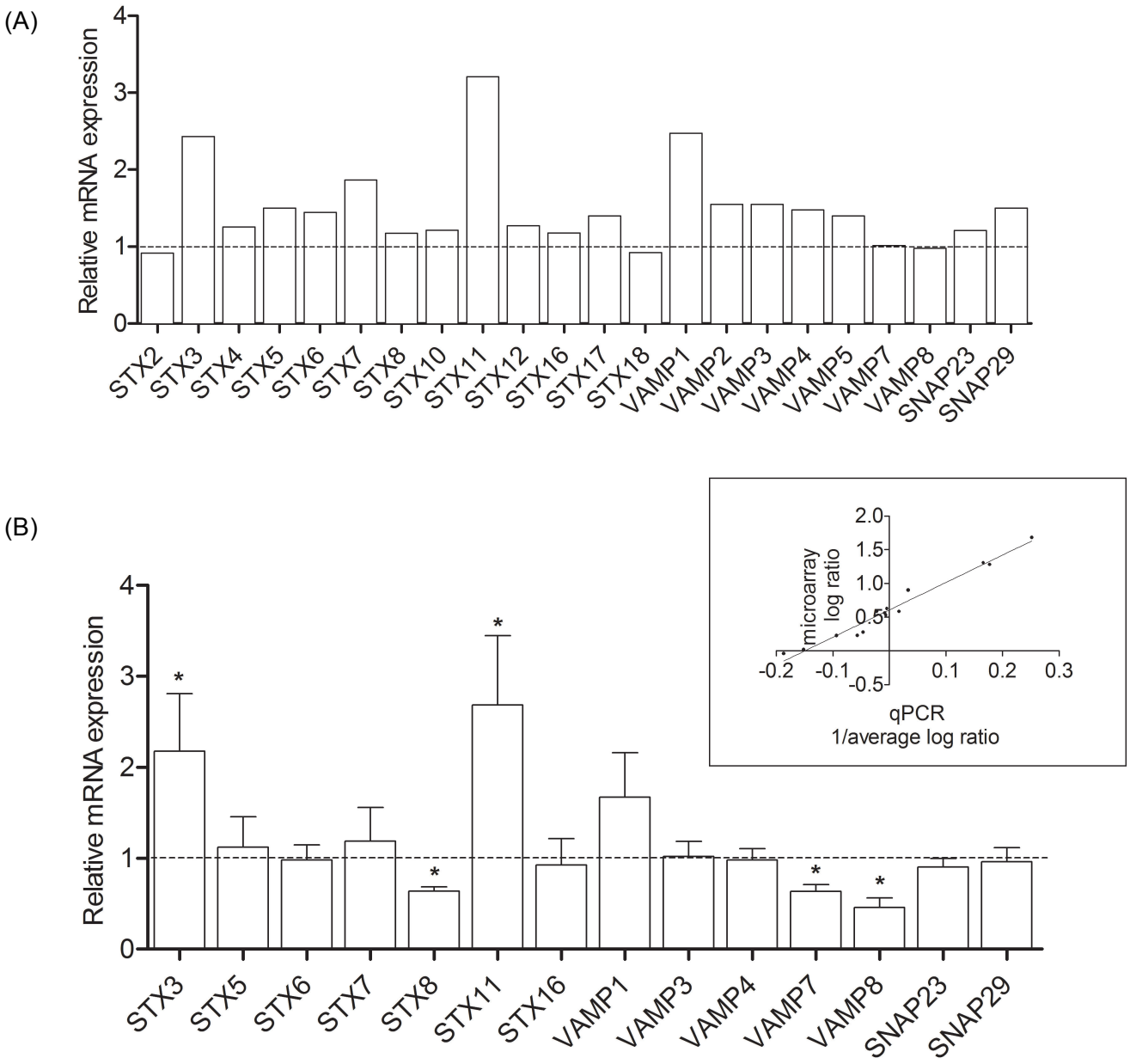

(A)
Relative mRNA expression
0
1
2
3
4
STX2
STX3
STX4
STX5
STX6
STX7
STX8
STX10
STX11
STX12
STX16
STX17
STX18
VAMP1
VAMP2
VAMP3
VAMP4
VAMP5
VAMP7
VAMP8
SNAP23
SNAP29
(B)
Relative mRNA expression
0
1
2
3
4
*
*
*
*
*
STX3
STX5
STX6
STX7
STX8
STX11
STX16
VAMP1
VAMP3
VAMP4
VAMP7
VAMP8
SNAP23
SNAP29
microarray log ratio
2.0
1.5
1.0
0.5
-0.5
-0.2
-0.1
0.1
0.2
0.3
qPCR
1/average log ratio

Figure 4

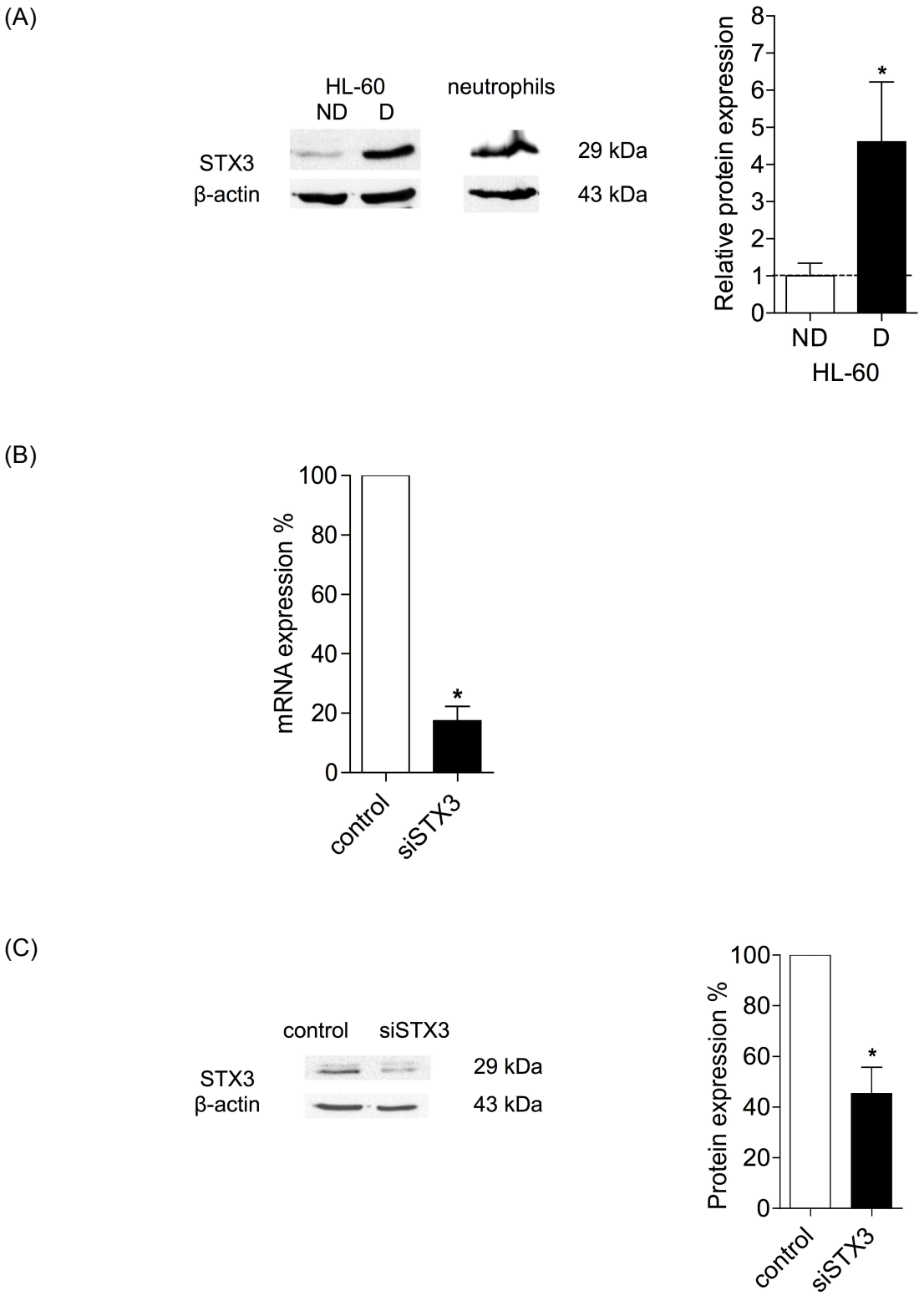

(A)
HL-60
ND D
neutrophils
STX3
β-actin
29 kDa
43 kDa
Relative protein expression
8
7
6
5
4
3
2
1
0
*
ND D
HL-60
(B)
mRNA expression %
100
80
60
40
20
0
*
control
siSTX3
(C)
control siSTX3
STX3
β-actin
29 kDa
43 kDa
Protein expression %
100
80
60
40
20
0
*
control
siSTX3

Figure 5

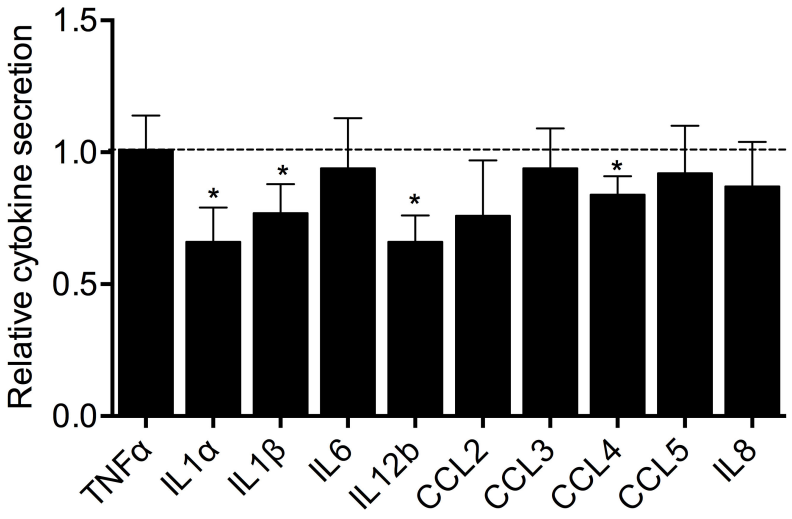
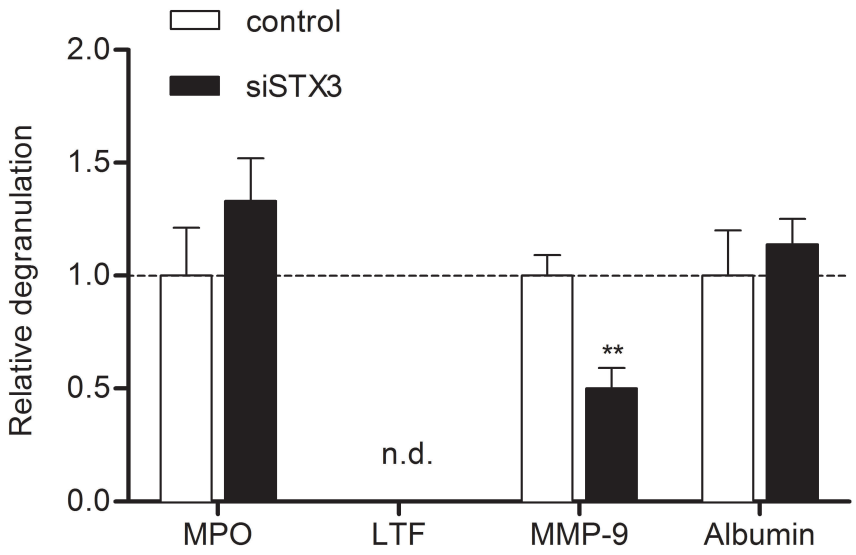

Relative cytokine secretion
1.5
1.0
0.5
0.0
*
*
*
*
TNFα
IL1α
IL1β
IL6
IL12b
CCL2
CCL3
CCL4
CCL5
IL8

Figure 6

(A)


control
siSTX3
Relative degranulation
2.0
1.5
1.0
0.5
0.0
**
n.d.
MPO
LTF
MMP-9
Albumin

(B)

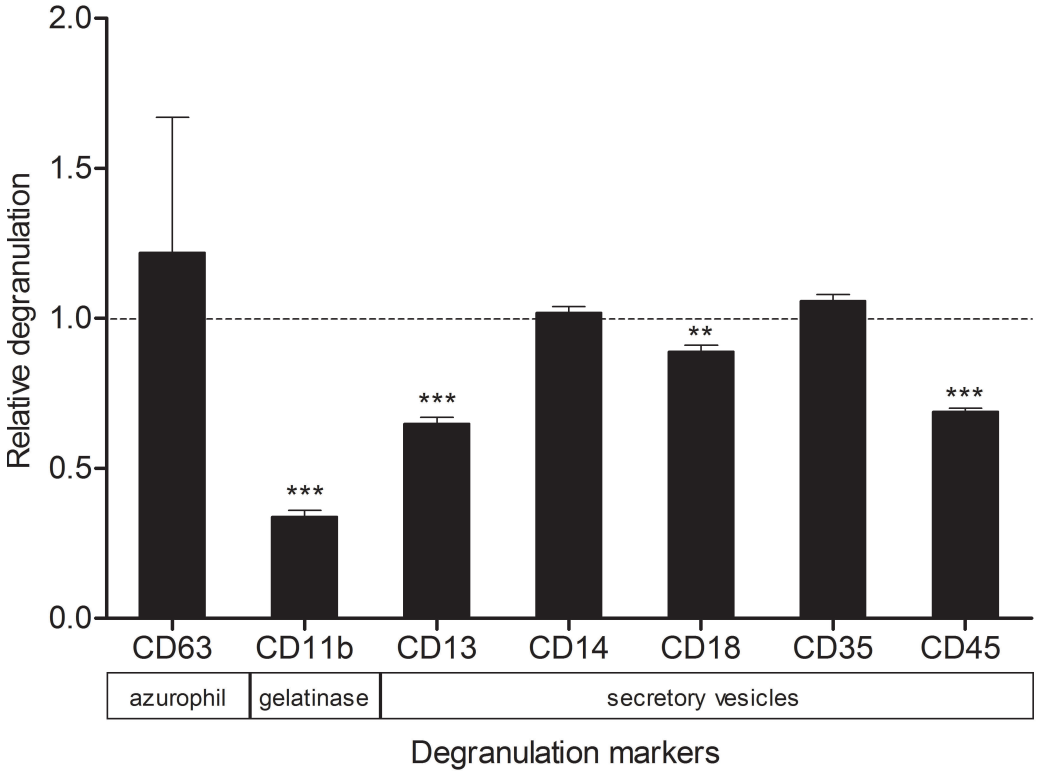
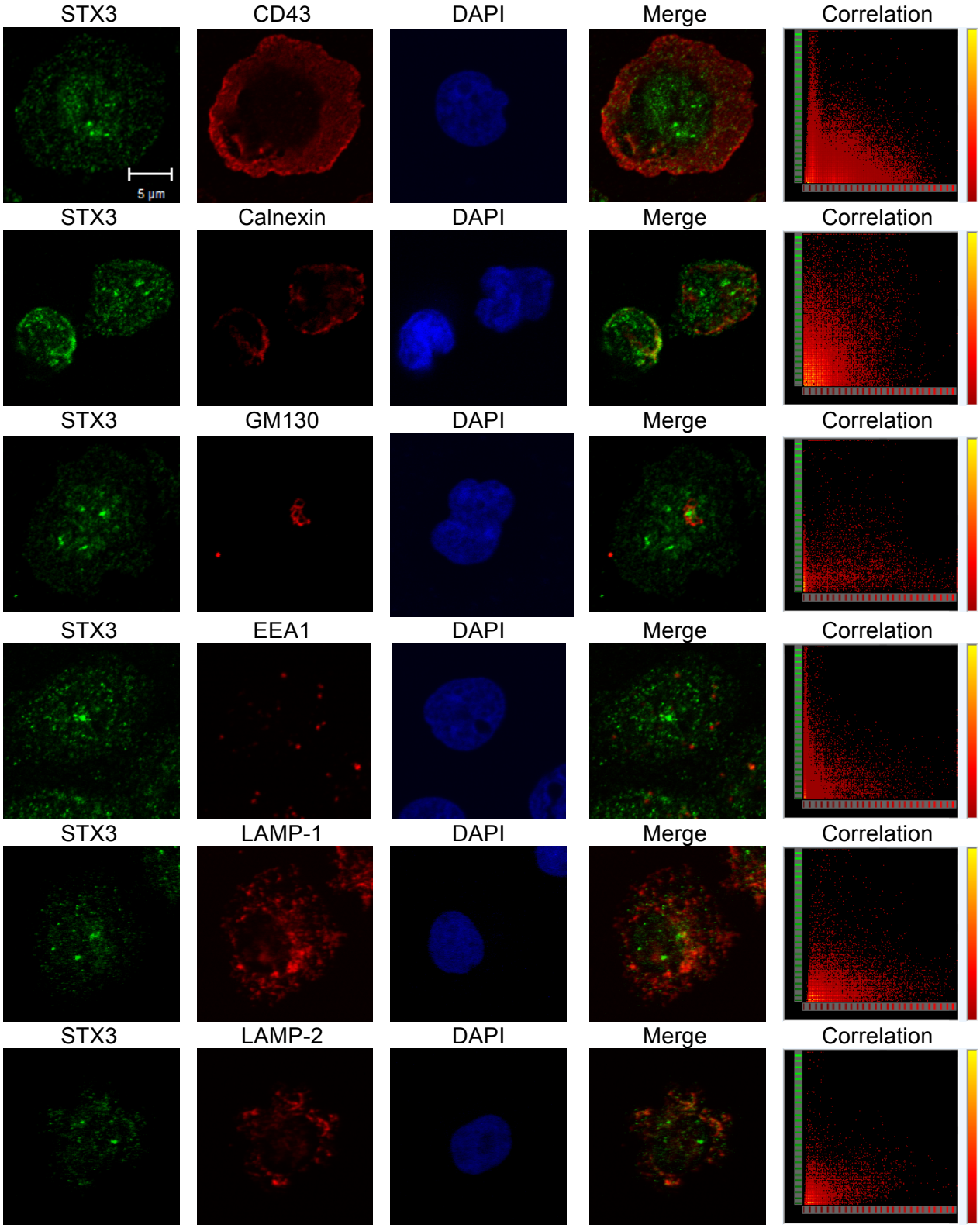
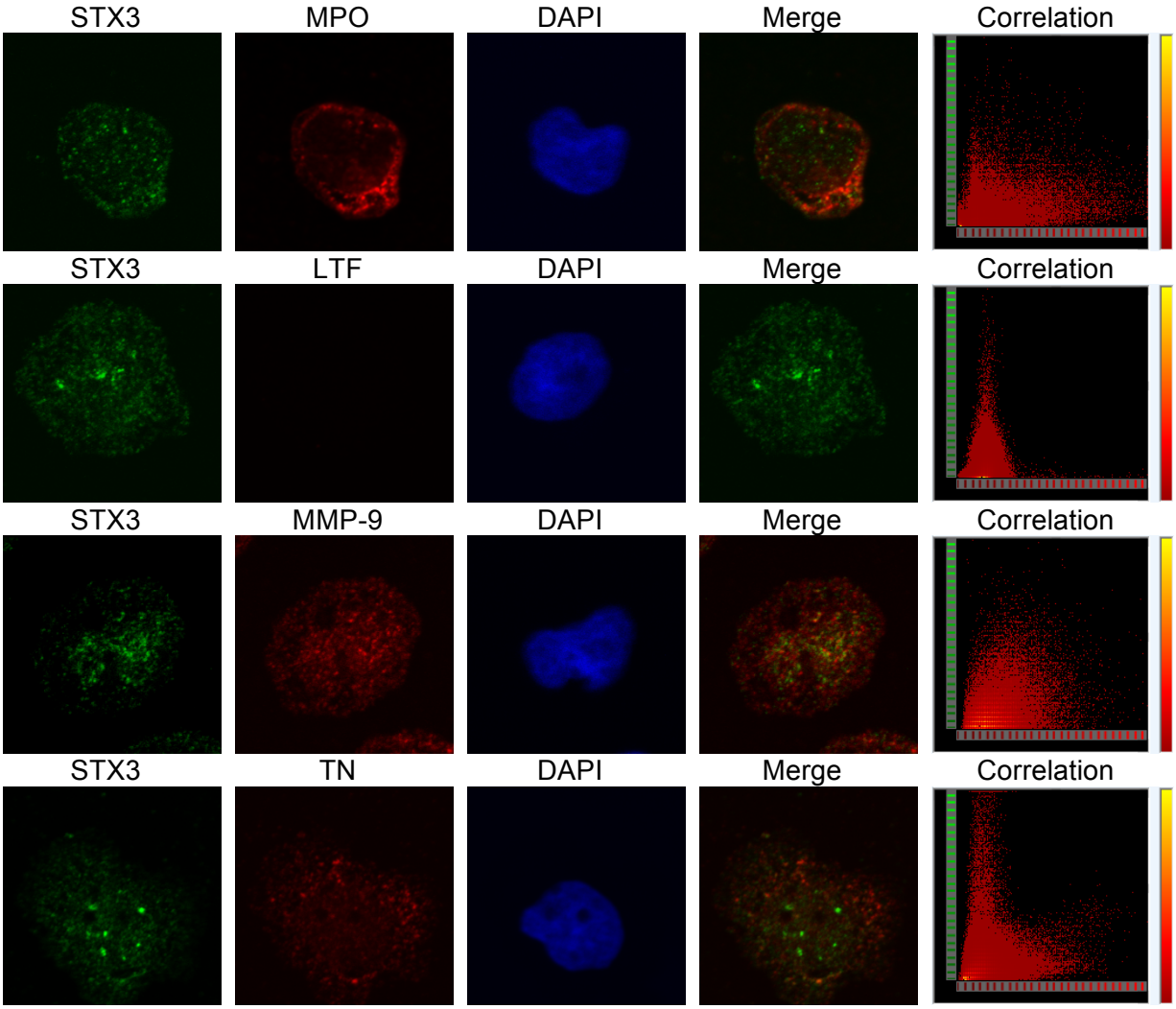

Relative degranulation
2.0
1.5
1.0
0.5
0.0
***
***
**
***
CD63
CD11b
CD13
CD14
CD18
CD35
CD45
azurophil
gelatinase
secretory vesicles
Degranulation markers

Figure 7

(A)


STX3
CD43
DAPI
Merge
Correlation
5 µm
STX3
Calnexin
DAPI
Merge
Correlation
STX3
GM130
DAPI
Merge
Correlation
STX3
EEA1
DAPI
Merge
Correlation
STX3
LAMP-1
DAPI
Merge
Correlation
STX3
LAMP-2
DAPI
Merge
Correlation

(B)


STX3
MPO
DAPI
Merge
Correlation
STX3
LTF
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Correlation
STX3
MMP-9
DAPI
Merge
Correlation
STX3
TN
DAPI
Merge
Correlation

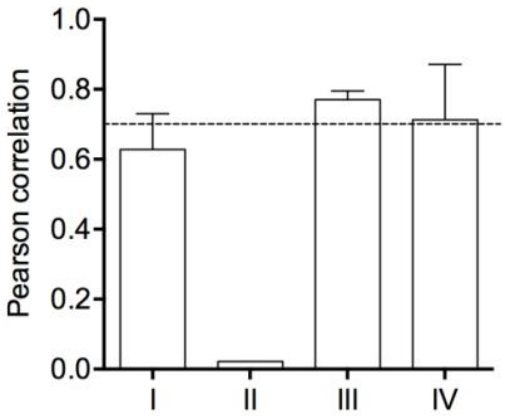
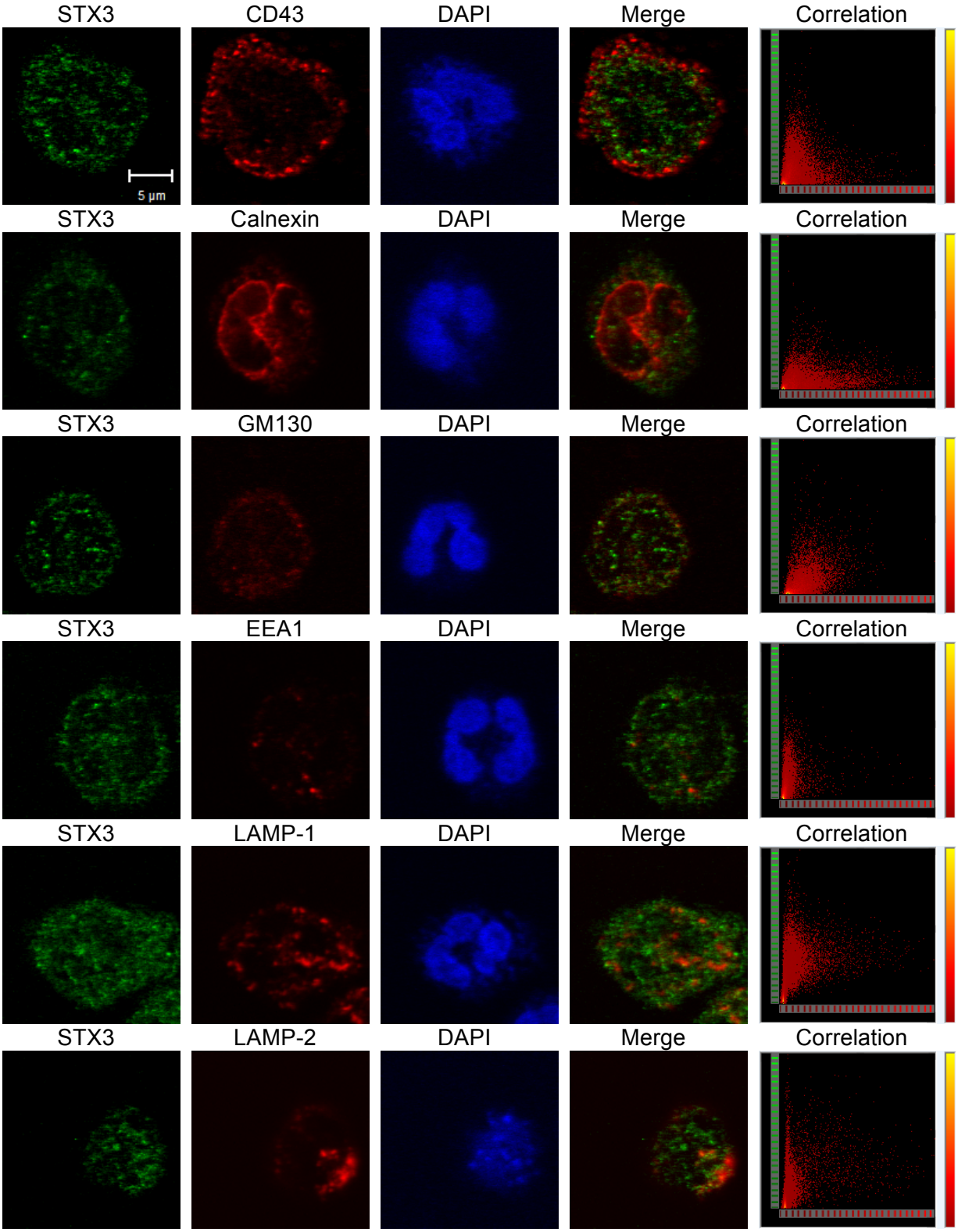
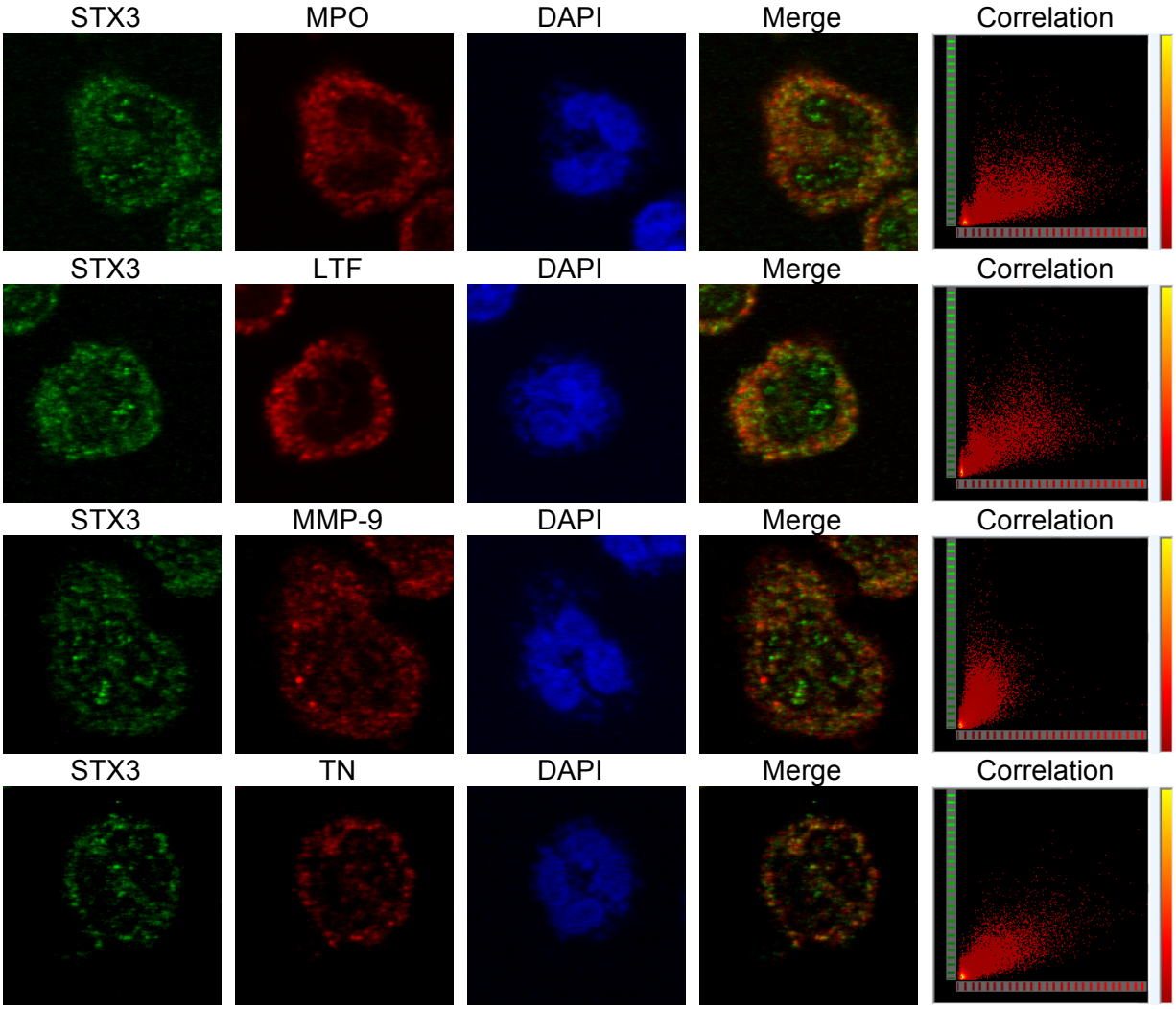

Pearson correlation
1.0
0.8
0.6
0.4
0.2
0.0
I
II
III
IV

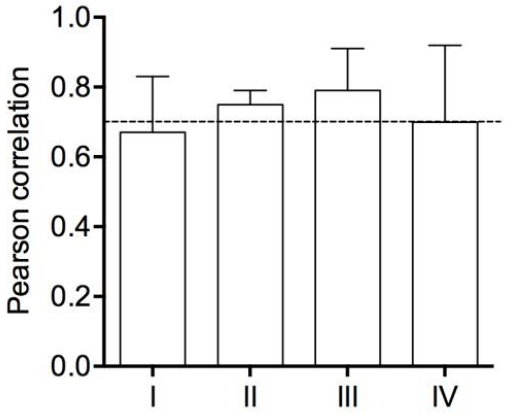
Figure 8

(A)


STX3
CD43
DAPI
Merge
Correlation
5 µm
STX3
Calnexin
DAPI
Merge
Correlation
STX3
GM130
DAPI
Merge
Correlation
STX3
EEA1
DAPI
Merge
Correlation
STX3
LAMP-1
DAPI
Merge
Correlation
STX3
LAMP-2
DAPI
Merge
Correlation

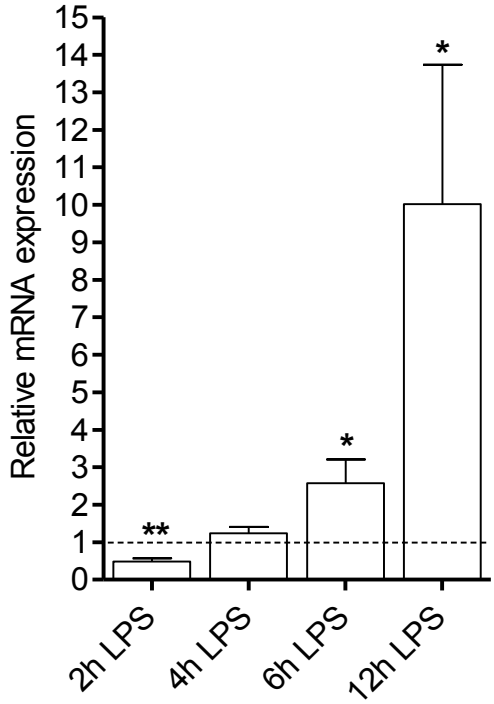
(B)


STX3
MPO
DAPI
Merge
Correlation
STX3
LTF
DAPI
Merge
Correlation
STX3
MMP-9
DAPI
Merge
Correlation
STX3
TN
DAPI
Merge
Correlation

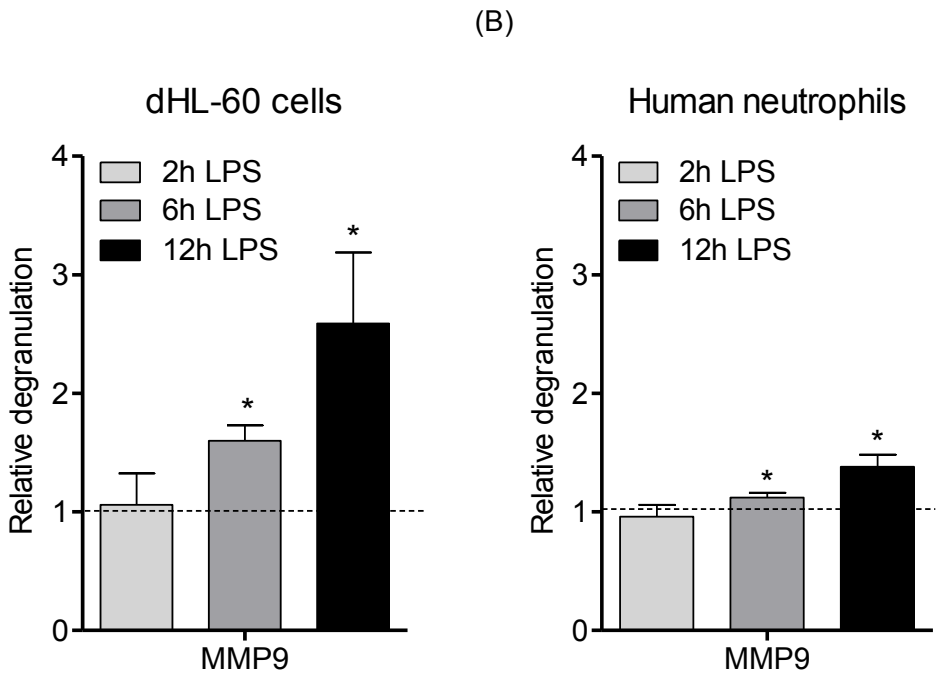

Pearson correlation
1.0
0.8
0.6
0.4
0.2
0.0
I
II
III
IV

1. Supplemental Data

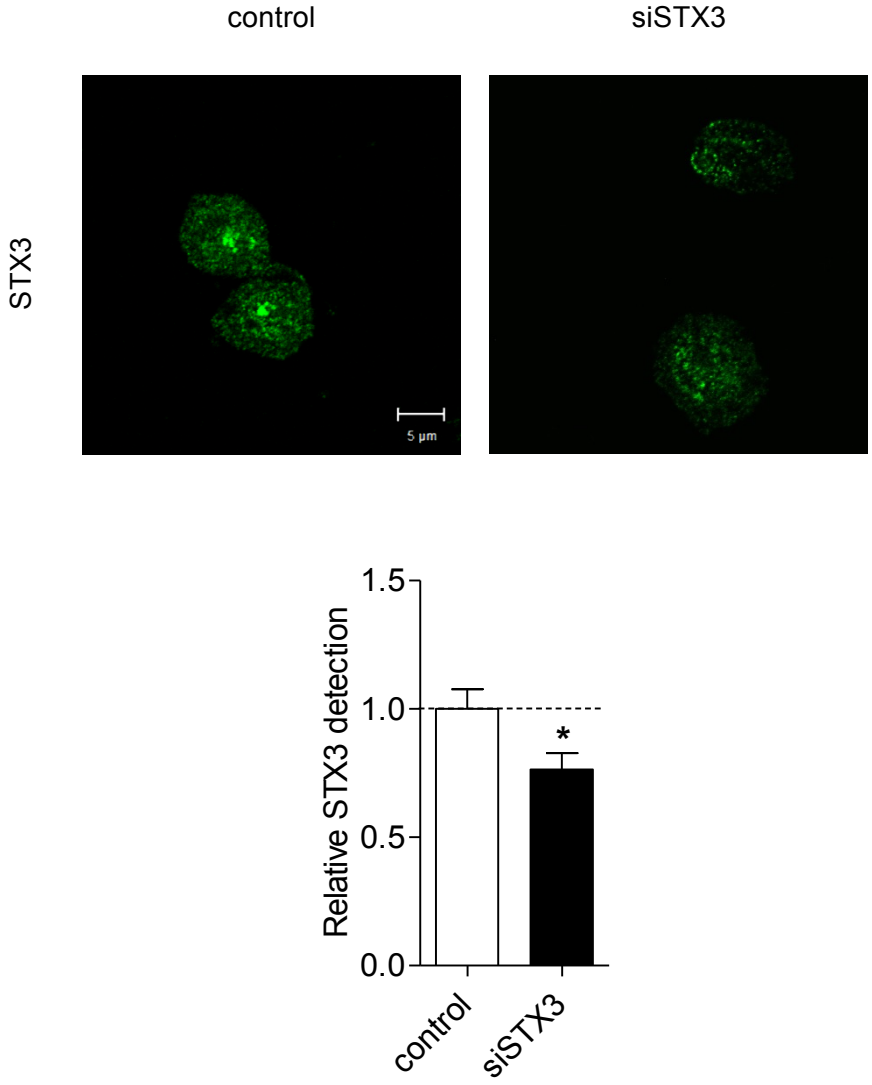
Suppl. Fig. 1 Time-dependent effect of LPS on MMP-9 mRNA expression in dHL-60 cells. Validation of MMP-9 mRNA expression was performed by real-time qPCR after treatment with 100 ng/mL LPS for 2, 4, 6, and 12 h. Data were normalized to β-actin and expressed relative to the calibrator (non-stimulated control) at the corresponding time ± SEM of at least four independent experiments. Significantly different from MMP-9 expression in the non-stimulated control: ** $p < 0.01$, * $p < 0.05$.



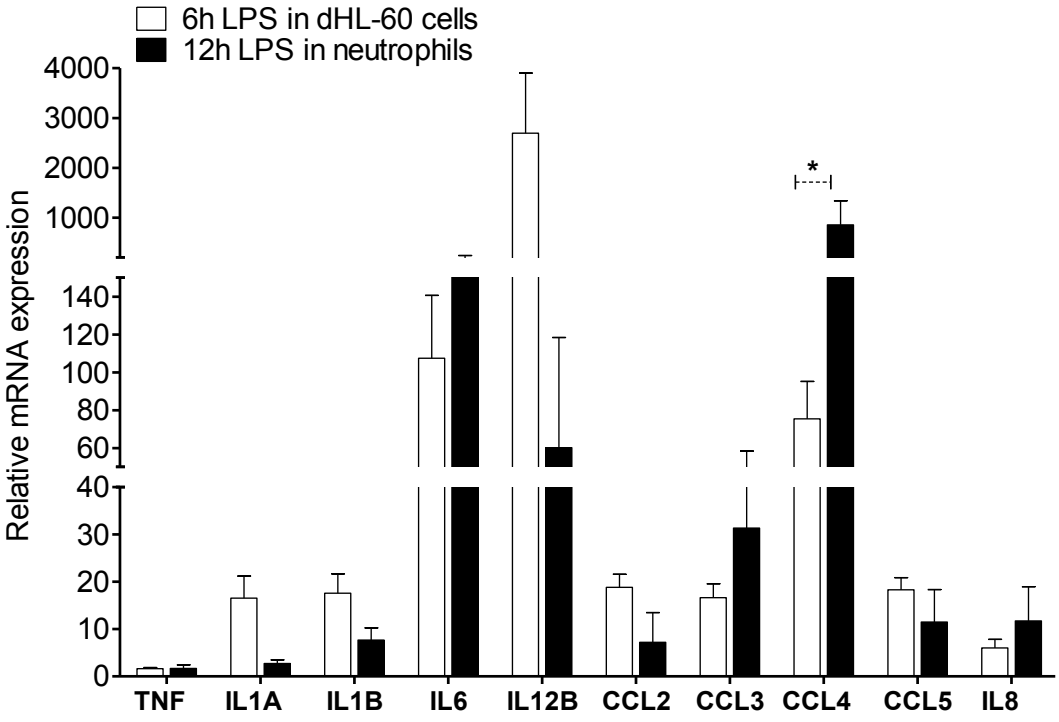
Suppl. Fig. 2 Granule exocytosis from (A) dHL-60 cells and (B) human neutrophils upon LPS stimulation. Cells were assessed for degranulation using MMP-9 ELISA kit after 2, 6 and 12 h stimulation by 100 ng/mL LPS. Supernatants from non-stimulated cells at the same time point served as control. ELISA results were normalized by LDH absorbance values. Results are expressed in relative degranulation meaning the ratio between LPS-stimulated samples and non-stimulated samples ± SEM of at least four independent experiments. Significantly different from non-stimulated control: * $p < 0.05$.



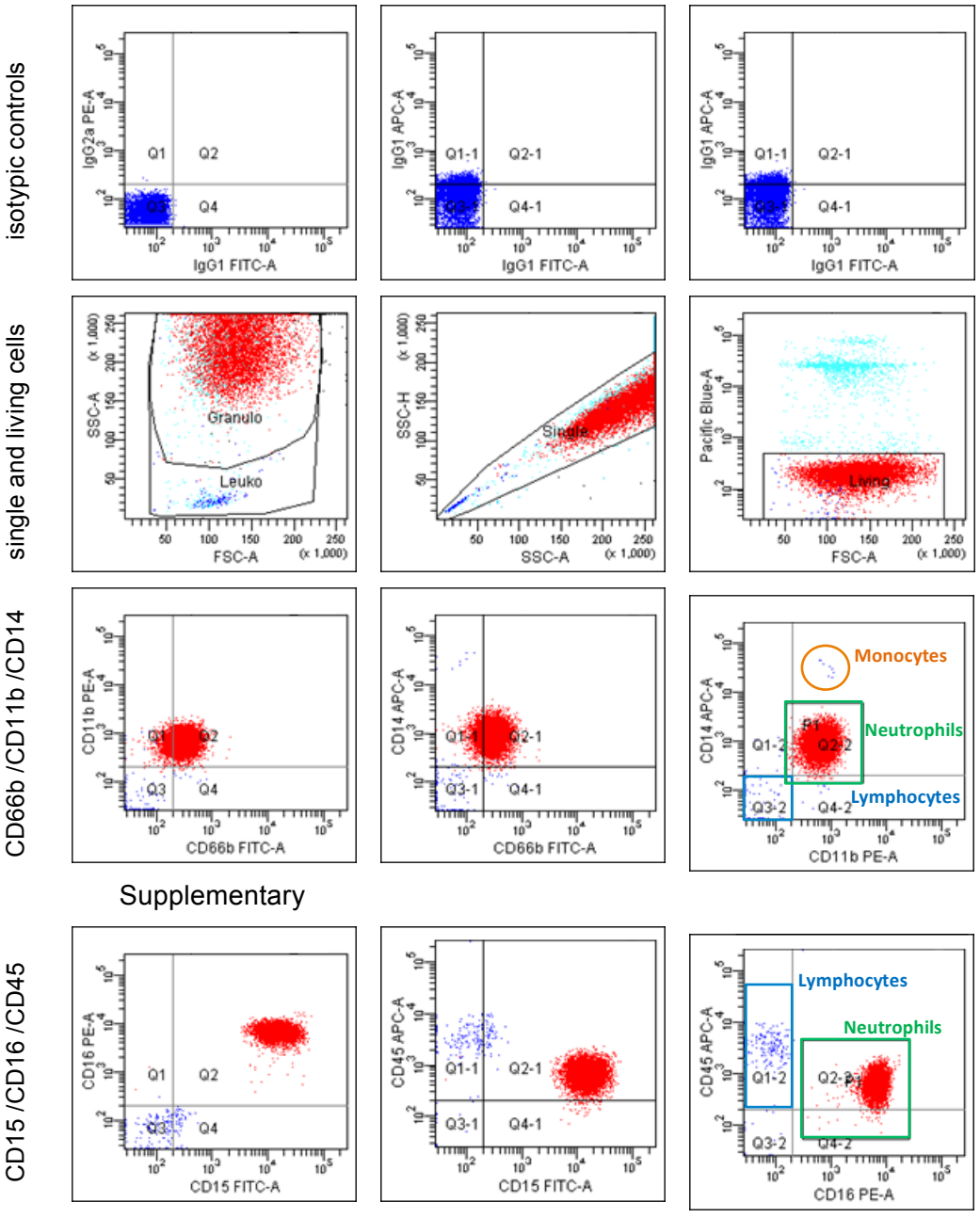
Suppl. Fig. 3 Immunofluorescence analysis of STX3 in dHL-60 cells treated with non-silencing siRNA (control) or STX3 siRNA and the corresponding densitometry evaluation. Results are given in ratio between STX3 siRNA- and non-silencing siRNA-treated (control) dHL-60 cells ± SEM of at least three independent experiments. Significantly different from non-silencing siRNA-treated dHL-60 cells (control): * $p < 0.05$. Scale bar, 5 µm.



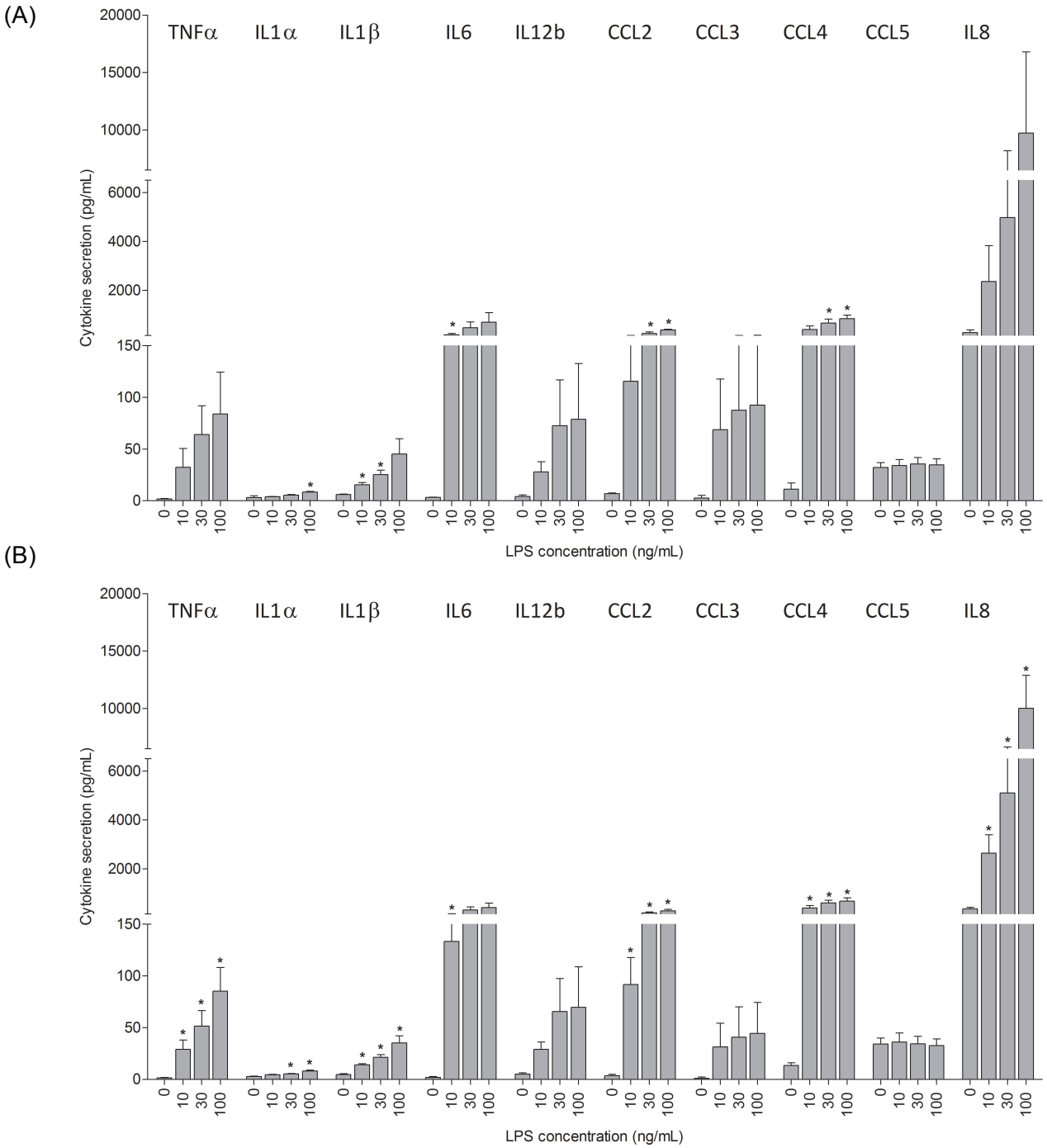
Suppl. Fig. 4 Only highest cytokine mRNA expression pattern of dHL-60 cells and freshly purified neutrophils. Data were normalized to β-actin and expressed relative to the non-stimulated control ± SEM of at least five independent experiments. Significantly different cytokine expression between dHL-60 cells and human neutrophils: * $p < 0.05$.



Suppl. Fig. 5 Representative evaluation of neutrophil purity in our cell preparations. To specifically identify neutrophils, purity of isolated neutrophils was analyzed by the BD FACSCanto™ II flow cytometer (BD Biosciences) using two mixtures of selection markers CD66b-FITC/CD11b-PE/CD14-APC and CD15-FITC/CD16-PE/CD45-APC on 10,000 events in the gated population of homogenous (FSC-A vs. SSC-A), single (SSC-A vs. SSC-H) and living cells (negative cells for Sytox Blue staining). In our evaluation, **neutrophils** are $CD11b^{+}CD66b^{+}CD14^{low}$ (98.1%), **monocytes** are $CD11b^{+}CD66b^{-}CD14^{high}$ (0.1%), and **lymphocytes** are $CD11b^{-}$ $CD66b^{-}CD14^{-}$ (1.8%). Eosinophils, that are $CD45^{+}$ $CD16^{-}$ and SSC^{high}, are embedded in the lymphocyte gate of the last plot.



Suppl. Fig. 6 Purity of neutrophil preparations and LPS-induced cytokine secretion. Cytokine secretion was measured upon 12 h stimulation with 0, 10, 30, and 100 ng/mL LPS by (A) ≥ 98% pure neutrophils (n=3) and (B) ≥ 90% pure neutrophils (n=5). Results are mean ± SEM of at least three independent experiments. Significantly different from cytokine secretion in non-stimulated control: * $p < 0.01$.



Manuscript III *to be submitted*

III STX3 and SNAP29 assemble in a SNARE complex to regulate IL-12b secretion and MMP-9 exocytosis

Previously, we identified the Q_a-SNARE STX3 as a major player in secretion of cytokines and granules. However, further investigations are still needed to elucidate the precise mechanism by which STX3 specifically regulates the trafficking pathway.
It is known that the first step in the SNARE assembly-disassembly cycle consists of the clustering of SNAREs into acceptor complexes and represents a rate-limiting step in the whole cycle. Hence, the aim of this part of the study was **to identify potential $Q_{b,c}$-SNARE partners interacting with STX3 which are specific for the pro-inflammatory mediator release.**

We investigated the expression of $Q_{b,c}$-SNAREs in our cells and identified SNAP23 and SNAP29 to be potential candidates of STX3 interaction partners. Functional analysis shows that SNAP23 did not affect neutrophil functions whereby SNAP29 knockdown significantly inhibited the release of IL-12b, CCL2 and IL-8 as well as MMP-9. Immunofluorescence studies as well as proximity ligation experiments confirmed the colocalizaton of STX3 and SNAP29.

Taken together, our findings point towards the formation of a cognate STX3-SNAP29 complex with functional impact on cytokine release and degranulation.

STX3 and SNAP29 assemble in a SNARE complex to regulate IL-12b secretion and MMP-9 exocytosis

Isabelle Naegelen, Sébastien Plançon, Fabrice Tolle, Eric J. Tschirhart, and Sabrina Bréchard

University of Luxembourg, Life Sciences Research Unit, Calcium Signaling and Inflammation, 162A avenue de la Faïencerie, L-1511 Luxembourg

Corresponding author:

Sabrina Bréchard, PhD

University of Luxembourg, Life Sciences Research Unit, Calcium Signaling and Inflammation

162A avenue de la Faïencerie

L-1511 Luxembourg, Luxembourg

Phone number: +352/466644-6434

Fax number: +352/466644-6435

E-mail address: sabrina.brechard@uni.lu

Running title: STX3 and SNAP29 regulate neutrophil exocytosis

Keywords: SNARE proteins, neutrophils, degranulation, cytokine secretion

Abbreviations: CBA, cytometric bead array; dHL-60, DMSO-differentiated HL-60 cells; MPO, myeloperoxidase; LTF, lactoferrin; MMP-9, gelatinase B; TN, tetranectin; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; STX3, syntaxin 3; SNAP23, synaptosomal-associated protein, 23kDa; SNAP29, synaptosomal-associated protein, 29kDa

Financial support was provided by the University of Luxembourg.

Abstract

During inflammatory processes, cascade of events leading to exocytosis in neutrophils need to be rigorously regulated in order to prevent chronic tissue injuries. Intracellular trafficking pathways are governed by components of the SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptor) fusion machinery. Formation of SNARE complexes mediates the fusion of two opposed membranes and, ultimately, the release of cargo proteins into the extracellular milieu.

We have recently shown that the Q_a-SNARE syntaxin 3 (STX3) is required for the release of IL-1α, IL-1β, IL-12b, CCL4 and MMP-9 upon LPS stimulation. Here, we analyze the involvement of $Q_{b,c}$-SNARE partners in exocytosis and their potential association with STX3. Determination of SNARE mRNA expression revealed that SNAP29 and SNAP23 are expressed in neutrophil-like HL-60 cells and neutrophils isolated from venous peripheral blood. Inhibition of SNAP29 using a target-specific RNAi approach decreased IL-8, IL-12b, CCL2 and MMP-9 release whereas SNAP23 knockdown altered IL-8 release. Additionally, immunofluorescence studies showed that SNAP29 colocalizes and interacts with STX3.

In summary, we provide evidence that SNAP29 in conjunction with STX3 are crucial for the release of IL-12b and MMP-9 and different combinations of SNARE proteins may adjust the secretion of other pro-inflammatory mediators.

Introduction

Upon infection by microbial agents, activation of neutrophils is pivotal for the onset of inflammatory responses and thus, for efficient host defense mechanisms. During these processes, neutrophils aim to kill invading pathogens and recruit other immune cells to the site of inflammation (Cassatella, 1995; Borregaard and Cowland, 1997). Among other functions, they are able to release a large plethora of granule proteins (*e.g.* antimicrobial proteins, alarmins and proteases) and pro-inflammatory mediators such as cytokines, which are critical events for antimicrobial activity and communication between the innate and adaptive immune systems. Preformed or *de novo* synthesized mediators are specifically transferred between the different intracellular compartments to be exocytosed to the extracellular milieu in order to ensure an appropriate and adapted response.

Because tissue integrity can be dramatically compromised by defective mechanisms of vesicle trafficking along the secretory pathway leading to the development of severe chronic inflammatory diseases such as inflammatory bowel disease (Radford-Smith and Jewell, 1996), or rheumatoid arthritis (Edwards and Hallett, 1997), mechanisms of secretion of the various proteins involved must be firmly controlled. The identification of molecules involved in exocytosis of mediators is a critical step to provide novel therapeutic targets for the modulation of immune responses by neutrophils and thus, prevent inflammatory disorders since these cells are directly involved in the exacerbation of inflammation.

In eukaryotic cells, vesicle-mediated trafficking and exocytosis are governed by key coordinators collectively called soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins (Sanderfoot and Raikhel, 1999; Chen and Scheller, 2001). Different SNAREs from two opposing membranes act together in order to form complexes in *trans*-configuration and lead to the release of cargo molecules *via* fusion of vesicle and plasma membranes. To date, the highly conserved SNARE superfamily consists of 38 mammalian members that are subdivided, accordingly to their central residue contributing to the SNARE complex formation, into Q_a-, $Q_{b,c}$- and R- SNAREs (Jahn and Sudhof, 1999). In general, one Q_a- (syntaxin family) and two $Q_{b,c}$-SNAREs (SNAP25 homologues) located on the target membrane generate so-called “acceptor complexes”. Previous study has shown that this step is rate-limiting for subsequent interaction with one R-SNARE (synaptobrevin family) located on the vesicle membrane in order to assemble to a twisted parallel 4-helical bundle and lead to the formation of the *trans*-SNARE complex (Jahn and Scheller, 2006). Many combinations between Q- and R-SNAREs exist in order to assure the specificity of intracellular trafficking and, ultimately, secretion.

While SNAREs have first been discovered and broadly described in neuronal cells, their specific role and functional combination in immune cells still remains largely unknown. In

neutrophils, the Q_a- and $Q_{b,c}$-SNAREs syntaxin 6 and SNAP23 have been shown to form a complex mediating the release of CD67-enriched specific granules (Martin-Martin *et al.*, 2000).

In a recent study, we determined the role of the Q_a-SNARE syntaxin 3 (STX3) in the exocytosis pathway of gelatinase B (MMP-9), as well as the cytokines IL-1α, IL-1β, IL-12b and CCL4 upon LPS stimulation (see *Manuscript II*). Here we aim to further investigate the molecular machinery implicated in the trafficking pathway of mediators in neutrophils. Our research emphasizes on the identification of potential binding partners of STX3 regulating specific protein secretion. Since the rate-limiting step in the SNARE assembly-disassembly cycle consists of the formation of acceptor complexes by Q_a- and $Q_{b,c}$-SNAREs, we speculate on the participation of SNAP25 homologues in the release of cytokines in human neutrophils and degranulation together with STX3. In this study, we report that SNAP29 is associated to STX3 and regulates IL-12b and MMP-9 secretion under pro-inflammatory conditions in DMSO-differentiated HL-60 cells, a neutrophil cell model.

Materials and Methods

Cell culture

The promyelocytic HL-60 cell line (Collins *et al.*, 1979) was purchased at ATCC-American Type Culture Collection (Manassas, VA, USA) (CCL-240) and was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and streptomycin (Lonza, Verviers, Belgium). HL-60 cells were differentiated into neutrophil-like cells by addition of 1.3% DMSO to the culture medium for 4.5 days.

Purification of human neutrophils

Peripheral blood of healthy volunteers was collected in EDTA-containing Vacutainer® tubes (BD Biosciences, Erembodegem, Belgium). Samples were collected in accordance with the good clinical practices and following the national and international ethical recommendations. Neutrophils were isolated from blood samples by Polymorphprep™ separation procedure (Axis-Shield, Dundee, Scotland) according to manufacturer's instructions. Remaining erythrocytes in the neutrophil cell suspension were lysed for 10 min with red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4). Neutrophils were washed and resuspended in PBS 1X (pH 7.4). Purity of isolated neutrophils was analyzed by the BD FACSCanto™ II flow cytometer (BD Biosciences) using two mixtures of selection markers CD66b-FITC (clone B13.9)/ CD11b-PE (clone MEM-174)/ CD14-APC (clone MEM-18) and CD15-FITC (clone MEM-158)/ CD16-PE (clone LNK16)/CD45-APC (clone MEM-28) (Immunotools, Friesoythe, Germany) on 10,000 events in the gated population of homogenous (FSC-A *vs.* SSC-A), single (SSC-A *vs.* SSC-H) and living cells (negative cells

for Sytox Blue staining (Life Technologies, Gent, Belgium)). Purified neutrophils were positive for all the selection markers used by flow cytometry. Human neutrophils were cultured in X-VIVO™ 15 medium with L-glutamine, and gentamicin (Lonza) at 37°C and 5% CO_2 up to 24 h after purification.

Total RNA extraction

RNA from dHL-60 cells was extracted using innuPrep RNA mini kit (Analytik, Jena, Germany) according to manufacturer's instructions. The quantity and quality of total RNA were evaluated by a NanoDrop® ND-2000 spectrophotometer (Thermo Scientific, Erembodegem, Belgium).

Quantitative real-time PCR (qPCR)

cDNA was synthesized from 2 µg total RNA by using the ThermoScript™ Reverse Transcriptase System (Life Technologies, Gent, Belgium). Forward and reverse primers (for SNAP23: forward: 5'-AAG-AAC-TTT-GAG-TCT-GGC-AAG-G-3'; reverse: 5'-TTG-CTG-AAG-CTG-ACC-ATT-TG-3'; for SNAP29: forward: 5'-CAA-AGC-AAA-GGA-AAC-CTC-CA-3'; reverse: 5'-GCC-TAT-GGA-GGC-TGT-GGA-TA-3') were designed with the aid of the GenScript Online PCR Primer Design Tool (www.genscript.com). Primer efficiency and amplicon integrity were controlled by analysis of melting-curves, agarose gel and sequencing. qPCR was performed with iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Nazareth, Belgium) for 40 cycles on a MyIQ single color real-time qPCR detection system. All data were analyzed in triplicates. Quantification of mRNA levels was carried out with Bio-Rad iQ5 software and normalization of mRNA levels was calculated with respect to the reference gene ß-actin according to the $2^{-\Delta\Delta CT}$ Livak method (Livak and Schmittgen, 2001).

Short interfering RNA assays

Double-stranded short interfering RNA (siRNA) of 19 nucleotides targeting human SNAP23 and SNAP29 (as well as a non-silencing scramble siRNA) were custom-ordered from Eurogentec (Seraing, Belgium). Three synthesized siRNA candidate duplexes were tested for silencing efficiency. The most efficient siRNA to mediate knockdown expression of SNAP23 and SNAP29 was chosen leading to ~40% protein expression inhibition and used for functional analysis. All experiments were performed with a siRNA duplex against SNAP23 (sense: 5'-GAGCAAAGAAACUCAUUGA-3'; antisense: 5'-UCAAUGAGUUUCUUUGCUC-3') or SNAP29 (sense: 5'- GCCCAACAACAGAUUGAAA-3', antisense: 5'- UUUCAAUCUGUUGUUGGGC-3'). The non-silencing control siRNA (sense: 5'-UGCGCUACGAUCGACGAUG-3', antisense: 5'-CAUCGUCGAUCGUAGCGCA-3') was used as a negative control. siRNA target sequences chosen in this study were screened by National Center for Biotechnology Information BLAST searches to avoid mismatches. dHL-60 cells ($2x10^6$) were transiently transfected with 2 µg of SNAP23 or SNAP29 siRNA or non-silencing control sequence at day 3 of differentiation using the Nucleofector apparatus

(Amaxa Biosystems, Cologne, Germany) and the Nucleofector V kit with program T-019 according to the manufacturer's protocol. Terminally differentiated cells were processed for further experiments upon 48 h post-transfection.

Western Blot analysis

Cells were lysed in a buffer containing 50 mM Hepes, pH 7.5; 150 mM NaCl; 10% glycerol; 1 mM EDTA; 10 mM NaF; 0.5 mM Na_3VO_4; 1% Triton X-100; 1.5 mM $MgCl_2$ supplemented with a mix of serine and cysteine protease inhibitors (cOmplete Protease Inhibitor Cocktail Tablets, Roche Molecular Biochemicals, Basel, Switzerland). For human neutrophil lysates, 30 µL of 0.05 M diisopropylfluorophosphate (D0879, Sigma) was added to the $40x10^6$ cell pellet prior to the addition of 500 µL lysis buffer. Lysates were centrifuged at 10 000 g at 4°C for 10 min and an equal volume of Laemmli 2X buffer was added to supernatants. After separating samples on 12% SDS-PAGE, proteins were transferred onto nitrocellulose (Hybond™-ECL, Amersham, GE Healthcare, Belgium) membranes, which were saturated with a blocking buffer containing 3% w/v non-fat milk. Immunodetection was realized by using rabbit polyclonal anti-human SNAP23 (1:500 dilution, HPA001214, Sigma), mouse monoclonal anti-human SNAP29 (1:500 dilution, 3E4-E6, Novus Biologicals, Cambridge, United Kingdom) and mouse monoclonal anti-β-actin (1:500 dilution, clone C4, Millipore, Bruxelles, Belgium) as primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies directed against rabbit IgGs (1:10000 dilution, AP132P, Millipore) or mouse IgGs (1:20000 dilution, AP124P, Millipore). Revelation of bands was visualized by ECL detection. Band intensities were quantified by densitometry using ImageJ densitometry software (National Institutes of Health, Bethesda, Maryland, USA). The integrated intensity of the target protein band was normalized to the reference protein (β-actin).

Enzyme-linked immunosorbent assay (ELISA)

To quantify the release of granule matrix proteins upon degranulation, supernatants from dHL-60 cells, stimulated with 10 ng/mL bacterial LPS from *E. coli* serotype 0111:B4 (Sigma, Bornem, Belgium) for 6 h, were analyzed *via* human myeloperoxidase (MPO) ELISA kit (BMS2038INST, eBioscience, Vienna, Austria), human matrix metallopeptidase 9 (MMP-9) ELISA kit (Cat#: ELH-MMP-9-001, RayBio, Boechout, Belgium) and bovine albumin ELISA kit (E11-113, Bethyl Laboratories, Antwerpen, Belgium) according to the manufacturer's instructions. Cell cytotoxicity upon treatments was examined using lactate dehydrogenase assay (LDH, CytoTox 96® Non-Radioactive Cytotoxicity Assay, G1780, Promega) according to manufacturer's instructions. For normalization, ELISA absorbance results were divided by LDH absorbance values. The relative degranulation was determined by calculating the ratio between absorbance values of (+LPS/-LPS) of SNAP23 or SNAP29 siRNA-treated dHL-60 cells and (+LPS/-LPS) of non-silencing siRNA-treated dHL-60 cells.

Measurement of cytokine secretion by cytometric bead array (CBA)

dHL-60 cell density was adjusted to $2x10^6$ cells *per* condition for subsequent quantitative measurement of cytokine secretion cells stimulated with 10 ng/mL bacterial LPS from *E. coli* serotype 0111:B4 for 6 h. Fresh supernatants were collected and used directly for cytometric bead array (CBA, BD Biosciences) analysis. The multiplex standard curve composed of mixed cytokine standards was set up by serial dilutions according to the manufacturer's instructions. Selected capture beads were prepared and added to supernatants. The following beads were used: CCL2 (MCP1, bead D8), CCL3 (MIP1α, bead B9), CCL4 (MIP1ß, bead E4), CCL5 (RANTES, bead D4), IL-1α (bead D6), IL-1ß (bead B4), IL-6 (bead A7), IL-8 (CXCL8, bead A9), IL-12b (bead E5), and TNFα (bead C4). After 1 h of incubation, detection reagent was added to each sample. After 2 h of incubation, samples were rinsed with wash buffer and centrifuged. Samples were washed again prior to flow cytometry analysis (BD FACSCanto™ II, BD Biosciences). Results were quantified using the standard curves and the Flow Cytometric Analysis Program (FCAP) Array software (Soft Flow, Minneapolis, USA).

Immunofluorescence analysis of STX3-SNAP29 localization

Cells were centrifuged for 5 min at 300 g to adhere to glass coverslips. After fixation with 3% w/v paraformaldehyde/2% w/v sucrose in PBS 1X, cells were permeabilized in a PBS 1X buffer containing 0.1% w/v saponine and 0.5% w/v BSA, then blocked with 5% human IgG (Sigma). Cells were immunostained with the following primary antibodies: rabbit anti-human STX3 (1:400 dilution, HPA002191, Sigma) and mouse anti-human SNAP29 (1:100 dilution, 3E4-E6, Novus Biologicals). In addition, immunostaining with each marker alone as positive controls served to detect and avoid any fluorescent signal bleed-through to other channels. The use of donkey serum (1:500 dilution, Jackson ImmunoResearch, Newmarket Suffolk, UK) served as negative control. After washing with blocking buffer, cells were incubated with cyanine 3-conjugated donkey anti-mouse (1:150 dilution, 715-165-150, Jackson ImmunoResearch), AlexaFluor488-conjugated donkey anti-rabbit (1:200 dilution, A21206, Life Technologies) antibodies as well as DAPI for nuclear staining (1μM, D1306, Life Technologies). After washing, samples were mounted on Moviol/DABCO and monitored by confocal microscopy using a 63×/1.4 oil objective with a 405 nm laser and a band pass BP 420-480 nm to excite and detect DAPI, a 488 nm laser and a BP 505-550 nm to excite and detect AlexaFluor488, and a 561 nm laser and a BP 575-615 nm to excite and detect cyanine 3, respectively (LSM 510 META; Zeiss, Zaventem, Belgium). Confocal 2D and z-stack images were deconvolved using AutoQuant X3 (Media Cybernetics, Rockville, USA). A minimum of 100 cells *per* condition from z-stack images only was evaluated for Pearson correlation (PCC). The PCC was calculated since it is a rapid, simple and most importantly, robust tool to investigate co-localization of two proteins (Dunn *et al.*, 2011). The PCC was

evaluated for each entire image according to the equation mentioned by Barlow *et al.* (Barlow *et al.*, 2010). In our case, we put the threshold of the PCC ≥ 0.7 in order to select only for highly positive correlation between green and red pixels.

In situ proximity ligation assay (PLA)

To investigate STX3 protein-protein interaction, we used the Duolink reagent kit (Sigma). Cell adherence, fixation, permeabilization, blocking steps and incubation with STX3 and SNAP29 primary antibodies were performed as described above. Then, procedures including PLA probes, hybridization, ligation, amplification, and detection were followed according to the manufacturer's protocol. Samples incubated with each primary antibody alone, as well as only PLA probes served as controls. After washing with wash buffer B (0.1M NaCl, 0.2M Tris base, 26g Tris-HCl, pH 7.5), samples were mounted on Duolink mounting medium (containing DAPI for nucleus staining) and monitored by confocal microscopy using a 63×/1.4 oil objective with a 405 nm laser and a band pass BP 420-480 nm to excite and detect DAPI, and a 561 nm laser and a long pass LP 575 nm to excite and detect orange fluorescent dots, respectively. A minimum of 100 cells *per* condition from confocal 2D images were evaluated using Duolink® ImageTool.

Statistical analysis

Statistical analysis was performed by using the 2-tailed unpaired Student's t-test in the Prism5 software (Graph Pad Software, La Jolla, CA, USA). The difference between the means are considered statistically significant when the p-value is * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Results

Expression of SNAP23 in HL-60 cells and human neutrophils

We have previously demonstrated that STX3 is expressed in both, human neutrophils and differentiated HL-60 cells, a cell model for neutrophils. By using the CBA technique, we showed that STX3 is involved in the exocytosis pathway of MMP-9 and secretion of cytokines IL-1α, IL-1β, IL-12b, and CCL4 upon LPS stimulation. Further, our microarray gene expression analysis (Arrayexpress, E-MTAB-2107) performed on HL-60 cells showed that SNAP23 and SNAP29 were the only $Q_{b,c}$-SNAREs expressed in native and differentiated cells. Based on these elements, we hypothesized that at least one of these two proteins participates with STX3 in IL-1α, IL-1β, IL-12b, and CCL4 secretion in differentiated HL-60 cells and MMP-9 release.

We assessed first the expression of SNAP23 in human neutrophils, promyelocytic native HL-60 cells and DMSO-treated HL-60 cells inducing terminal differentiation along granulocyte lineage (Collins, 1987).

To confirm microarray results, we used qPCR to determine the relative expression of detected SNAP23 mRNA. In both cell types, SNAP23 mRNA was similarly expressed and no modification during differentiation of HL-60 cells was observed (**Fig. 1A**). Because the mRNA level does not necessarily correlate to the protein level, Western blot analysis was carried out to quantify SNAP23 in native and differentiated HL-60 cells. In contrast to transcript expression, SNAP23 protein expression was more than 2-fold upregulated upon cell differentiation (**Fig. 1B**). The expression of SNAP23 protein was checked and confirmed in human neutrophils (**Fig. 1B**).

Downregulation of SNAP23 inhibits LPS-mediated IL-8 secretion

To address the issue whether SNAP23 is functionally involved in the cytokine secretion under pro-inflammatory conditions in neutrophils, we used siRNA sequences to selectively suppress SNAP23 proteins. Specific siRNA sequences led to effective knockdown of the respective target molecules compared to non-silencing siRNA; SNAP23 protein was reduced up to 40% (**Fig. 1C**).

Unexpectedly, after 6 h stimulation of dHL-60 cells with 10 ng/mL LPS, we found that downregulation of SNAP23 decreased secretion of IL-8 of about 30% (**Fig. 2A**) without affecting the release of other cytokines, except for TNFα. Because adapted SNARE complexes with different sets of SNARE partners are probably associated with distinct inflammatory responses in neutrophils and since STX3 has also been demonstrated to regulate MMP-9 release, we approach the role of SNAP23 in the process of degranulation. To this end, supernatants of dHL-60 cells stimulated by 10 ng/mL LPS for 6 h were tested by ELISA for secretion of soluble mediators that have been described characteristic for the different granule types: myeloperoxidase (MPO) for azurophil granules, matrix metalloproteinase-9 (MMP-9) for gelatinase granules, and serum albumin for secretory vesicles (Borregaard and Cowland, 1997). No marker of specific granules has been used since dHL-60 cells are known to lack this type of granules (Harris and Ralph, 1985). SNAP23 siRNA does not affect the capacity of dHL-60 cells to secrete MMP-9 (**Fig. 2B**). In addition, release of MPO, and albumin was also not altered.

SNAP29 knockdown affect LPS-mediated IL-12b secretion and MMP-9 exocytosis

Beside SNAP23, our microarray data showed that SNAP29, a second homolog of SNAP25, was also expressed in HL-60 cells. This result was confirmed by qPCR and western blotting, although no upregulation during the differentiation process was observed (**Fig. 3A** and **3B**). As expected, SNAP29 expression was also found in human neutrophils (**Fig. 3B**).

Since SNAP29 has been reported to bind to a broad variety of syntaxin isoforms in *in vitro* studies (Steegmaier *et al.*, 1998), we next examined if SNAP29 does possess a role in the intracellular signaling trafficking in combination with STX3. dHL-60 cells were transfected

with specific SNAP29 siRNA, which inhibited approximately 40 % of the protein level (**Fig. 3C**). Upon LPS stimulation, SNAP29 knockdown reduced the release of IL-12b by almost 40%, IL-8 by 33% and CCL2 by 30% (**Fig. 4A**). As previously observed for SNAP23, inhibition of SNAP29 triggered an increase of TNFα and IL-1β secretion. As shown in **Fig. 4B**, SNAP29 siRNA led to a reduction of MMP-9 release by dHL-60 cells. MPO and albumin secretion was unchanged by the downregulation of SNAP29 expression. Because SNAP29 and STX3 affected the release of MMP-9 and IL-12b we postulate that STX3 and SNAP29 are involved in the formation of the same complex for the intracellular transport of MMP-9 and IL-12b.

SNAP29 colocalizes with STX3

To assess our hypothesis, we investigated if SNAP29 and STX3 colocalize in dHL-60 cells and human neutrophils at the resting level. For that, cells were double-labeled with primary antibodies against STX3 and SNAP29 and the localization of endogenous proteins was examined by immunofluorescence microscopy. According to the pixel-based evaluation of our confocal images by Pearson correlation, we found a colocalization of STX3 with SNAP29 in cytoplasmic granules of dHL-60 cells and human neutrophils (**Fig. 5**).

Similar labeling procedures were performed in dHL-60 cells stimulated for 6 h with 100 ng/mL LPS and neutrophils stimulated for 12 h with 100 ng/mL LPS. Interestingly, Pearson correlation coefficients were unchanged upon LPS stimulation (*data not shown*). These results are in accordance to our previous study in which STX3 did not relocalize to another cellular compartment upon LPS stimulation. Altogether, these data suggest that STX3 and SNAP29 participate to the regulation of the fusion events between individual cellular compartments before exocytosis.

SNAP29 interacts with STX3

In order to confirm that SNAP29 is a binding partner of STX3 involved in the secretory pathway, we performed interaction studies. First, interactions were examined *via* co-immunoprecipitation experiments in total lysates of dHL-60 cells and human neutrophils. Our preliminary results suggested that STX3 was able to interact with SNAP29. However, probably due to the low expression level of SNAP29, only weak signals were detected and experiments were difficult to reproduce with reliability (*data not shown*). Consequently, to directly examine SNAP29 and STX3 interactions, we decided to use the *in situ* proximity ligation assay (PLA) technology which detects protein-protein interactions quantitatively as fluorescent distinct punctate spots by rolling-circle amplification reactions dependent on the close proximity of the both proteins (Soderberg *et al.*, 2006). dHL-60 cells were probed with a combination of rabbit anti-human STX3 and mouse anti-human SNAP29 as primary antibodies as described in *Materials and Methods*. As shown in **Fig. 6**, red fluorescent spots

indicated close proximity of SNAP29 and STX3 heightening the fact that SNAP29 and STX3 interact at the subcellular localization in our cells.

Discussion

In eukaryotic cells, SNARE proteins are known to be the major players of membrane fusion events. Initially, the involvement of SNAREs in the process of exocytosis has been extensively studied in neuronal cells. Over the years, studies underlining the role of these exocytotic proteins have been expanded to other cell types including immune cells. However, most research efforts focused on mast cells and macrophages and the regulation of protein trafficking by SNAREs in neutrophils remains largely elusive. It has been shown that Q_a-SNARE STX4, located at the plasma membrane, is involved in the secretion of tertiary and specific granules in association with the $Q_{b,c}$-SNARE SNAP23 and R-SNAREs VAMP1 and VAMP2 (Mollinedo *et al.*, 2006; Logan *et al.*, 2006). STX4 may also mediate the secretion of azurophil granules with R-SNAREs VAMP1 and VAMP7. Moreover, STX6 present at the plasma membrane has been described as target SNARE for CD66b-positive specific granules containing SNAP23 (Martin-Martin *et al.*, 2000). To our knowledge, the role of SNAREs has been investigated only in degranulation and has not yet been reported to be involved in cytokine secretion by neutrophils. We previously reported that the Q_a-SNARE STX3 is expressed in neutrophils and knockdown of STX3 inhibited degranulation of MMP-9 and secretion of IL-12b, CCL4, IL-1α and IL-1β. However, it is unknown which SNARE proteins interact with STX3 to ensure this specific regulation.

In this study, we aim to identify STX3 partners, which are implicated in the regulation of the same protein trafficking pathway in a model of neutrophils. We show that $Q_{b,c}$-SNARE SNAP29, a homologue of SNAP25 present in non-neuronal cells, is required for IL-12b and MMP-9 release in association with STX3. SNAP29 has been considered to be ubiquitously located on multiple membranes (Steegmaier *et al.*, 1998) and to bind to a variety of syntaxin isoforms that reside on distinct intracellular organelles. In accordance with the literature (Steegmaier *et al.*, 1998), we found that STX3 and SNAP29 also interact in dHL-60 cells. In addition, these results are the first documentation that SNAP29 is present in secretory compartments of neutrophil-like HL-60 cells, colocalizes with STX3 and participates in trafficking events leading to cytokine release.

Interestingly, our results also showed that inhibition of SNAP29 mediated an increase of TNFα and IL-1β secretion. Downregulation of SNAP29 could disturb the steady state between the different SNAREs or regulatory factors leading to the formation or/and reorganization of specific SNARE complexes.

In addition, we cannot exclude the possibility that the downregulation of SNAP29 is compensated by other $Q_{b,c}$-SNAREs. In this respect, also SNAP23 has been found colocalized with STX3 at the level of cytoplasmic granules (*data not shown*). However, inhibition of SNAP23 affected only the secretion of IL-8 and not IL-12b, CCL4, IL-1α and IL-1β release as previously observed for STX3 (see *Manuscript II*). In accordance with the SNARE hypothesis, neutrophils possess distinct secretory compartments whose exocytosis is regulated by different SNARE complexes constituted of different SNARE isoforms. In this regard, SNAP23 could interact with STX3 but depending on the other binding partners involved in the complex, different fusion steps and trafficking pathways could be regulated. Alternatively, SNAP23 could form "non-cognate" STX3-SNAP23 complex since SNARE proteins concentrated in overlapping membrane microdomains have been reported to form interactions with no functional cross-talk (Bethani *et al.*, 2007). It is important to note that non-cognate SNARE complexes are able to fine-tune the fusion machinery and the specificity of vesicle trafficking by regulating the different levels of SNAREs available for *trans*-complex formation (Varlamov *et al.*, 2004). In this sense, the purpose of STX3-SNAP23 complexes in dHL-60 cells would result in the decrease of individual SNAP23 protein and thus, downregulate the number of its cognate complexes and functional role.

Further, while the formation of acceptor complexes by Q_a- and $Q_{b,c}$-SNAREs represent the rate-limiting step in the SNARE assembly-disassembly cycle, R-SNAREs have been proposed to have a role for specificity (Bennett, 1995). This specificity is probably also ensured by the assistance of other regulatory proteins (*e.g.* Sec1/Munc18-like proteins, complexins), which restrict the activity of interacting SNARE proteins in a spatial or temporal fashion (McNew *et al.*, 2000).

It will be now important to identify which other SNARE is associated to SNAP29-STX3 to mediate IL-12b and MMP-9 secretion. Under the assumption that a SNARE complex may generally consist of a bundle of four α-helices of three Q-SNAREs and one R-SNARE (Hong, 2005), several possibilities of SNARE association can be envisaged with the general idea that different associations could direct the secretion of specific cytokines. It is given that synaptobrevins attach to SNAP29-STX3 for the formation of the functional *trans*-SNARE complex. Moreover, SNAP29 could be present in multiple complexes and interact with different syntaxin isoforms to regulate diverse pathways of secretion and release of other cytokines (*e.g.* IL-8 and CCL2).

To date, the specificity of vesicle-mediated transport pathways in neutrophils is still elusive. However, since we provide evidence that SNAP29 in association with STX3 are crucial for the release of IL-12b and MMP-9 and different combinations of SNARE proteins, the next steps are to identify R-SNAREs that interact with the STX3-SNAP29 complex and confirm

their common impacts to improve our understanding on cytokine secretion and degranulation.

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Figure Legends

Figure 1 Endogenous SNAP23 expression.

(A) SNAP23 mRNA expression was validated by qPCR in dHL-60 cells. Data were normalized to β-actin and expressed relative to the SNAP23 expression in native HL-60 cells ± SEM of at least three independent experiments.

(B) SNAP23 protein expression in native HL-60 cells, DMSO-differentiated HL-60 (dHL-60) cells and neutrophils was determined by Western blotting. Proteins were detected with specific anti-SNAP23 antibodies. A western blot representative of four independent experiments is shown. Histogram representing the densitometry evaluation of western blots was performed by Image J software. Protein level was normalized to β-actin. Results are given in % ± SEM of at least four independent experiments. Significantly different from native HL-60 cells: * $p < 0.05$.

(C) Efficiency of SNAP23 knockdown in dHL-60 cells. Differentiated HL-60 cells were transiently transfected with SNAP23 siRNA. The efficiency of SNAP23 protein knockdown is given in % ± SEM from at least three independent experiments, respectively. Significant differences in expression relative to the non-silencing siRNA control: * $p < 0.05$.

Figure 2 Effect of SNAP23 knockdown in dHL-60 cells on cytokine secretion and granule exocytosis. Transfected cells were assessed after stimulation by 6 h LPS (10 ng/mL) for (A) cytokine secretion using CBA, and (B) for degranulation using MPO, MMP-9, and albumin ELISA kits. Cells transfected with a non-silencing sequence were used as control. Results are expressed in ratio of SNAP23 siRNA and non-silencing siRNA-transfected cells ± SEM of at least three independent experiments. Significantly different from non-stimulated dHL-60 cells: * $p < 0.05$.

Figure 3 Endogenous SNAP29 expression.

(A) SNAP29 mRNA expression was validated by qPCR in the dHL-60 cells. Data were normalized to β-actin and expressed relative to the SNAP29 expression in native HL-60 cells ± SEM of at least three independent experiments. Significantly different from native HL-60 cells: * $p < 0.05$.

(B) SNAP29 protein expression in native HL-60 cells, DMSO-differentiated HL-60 (dHL-60) cells and neutrophils was determined by Western blotting. Proteins were detected with specific anti-SNAP29 antibodies. A western blot representative of four independent experiments is shown. Histogram representing the densitometry evaluation of western blots was performed by Image J software. Protein level was normalized to β-actin. Results are given in % ± SEM of at least four independent experiments.

(C) Efficiency of SNAP29 knockdown in dHL-60 cells. Differentiated HL-60 cells were transiently transfected with SNAP29 siRNA. The efficiency of SNAP29 protein knockdown is

given in % ± SEM from at least three independent experiments, respectively. Significant differences in expression relative to the non-silencing siRNA control: * $p < 0.05$.

Figure 4 Effect of SNAP29 knockdown in dHL-60 cells on cytokine secretion and granule exocytosis. Transfected cells were assessed after stimulation by 6 h LPS (10 ng/mL) for (A) cytokine secretion using CBA, and (B) for degranulation using MPO, MMP-9, and albumin ELISA kits. Results are expressed in ratio of siRNA SNAP29 and non-silencing siRNA-transfected cells ± SEM of at least three independent experiments. Significantly different from non-stimulated dHL-60 cells: * $p < 0.05$, ** $p < 0.01$.

Figure 5 STX3 colocalizes with SNAP29.

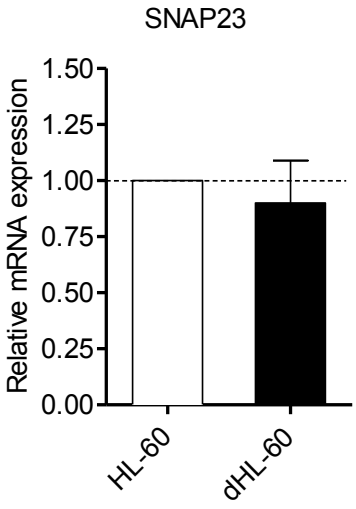
Intracellular localization of STX3, and SNAP29 in dHL-60 cells and human neutrophils. Colocalization experiments by immunofluorescence staining were performed with STX3 (green), SNAP29 (red) and DAPI for cell nucleus (blue). Corresponding correlation histogram is depicted for the entire overlay image. Scale bar, 5 µm. Pearson correlation coefficients were calculated for red and green channels in z-stack confocal images by AutoQuant. Values between 0.4 and 0.7 were considered positive, whereas ≥ 0.7 were considered highly positive for the co-localisation.

Figure 6 Intracellular interaction between STX3 and SNAP29.

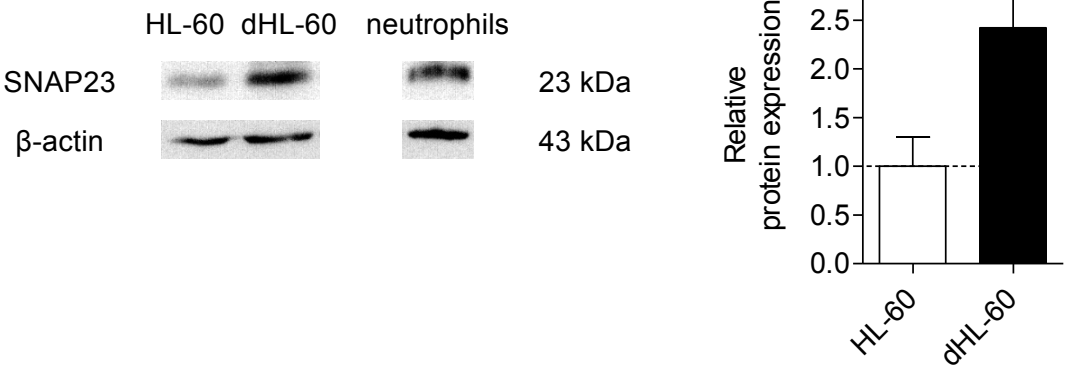
Protein interaction experiments by proximity-ligation assay were performed with primary anti-STX3, anti-SNAP29, and Duolink kit on dHL-60 cells. DAPI in the mounting medium served to stain the nucleus (blue). Only interacting STX3 proteins with SNAP29 result in fluorescent dots as depicted in merge images. Scale bar, 5 µm. Negative control samples were only incubated with PLA probes, anti-STX3 alone, or anti-SNAP29 alone, respectively. Quantification of protein interactions was performed *via* Duolink Image Tool. Results are given in signals in the cytoplasm per cell ± SD from at least three independent experiments.

Figure 1

(A)


SNAP23
Relative mRNA expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
HL-60
dHL-60

(B)


HL-60
dHL-60
neutrophils
SNAP23
β-actin
23 kDa
43 kDa
SNAP23
Relative protein expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
HL-60
dHL-60

(C)

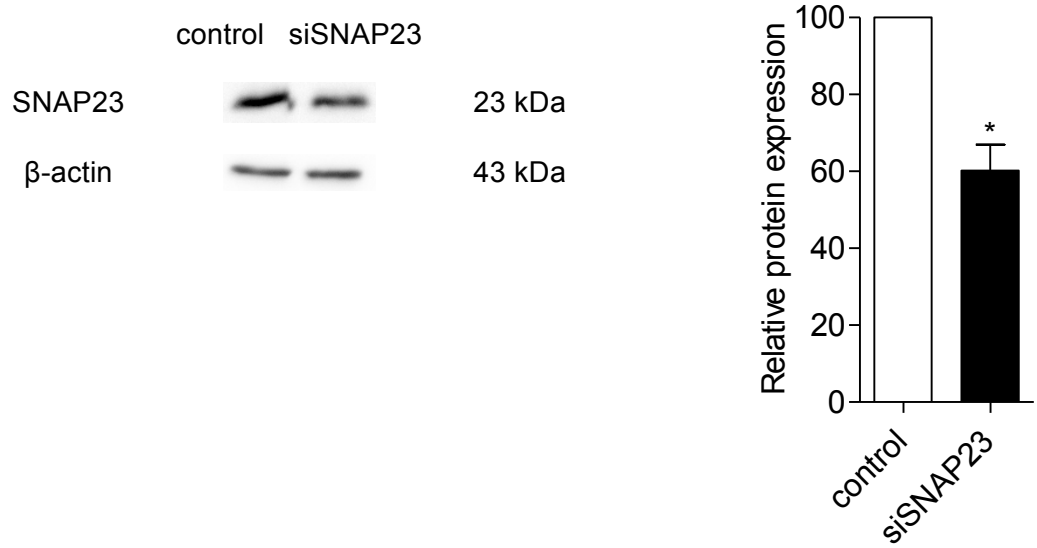
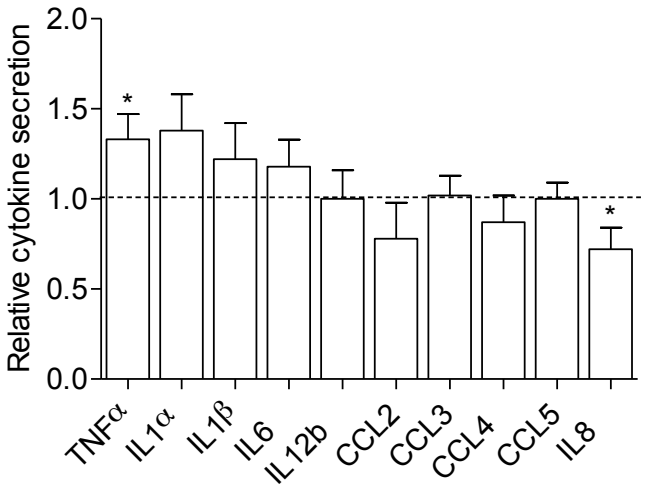

control
siSNAP23
SNAP23
β-actin
23 kDa
43 kDa
Relative protein expression
100
80
60
40
20
0
*
control
siSNAP23

Figure 2

(A)


Relative cytokine secretion
2.0
1.5
1.0
0.5
0.0
*
*
TNFα
IL1α
IL1β
IL6
IL12b
CCL2
CCL3
CCL4
CCL5
IL8

(B)

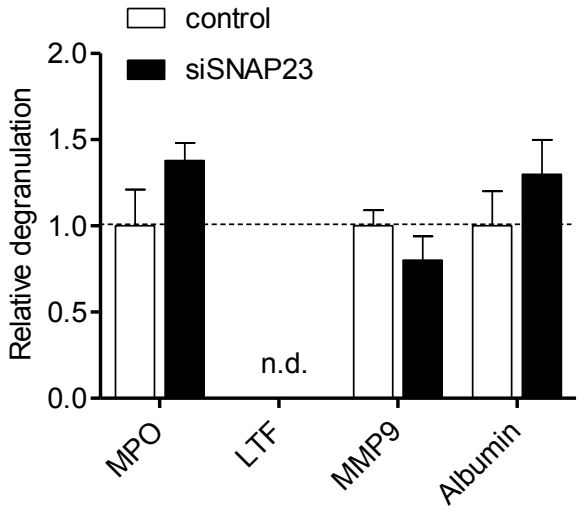
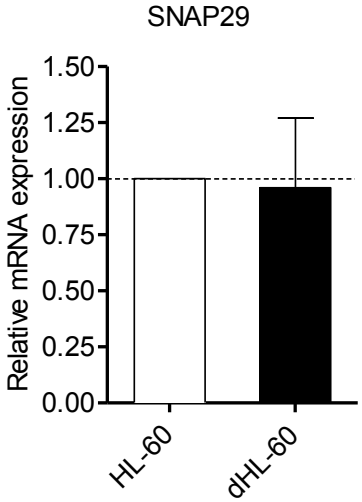
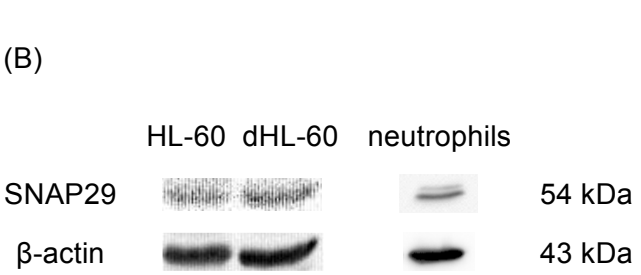

control
siSNAP23
Relative degranulation
2.0
1.5
1.0
0.5
0.0
n.d.
MPO
LTF
MMP9
Albumin

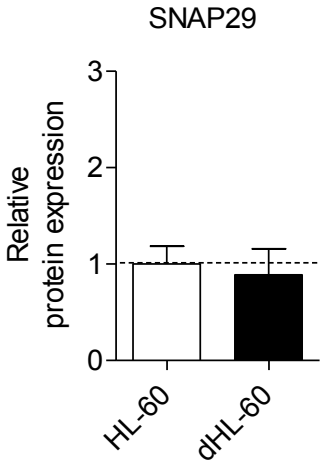
Figure 3

(A)


SNAP29
Relative mRNA expression
1.50
1.25
1.00
0.75
0.50
0.25
0.00
HL-60
dHL-60

(B)


HL-60
dHL-60
neutrophils
SNAP29
β-actin
54 kDa
43 kDa


SNAP29
Relative protein expression
3
2
1
0
HL-60
dHL-60

(C)


control
siSNAP29
SNAP29
β-actin
54 kDa
43 kDa

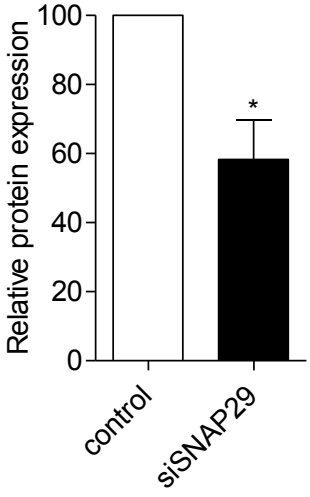
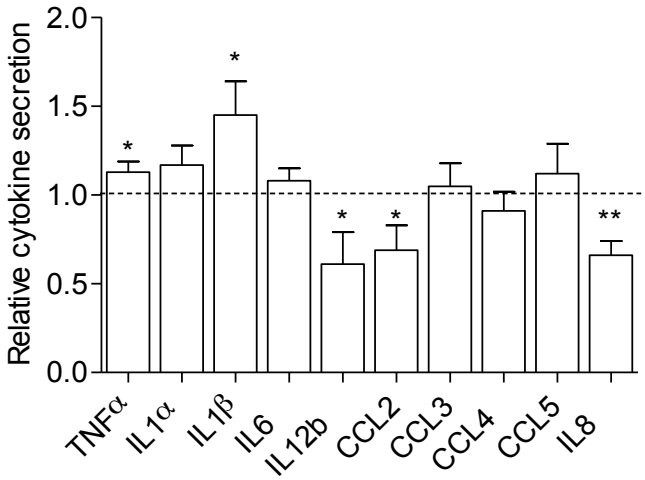

Relative protein expression
100
80
60
40
20
0
*
control
siSNAP29

Figure 4

(A)


Relative cytokine secretion
2.0
1.5
1.0
0.5
0.0
*
*
*
*
**
TNFα
IL1α
IL1β
IL6
IL12b
CCL2
CCL3
CCL4
CCL5
IL8

(B)

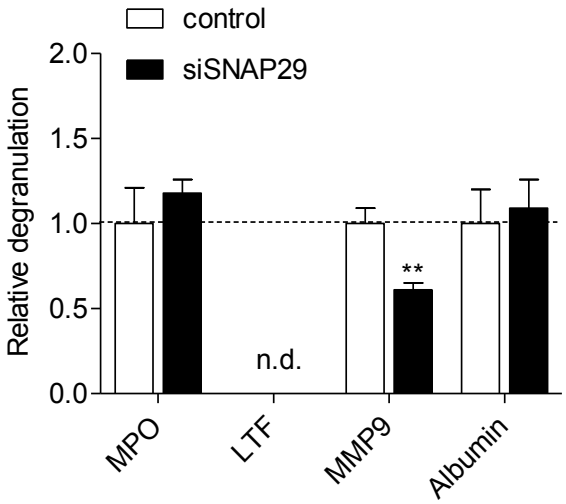
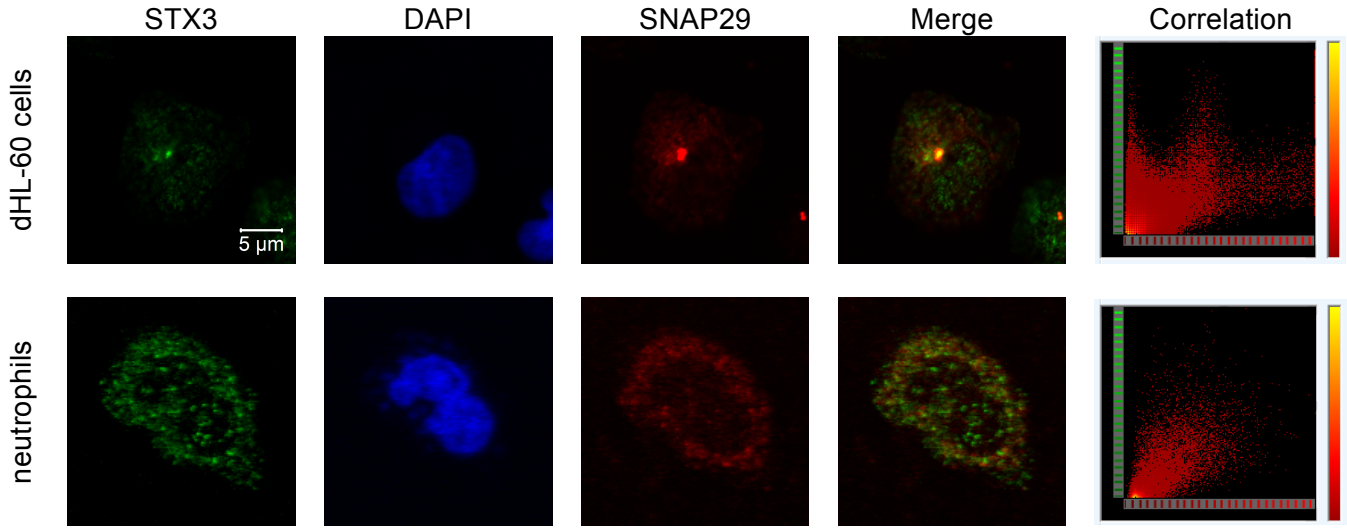

control
siSNAP29
Relative degranulation
2.0
1.5
1.0
0.5
0.0
**
n.d.
MPO
LTF
MMP9
Albumin

Figure 5


STX3
DAPI
SNAP29
Merge
Correlation
dHL-60 cells
neutrophils
5 µm

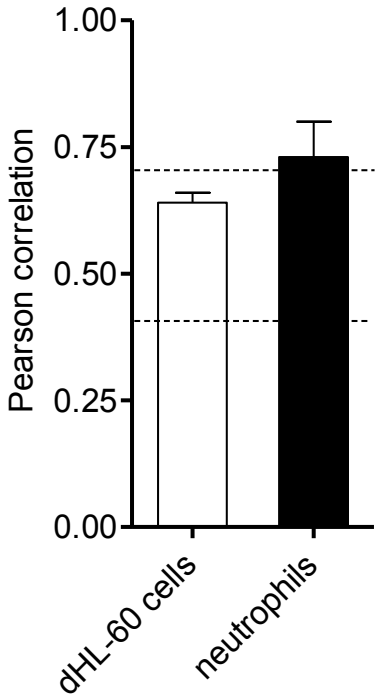
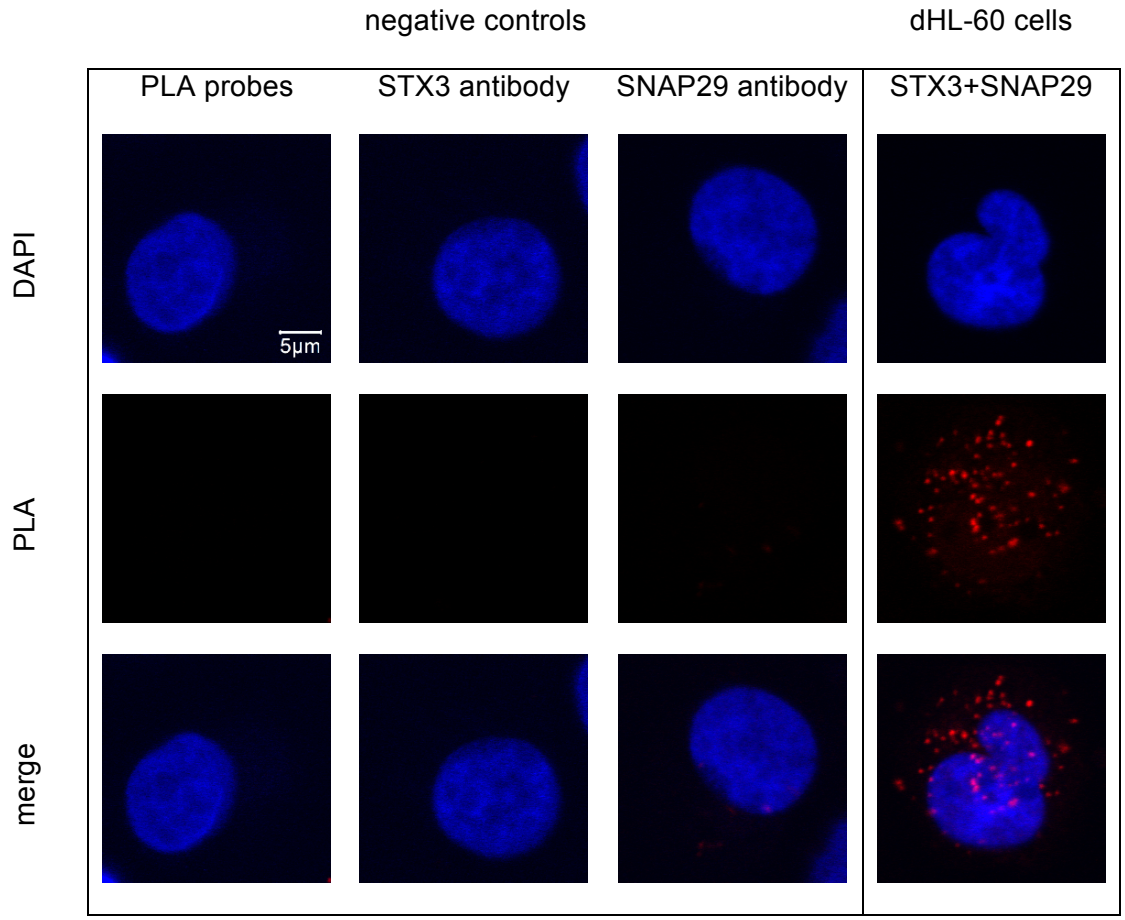
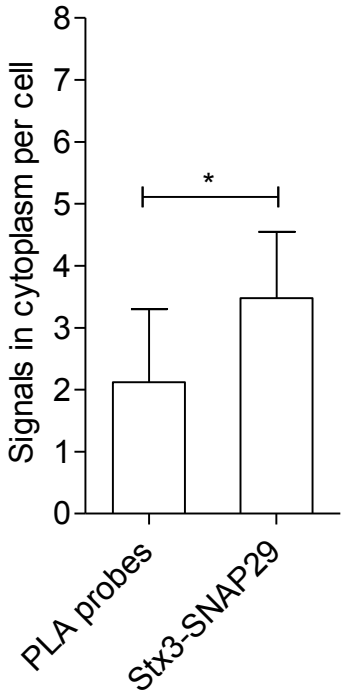

Pearson correlation
1.00
0.75
0.50
0.25
0.00
dHL-60 cells
neutrophils

Figure 6


negative controls
dHL-60 cells
PLA probes
STX3 antibody
SNAP29 antibody
STX3+SNAP29
DAPI
PLA
merge
5µm


Signals in cytoplasm per cell
0
1
2
3
4
5
6
7
8
*
PLA probes
Stx3-SNAP29

Additional Results

IV VAMP3 R-SNARE-mediated secretion of IL-8 and MMP-9

Previously, we found that the Q_a-SNARE STX3 and the $Q_{b,c}$-SNARE SNAP29 are co-localized in cytoplasmic granules and are able to form acceptor complexes. However, specificity of vesicle transport is concentrated on the interaction of the *trans*-SNARE complex with the R-SNARE on the vesicle membrane. We therefore aimed **to find a potential R-SNARE candidate for the STX3-SNAP29-SNARE complex**.

Endogenous expression level of R-SNARE VAMP3

To investigate which R-SNAREs are expressed in human neutrophils and our cellular model, whole-transcript expression was analysed. Microarray expression data for SNARE expression from native HL-60 cells, dHL-60 cells and human neutrophils are available at ArrayExpress (www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-2107. In our cells, we determined the expression of 7 R-SNAREs: VAMP1-4 and VAMP7-8. Results showed that HL-60 cells express VAMP1 to VAMP8. VAMP5 is the only one not expressed in human neutrophils. While VAMP1 seems to be the only R-SNARE whose expression is upregulated upon DMSO differentiation, all other VAMPs are equally expressed in native as well as in dHL-60 cells.

Literature-based analysis *via* Ingenuity showed that potential STX3 interaction partners are VAMP2, VAMP3, VAMP7 and VAMP8 (*data not shown*). Because *i)* VAMP1, VAMP2 and VAMP7 have already been largely described to act during degranulation within a SNARE complex involving STX4 and SNAP23 (Mollinedo *et al.*, 2003; Mollinedo *et al.*, 2006; Logan *et al.*, 2006), and *ii)* VAMP8 mRNA expression was downregulated upon HL-60 differentiation, we focused on the potential role of VAMP3 in neutrophil degranulation and cytokine secretion and correlated to the functional analysis already performed on STX3- and SNAP29-mediated effects in neutrophils under pro-inflammatory conditions.

In platelets, VAMP3 has been found in α-granules (Polgar *et al.*, 2002). Regarding cytokine secretion pathways, VAMP3 is required for *trans*-Golgi transport of TNFα, together with STX4 and STX6, in macrophages (Murray *et al.*, 2005). Interestingly, VAMP3 overexpression results in increased IL-6 secretion in macrophages (Manderson *et al.*, 2007).

To verify the transcript expression level of VAMP3, we performed qPCR and found that VAMP3 was equally expressed in native HL-60 as well as in dHL-60 cells (*data not shown*). Western blot analysis using rabbit polyclonal antibodies targeted against human VAMP3 (1:1000 dilution, NB300-510, Novus Biologicals) revealed that VAMP3 protein expression was however upregulated upon DMSO differentiation (**Fig. 1A**). The upregulated expression of VAMP3 might point towards a functional role.

VAMP3 knockdown affects secretion of the cytokines IL-8 and CCL4 as well as gelatinase degranulation

To investigate the functional implication of VAMP3 in vesicle trafficking and exocytosis in neutrophils, we used an RNAi approach (sense: 5'-GCCAAGUUGAAGAGGAAAU-3', antisense: 5'-AUUUCCUCUUCAACUUGGC-3') specifically targeting VAMP3 transcript. Efficient knockdown of VAMP3 was observed on the mRNA level with more than 70% inhibition (**Fig. 1B**), and on protein level with more than 50% inhibition (**Fig. 1C**). The knockdown of VAMP3 was also observed in VAMP3 siRNA-treated dHL-60 cells (**Fig. 1D**) upon immunofluorescence staining with primary sheep antibodies targeted against human VAMP3 (1:50 dilution, Novus Biologicals) and secondary AlexaFluor647-conjugated donkey anti-sheep (1:200 dilution, 713-605-147, Jackson ImmunoResearch) antibodies as well as DAPI for nuclear staining (1μM, D1306, Life Technologies).

To analyze the functional role of VAMP3 in cytokine secretion, we transfected the cells and stimulated them with 10 ng/mL LPS for 6 h. Compared to the non-silencing control, VAMP3 siRNA affected the release of IL-8 and CCL4 of more than 50% upon LPS stimulation in the cell supernatants (**Fig. 2A**). However, we determined the STX3-SNAP29 couple to act in IL-12b and MMP-9 release, and not IL-8 and CCL4. STX3 knockdown affected also CCL4 release, and SNAP29 knockdown inhibited IL-8 release. In order to determine if VAMP3 might act in a different SNARE complex with STX3 (with another $Q_{b,c}$-SNARE) or SNAP29 (with another Q_a-SNARE), we further investigated the impact of VAMP3 knockdown on the release of different granule types upon LPS stimulation. ELISA results show that VAMP3 knockdown specifically affects MMP-9 release about 50% in LPS-stimulated dHL-60 cells but leaves the release of MPO, and albumin unchanged (**Fig. 2B**). These results are in concordance with the effect of STX3- and SNAP29-mediated knockdown on gelatinase degranulation.

VAMP3 does not colocalize or interact with SNAP29

As VAMP3 exerts a similar inhibitory effect on MMP-9 release, compared to STX3 and SNAP29, we investigated the intracellular localization of VAMP3 in order to conclude on a possible SNARE interplay with STX3 and SNAP29. Therefore, we labeled VAMP3 and performed immunofluorescence analysis. Previously, we demonstrated that SNAP29 is colocalized with STX3 in cytoplasmic granules. However, confocal images show that VAMP3 does not colocalize with SNAP29 (**Fig. 3A**). The same observation could be made with dHL-60 samples stimulated with LPS (*data not shown*).

To confirm our findings, we performed proximity-ligation studies on dHL-60 cells labeling VAMP3, and SNAP29. Cells incubated with PLA probes only, or each of the primary antibodies alone served as negative controls. Additionally, samples incubated with mouse anti-human STX6 (1:100 dilution, clone 30, BD Biosciences) and sheep anti-human STX6 (1:40 dilution, AF5664, R&D Systems) served as positive control (**Fig. 3B**). Our results show that dHL-60 cells incubated with VAMP3 and SNAP29 primary antibodies did not result in any fluorescent spots visible in our confocal images. This means that VAMP3 does not interact with SNAP29 in dHL-60 cells in non-stimulated or LPS-stimulated conditions, and is thus excluded as a candidate in the STX3-SNAP29 complex.

VAMP3 is localized at the plasma membrane

As VAMP3 does not colocalize with SNAP29, we want to determine the exact intracellular location of VAMP3 in our cells, and performed further labeling experiments. Confocal images of dHL-60 cells show that VAMP3 does not colocalize with the different markers of granule types, MPO, LTF, MMP-9 and TN (**Fig. 4A**). These results confirm that VAMP3 is not colocalized with SNAP29 (**Fig. 3A**), and thus not with cytoplasmic granules.

In addition, VAMP3 was not observed to share same location with markers of the ER, Golgi, endosomes or lysosomes (**Fig. 4B**). The strongest colocalization could be found for VAMP3 and CD43, marker of the plasma membrane. These results confirm that VAMP3 is not implicated in the STX3-SNAP29 complex situated at the membrane of cytoplasmic granules.

Conclusion

In the SNARE assembly-disassembly cycle, the interaction of the acceptor SNARE complex (consisting of Q_a- and $Q_{b,c}$-SNAREs) with the R-SNAREs confers to the specificity of the intracellular vesicle transport. In previous studies, we have already determined that *i)* the Q_a-SNARE STX3 is involved in the release of cytokines IL-1α, IL-1β, CCL4 and IL-12b, as well as gelatinase degranulation, *ii)* the $Q_{b,c}$-SNARE SNAP29 represents a potential STX3 interaction partner, while it colocalizes with STX3 on cytoplasmic granules. In this part of the study, we questioned whether the R-SNARE VAMP3 is forming cognate complexes with STX3-SNAP29 to mediate neutrophil functions.

Using an RNAi approach in our neutrophil-like HL-60 cell model, we show that VAMP3 knockdown affects the secretion of the cytokines IL-8 and CCL4 upon LPS stimulation. Since IL-8 and CCL4 are known to be stored and to be constitutively secreted in our cells (Pellme *et al.*, 2006), the specific inhibition of these cytokines points towards a key role of VAMP3 in constitutive exocytosis.

Furthermore, VAMP3 also affects the release of MMP-9, the characteristic matrix protein of gelatinase granules. As we have identified STX3 and SNAP29 to affect specifically the same type of granules, VAMP3 could have been seen as a potential candidate for the STX3-SNAP29 complex. Immunofluorescence and proximity ligation studies nevertheless demonstrated that VAMP3 and SNAP29 are not localized at the same intracellular level. While STX3 and SNAP29 are present at cytoplasmic granules, further results revealed that VAMP3 is partly localized at the plasma membrane. Given our data, it is tempting to speculate that VAMP3 is implicated at the very end of the exocytotic event of gelatinase granules while STX3 and SNAP29 rather act on the level of cytoplasmic granules deeper inside the cell.

Figure Legends

Figure 1 VAMP3 protein expression.

(A) VAMP3 protein expression in undifferentiated HL-60 cells (ND), differentiated HL-60 cells (D) and neutrophils was determined by western blotting. Proteins were detected with specific rabbit polyclonal anti-VAMP3. A western blot representative of four independent experiments is shown. Histogram representing the densitometry evaluation of western blots was performed by Image J software. Protein level was normalized to β-actin. Results are given in ratio between differentiated and undifferentiated HL-60 cells ± SEM of at least four independent experiments.

(B) Efficiency of VAMP3 siRNA was determined by qPCR analysis. Differentiated HL-60 cells were transiently transfected with VAMP3 siRNA. The amount of VAMP3 mRNA in cells was normalized to β-actin and expressed as % of VAMP3 mRNA obtained from cells transfected with a non-silencing sequence used as control. Results are expressed as % ± SEM of at least seven independent experiments. Significantly different from control: * $p < 0.05$.

(C) Specificity of VAMP3 antibody was determined by western blot analysis. A western blot representative of three independent experiments is shown. Histogram shows efficiency of VAMP3 siRNA on protein expression. The integrated intensity of the target protein band was normalized to β-actin and expressed as % of control (non-silencing siRNA sequence). Results are expressed as % ± SEM of at least three independent experiments. Significantly different from control: * $p < 0.05$.

(D) Immunofluorescence analysis of VAMP3 in dHL-60 cells treated with non-silencing siRNA (control) or VAMP3 siRNA. Scale bar, 5 µm.

Figure 2 Functional role of VAMP3.

(A) Effect of VAMP3 knockdown in dHL-60 cells on cytokine secretion and granule exocytosis. Differentiated HL-60 cells were transiently transfected with VAMP3 siRNA. Transfected cells were assessed for cytokine secretion using CBA after 6 h stimulation by LPS (10 ng/mL). Results are expressed in relative cytokine secretion meaning the ratio between siRNA VAMP3 (+LPS/-LPS) and control-transfected cells (+LPS/-LPS) ± SEM of at least three independent experiments. Significantly different from non-silencing siRNA: * $p < 0.05$.

(B) Transfected cells were assessed for degranulation using myeloperoxidase (MPO), MMP-9, and albumin ELISA kits after 6 h stimulation by LPS (10 ng/mL). Cells transfected with a non-silencing sequence were used as control. Results are expressed in relative degranulation meaning the ratio between siRNA VAMP3 (+LPS/-LPS) and control-transfected cells

(+LPS/-LPS) ± SEM of at least three independent experiments. Significantly different from non-silencing siRNA: ** $p < 0.01$.

Figure 3 Colocalization and intracellular interaction between VAMP3 and SNAP29.

(A) Colocalization experiments by immunofluorescence staining were performed with VAMP3 (green), SNAP29 (red) and DAPI for cell nucleus (blue). Scale bar, 5 µm. Merge confocal images only represent green and red channels in order to conclude for co-localization. Corresponding scatter plots of the paired intensities of the green (y-axis for VAMP3 pixels) and red (x-axis for SNAP29 pixels) channels are depicted on the right column for the entire overlay image.

(B) Protein interaction experiments by proximity-ligation assay were performed with primary anti-VAMP3, anti-SNAP29, and Duolink kit on dHL-60 cells. DAPI in the mounting medium served to stain the nucleus (blue). Only interacting VAMP3 proteins with SNAP29 result in fluorescent dots as depicted in merge images. Scale bar, 5 µm. Quantification of protein interactions *via* Duolink Image Tool.

Figure 4 Intracellular VAMP3 localization in dHL-60 cells.

Colocalization experiments by immunofluorescence staining were performed with VAMP3 (green), (A) MPO for azurophil granules, lactoferrin (LTF) for specific granules, MMP-9 for gelatinase granules, tetranectin (TN) for secretory vesicles (red), (B) CD43 for plasma membrane, calnexin (CNX) for endoplasmic reticulum, GM130 for Golgi, EEA1 for early endosomes, LAMP-2 for late endosomes/lysosomes, and DAPI for cell nucleus (blue). Scale bar, 5 µm. Merge confocal images only represent green and red channels in order to conclude for co-localization. Corresponding scatter plots of the paired intensities of the green (y-axis for VAMP3 pixels) and red (x-axis for CD43, CNX, GM130, EEA1, LAMP-2, MPO, LTF, MMP-9, or TN pixels) channels are depicted on the right column for the entire overlay image. Pearson correlation coefficients were calculated for both channels in deconvolved z-stack confocal images by AutoQuant X3. Values ≥ 0.7 were considered strongly positive for co-localization. I, II, III and IV represent the azurophil, specific and gelatinase granules as well as secretory vesicles, respectively.

Figure 1

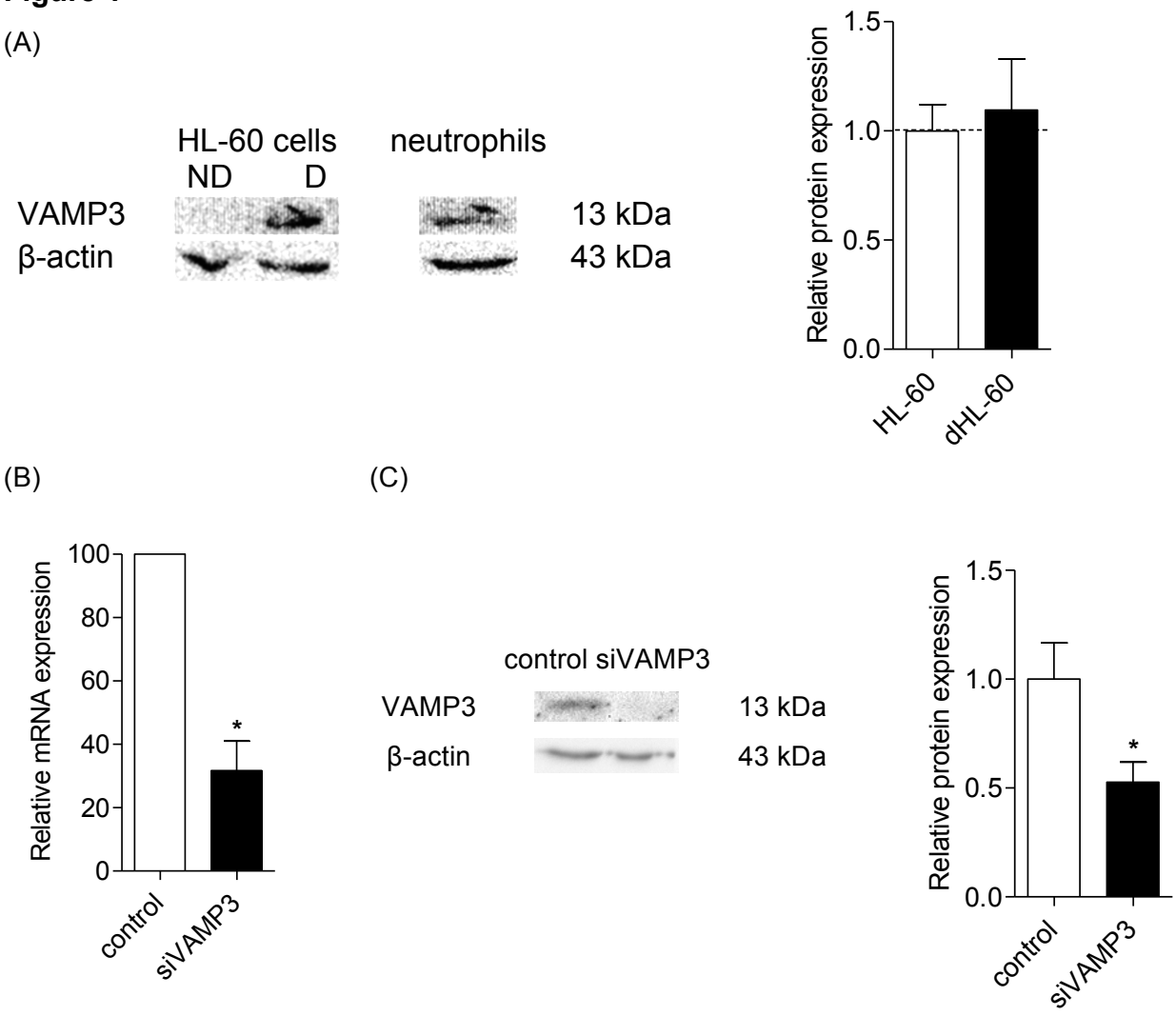
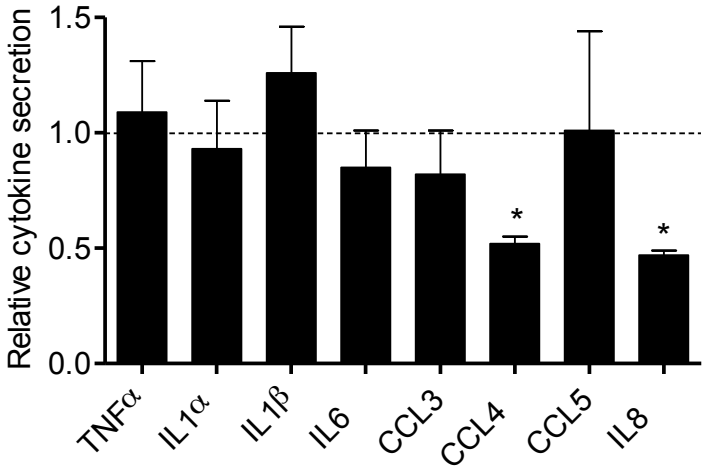

(A)
HL-60 cells
ND
D
neutrophils
VAMP3
β-actin
13 kDa
43 kDa
Relative protein expression
1.5
1.0
0.5
0.0
HL-60
dHL-60
(B)
Relative mRNA expression
100
80
60
40
20
0
*
control
siVAMP3
(C)
control siVAMP3
VAMP3
β-actin
13 kDa
43 kDa
Relative protein expression
1.5
1.0
0.5
0.0
*
control
siVAMP3
(D)
VAMP3
DAPI
merge
control
VAMP3 siRNA

Figure 2

(A)


Relative cytokine secretion
1.5
1.0
0.5
0.0
*
*
TNFα
IL1α
IL1β
IL6
CCL3
CCL4
CCL5
IL8

(B)

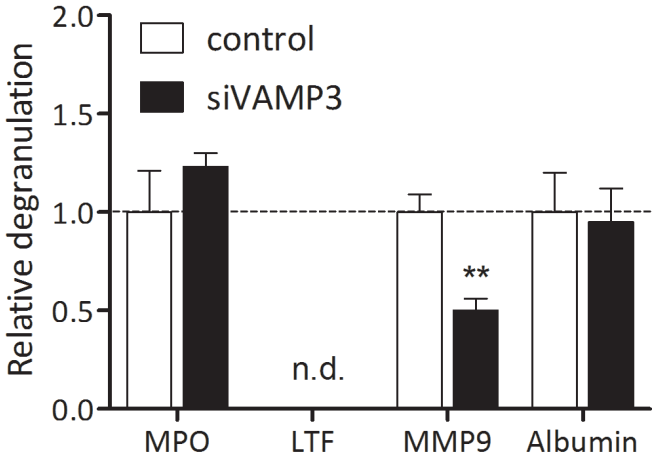
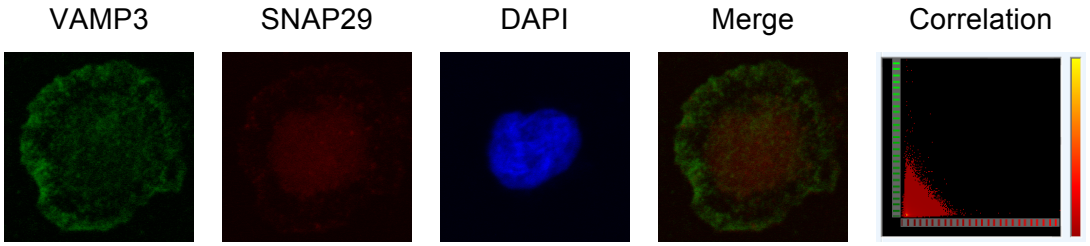

Relative degranulation
2.0
1.5
1.0
0.5
0.0
control
siVAMP3
**
n.d.
MPO
LTF
MMP9
Albumin

Figure 3

(A)


VAMP3
SNAP29
DAPI
Merge
Correlation

(B)

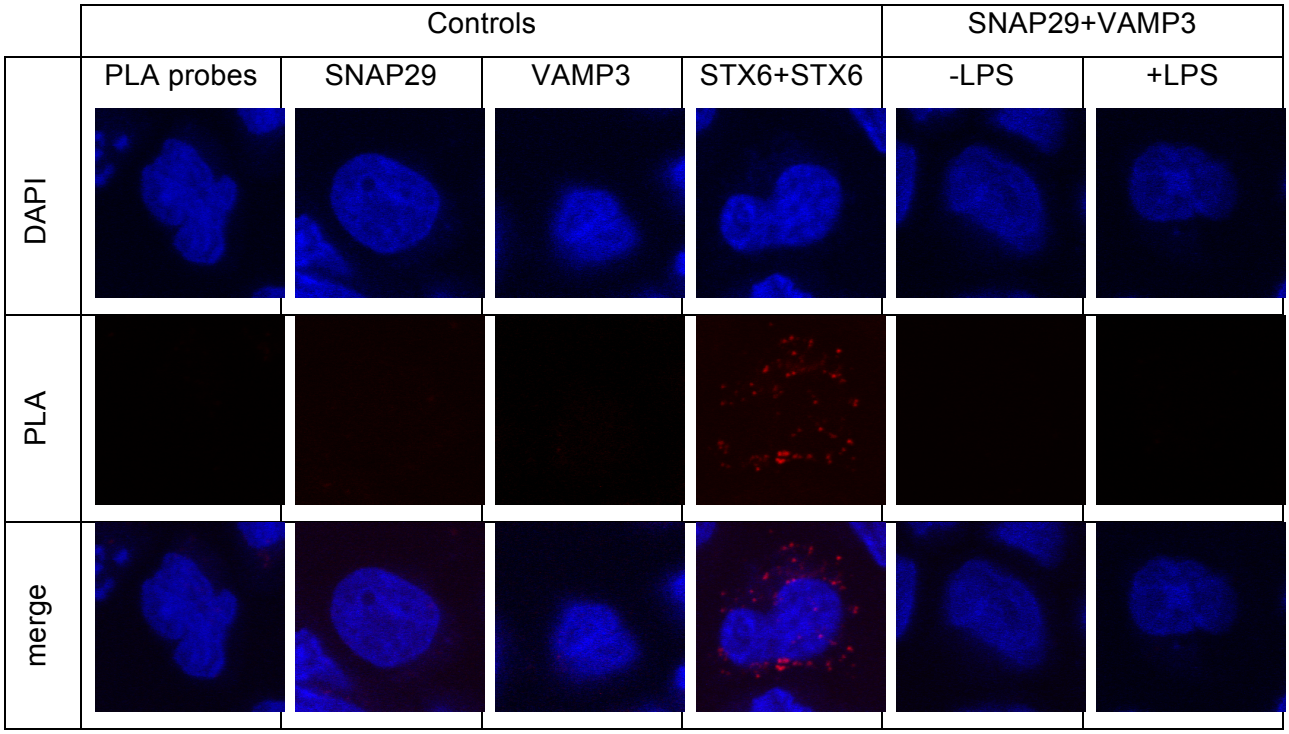
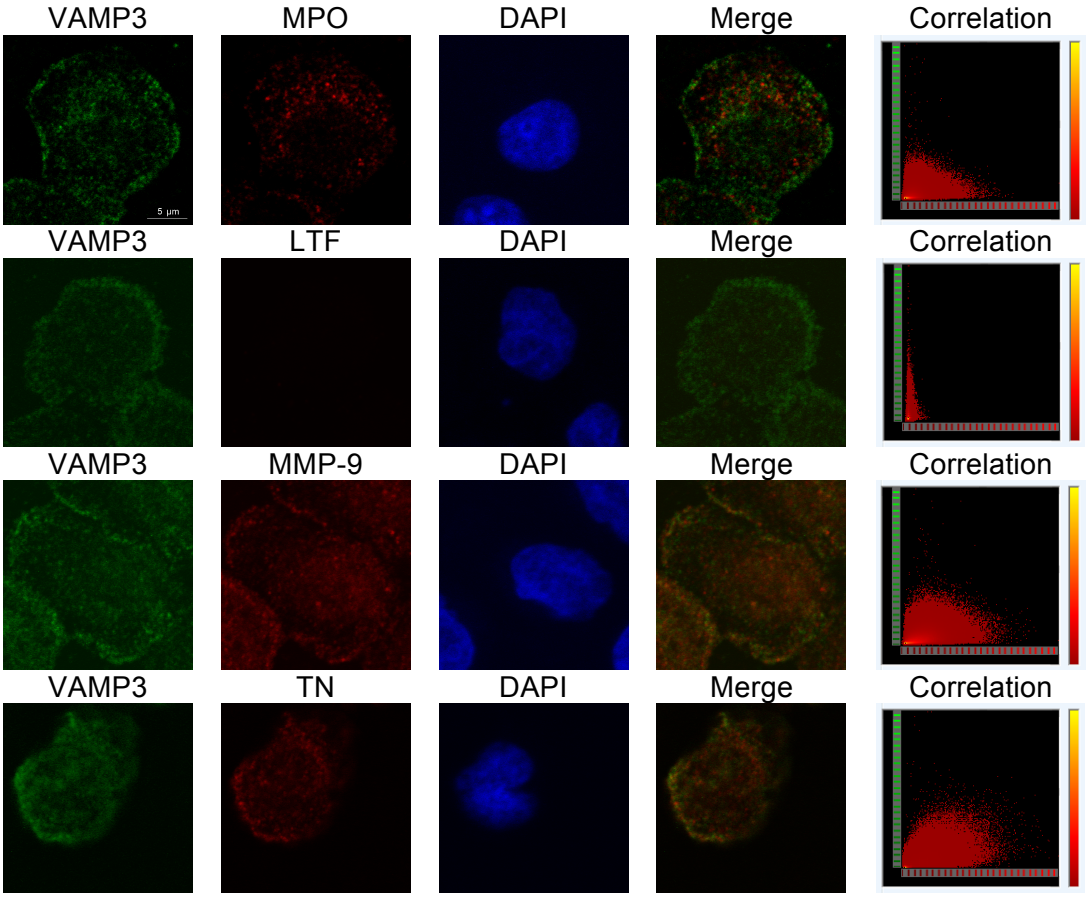
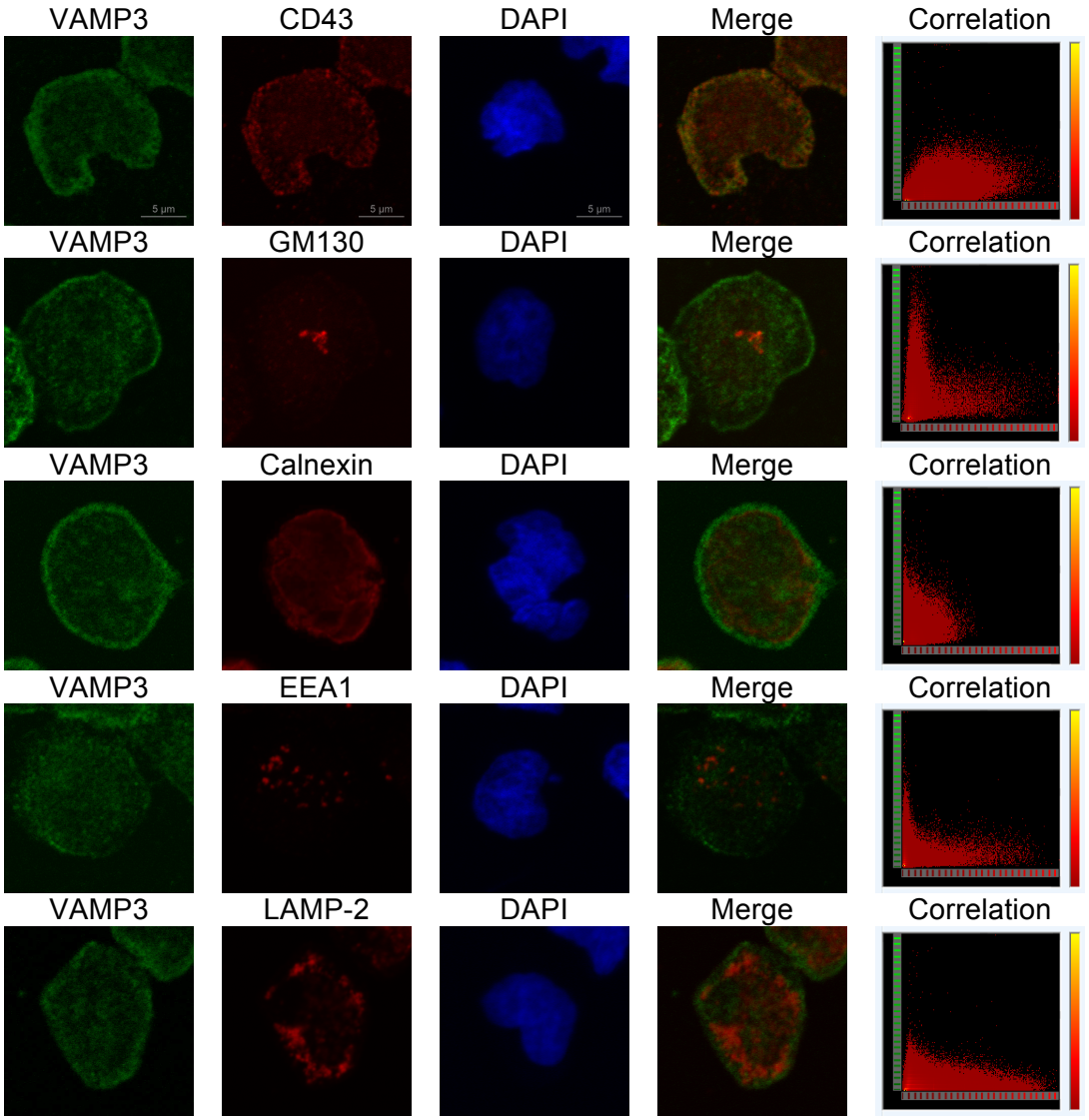

Controls
SNAP29+VAMP3
PLA probes
SNAP29
VAMP3
STX6+STX6
-LPS
+LPS
DAPI
PLA
merge

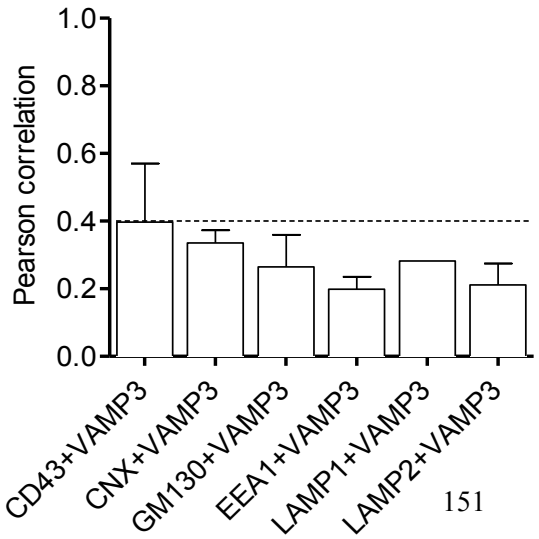
Figure 4

(A)


VAMP3
MPO
DAPI
Merge
Correlation
5 µm
VAMP3
LTF
DAPI
Merge
Correlation
VAMP3
MMP-9
DAPI
Merge
Correlation
VAMP3
TN
DAPI
Merge
Correlation

(B)


VAMP3
CD43
DAPI
Merge
Correlation
VAMP3
GM130
DAPI
Merge
Correlation
VAMP3
Calnexin
DAPI
Merge
Correlation
VAMP3
EEA1
DAPI
Merge
Correlation
VAMP3
LAMP-2
DAPI
Merge
Correlation


Pearson correlation
1.0
0.8
0.6
0.4
0.2
0.0
CD43+VAMP3
CNX+VAMP3
GM130+VAMP3
EEA1+VAMP3
LAMP1+VAMP3
LAMP2+VAMP3

Discussion

I Neutrophil-like HL-60 cells as experimental model

Neutrophils are terminally differentiated cells with a very short lifespan in the blood circulation as well as in *in vitro* culture. Hence, manipulations on primary neutrophils are challenging and long-term transfections for investigations are merely impossible. Further, isolated neutrophil preparations from the blood samples of different donors can be highly variable (*e.g.* alcohol consumption, smokers). The conditions of specimen handling (*e.g.* anticoagulant, buffy coat or fresh blood) have substantial impact on the outcome. Also, the preparation of ultra-pure neutrophil samples from whole blood is time-consuming, highly sensitive to temperature, can easily affect cell viability and lead to cellular pre-activation.

Therefore, cell lines represent an asset to analyze neutrophil functions due to their unlimited supply of cell material and availability of more homogeneous cell populations. To date, conditional immortalization of human neutrophil precursor cells has been unsuccessful and has only been achieved in murine cells (McDonald *et al.*, 2011). However, murine cells do not appropriately reflect the behavior of human neutrophils due to interspecies variability. For example, the azurophil granule protein defensin is present in human neutrophils but absent in murine neutrophils. Also while neutrophils represent 50-70% of the human peripheral white blood cells, they account only for 10-25% in the murine organism (Doeing *et al.*, 2003).

Since many years, the most extensively used cell line for investigating human neutrophil function has been the HL-60 cell line (Collins *et al.*, 1978; Drexler *et al.*, 1995), derived from a 36 years old female patient with acute promyelocytic leukemia, and differentiated by the use of chemicals (mostly DMSO) into neutrophil-like cells. Several reports have characterized the morphological and functional changes upon differentiation in HL-60 cells (dHL-60 cells) (Gallagher *et al.*, 1979; Mollinedo *et al.*, 2008). The differentiation status of HL-60 cells upon DMSO treatment can effectively be monitored by the upregulation of CD11b (Carrigan *et al.*, 2005), and downregulation of transferrin receptor (CD71) at the plasma membrane (Mora-Jensen *et al.*, 2011). The HL-60 cell line is easily transfectable and has been regarded as a very suitable model for studies on respiratory burst activity (Teufelhofer *et al.*, 2003), phagocytosis (Fleck *et al.*, 2005), and cell migration (Millius and Weiner, 2010).

However, early reports on dHL-60 cells demonstrated a lack in granule maturation and the absence of specific granules (Collins, 1987; Parmley *et al.*, 1987; Gallagher *et al.*, 1979; Johnston *et al.*, 1992; Le Cabec *et al.*, 1997). In our studies, the characteristic protein for specific granules, LTF, was indeed undetectable by immunofluorescence microscopy or ELISA.

The absence of LTF however might not be a consequence of a genetic aberration, as the LTF gene was reported to be structurally intact and the sequence of the 5' region completely normal (Johnston *et al.*, 1992). Therefore, Johnston *et al.* only questioned the absence or dysfunction of an essential factor in the induction of mRNA transcription. Alternative cell lines such as PLB-985 cells have similarly been reported to lack this type of granules (Pivot-Pajot *et al.*, 2010) and thus do not represent a more suitable model for neutrophils.

dHL-60 cells have been claimed to exclusively express azurophil granules (Dahlgren, 1989). The amount of the matrix protein MPO (4.4 µg *per* 10^6 cells) in azurophil granules was found to be similar in dHL-60 cells and in mature neutrophils. We provide evidence *via* immunofluorescence microscopy that MPO is present and detectable in dHL-60 cells. In the same line, azurophil granules were also determined by the presence of the CD63 granule marker *via* flow cytometry. However, MPO release and CD63 mobilization to the plasma membrane were both not significantly changed upon LPS stimulation. This might be explained by the fact that azurophil granules can only partially be mobilized (Sengelov *et al.*, 1993; Nusse *et al.*, 1998; Faurschou *et al.*, 2002).

Even though several reports also questioned the formation of gelatinase granules along HL-60 differentiation (Lubbert *et al.*, 1991; Newburger *et al.*, 1979; Borregaard and Cowland, 1997), our data demonstrate that MMP-9 mRNA is expressed in dHL-60 cells (see *Supplementary Data Manuscript II*) and supports the first reports from Davis *et al.*, in which MMP-9 is synthesized in HL-60 cells (Davis and Martin, 1990). Another report suggested that the continuous MMP-9 mRNA synthesis in HL-60 cells is regulated *via* ERK1/2 activity (Heidinger, 2006). On the protein level, a previously published report from our group already detected basal MMP-9 activity in supernatants from DMSO-differentiated HL-60 cells *via* gelatinase zymography (Bernard *et al.*, 2008). Ries *et al.* suggested that basal MMP-9 release is dependent on the TNFα-induced protein kinase C signaling pathway (Ries *et al.*, 1994), and that other biologically active agents such as growth factors (*e.g.* EGF) and cytokines (*e.g.* IL-1, TNFα) might induce MMP-9 production in HL-60 cells in a para- or autocrine pathway (Gazzanelli *et al.*, 2000). In addition, ELISA results showed that MMP-9 release levels from human neutrophils and dHL-60 cells were time-dependently increased upon LPS stimulation in a similar fashion (see *Supplementary Data Manuscript II*).

Our report proves that the serum protein tetranectin is detectable in dHL-60 cells *via* immunofluorescence microscopy. These results are in consistence with the report from Clemmensen *et al.,* which describes the low expression of tetranectin in HL-60 cells

(Clemmensen *et al.*, 1991). Although Gallagher *et al.* argumented that secretory vesicles might be absent due to the lack of alkaline phosphatase (Gallagher *et al.*, 1979; Garattini and Gianni, 1996), other reports indicated that granulocyte-colony stimulating factor and all-*trans* retinoic acid (ATRA) synergistically induce expression of alkaline phosphatase in a protein kinase C-dependent manner (Taoka *et al.*, 1993) in HL-60 cells (Gianni *et al.*, 1994; Garattini and Gianni, 1996; Tsuruta *et al.*, 1999). Moreover, we provide first evidence *via* ELISA that serum albumin was present in supernatants from LPS-stimulated dHL-60 cells.

Typically, we were able to detect an overall higher level of degranulation in primary cells than in dHL-60 cells upon LPS treatment. In both cells, the peak of degranulation was detected upon 12h of LPS stimulation pointing out that dHL-60 cells represent a suitable model for investigating neutrophil functions. Our flow cytometry results are in line with the statement that in general, a wide range of different stimuli (*e.g.* fMLF, A23187, or PMA) has been shown to induce a reasonably normal degranulation response in dHL-60 cells (Meagher and Cotter, 1988), although less high in performance compared to primary neutrophils (Newburger *et al.*, 1979).

In our study, we clearly demonstrated that the HL-60 cell model also has the capacity for cytokine production and release upon pro-inflammatory conditions. Regarding the cytokine response in neutrophil-like HL-60 cells, surprisingly only a few reports are known so far. The fact that Grande *et al.* discovered that ATRA-treated HL-60 cells are not only differentiated but already highly express cytokines, similar to LPS treatment (Grande *et al.*, 1995), strongly supports the use of DMSO-differentiated HL-60 cells in our experiments and to analyze the effect of LPS. The cytokines investigated were notably IL-1α, IL-1β, IL-6, IL-8, IL-12b, CCL2, CCL3, CCL4, CCL5, as well as TNFα. In accordance with our results, Spear *et al.* indicated that IL-1 production and secretion is inducible in dHL-60 cells (Spear *et al.*, 1988). Also, Mollinedo *et al.* described the expression of a variety of cytokines and cytokine receptors, *i.e.* CCL3, IL-1β, and IL-8, in DMSO-treated HL-60 cells (Mollinedo *et al.*, 2008).

While cytokine peak expression was detected after 6h LPS in dHL-60 cells but after 12h LPS in primary cells, similar expression levels of the major neutrophil-derived cytokines were profiled in both cells (see *Supplementary Data Manuscript II*). Our study revealed, for the very first time, the similar behavior of human neutrophils and HL-60 cells in the cytokine response under pro-inflammatory conditions. As the question of neutrophil purity in primary cell preparations is constantly raised, HL-60 cells are a suitable model to facilitate investigations on cytokine responses.

II Controversies on neutrophil-derived cytokines

Several reports have questioned the cytokines actually released by neutrophils due to many challenges in the purification steps of neutrophil preparations. Therefore, the evaluation of the purity needs to be highly accurate and representative of the population. Out-dated studies, in which the purity of only a low number of the whole population was evaluated by Giemsa staining is not acceptable anymore (Bazzoni *et al.*, 1991; Melani *et al.*, 1993; Retini *et al.*, 1996). Up-to-date technologies, such as multicolor flow cytometry or fluorescence-activated cell sorting (Dorward *et al.*, 2013), enable the highly accurate analysis of several thousand cells *per* sample.

In our studies, neutrophils isolated from the whole peripheral blood of healthy donors were obtained with an elevated purity of 98.1% according to our selection criteria $CD11b^{+}CD66b^{+}CD14^{low}$ (see *Supplementary Data Manuscript II*). Monocytes and lymphocytes in our preparations were identified by the markers $CD11b^{+}CD66b^{-}CD14^{high}$ (0.1%) and $CD11b^{-}CD66b^{-}CD14^{-}$ (1.8%), respectively. Contrary to recent reports that highlight the dramatic impact of very low percentages of monocyte contamination on cytokine secretion (Tamassia *et al.*, 2014), we observed that levels of secreted cytokines are similar between neutrophils highly purified (≥ 98%) and those purified to ≥ 90% (see *Supplementary Data Manuscript II*). These data underline the fact that 0.1% contamination of our preparation by monocytes does not significantly impact the high level of secretion observed for certain cytokines (*e.g.* IL-6, TNFα). This conclusion is additionally supported by the results obtained in our neutrophil-like HL-60 cell model for which we found comparable levels of cytokine secretion.

CBA experiments illustrate that our neutrophil preparations released IL-6 up to 600 pg/mL upon 12h of LPS treatment. IL-6 is one of the cytokines that has been regarded as a sign of monocyte contamination in the neutrophil preparation (Cassatella, 1995). However, many reports showed that human neutrophils are able to produce IL-6 (Ericson *et al.*, 1998). Some studies using buffy coats with concentrated content of leukocytes for neutrophil preparations detected IL-6 expression (Palma *et al.*, 1992; Melani *et al.*, 1993). Other reports argumented that *i)* IL-6 gene expression is only induced upon LPS stimulation in peripheral granulocytes (Grande *et al.*, 1995), *ii)* IL-6 is produced upon GM-CSF and TNFα stimulation (Cicco *et al.*, 1990) or upon IL-1 induction of the IL-6 promoter (Cahill and Rogers, 2008) implying an auto- or paracrine fashion, *iii)* a plasma component might be essential for IL-6 secretion (Oishi and Machida, 1997), and *iv)* intracellular IL-6 protein is induced after cross-linking FcγRI or FcγRII (Ericson, 1998). Melani

et al. reported that levels of IL-6 are variable depending on the donor, the time-lag between donation and purification (possibly due to the short half-life of cytokines), and that abolished IL-6 expression in ultra-pure neutrophil preparations can be re-activated by addition of growth factors to the culture medium (Melani *et al.*, 1993; Ericson *et al.*, 1998). IL-6 synthesis is known to be inducible *via* the NF-κB signaling pathway (Libermann and Baltimore, 1990), which is generally activated upon LPS stimulation. In addition, variations of measured IL-6 levels can largely depend on the fact that many immunoassays use antibodies generated against the recombinant non-glycosylated form of IL-6, and thus not its glycosylated form (Taupin *et al.*, 1997; Van den Steen *et al.*, 1998). Taken together, IL-6 detection in neutrophil preparations might be highly variable due to the low production level, the need of various activation stimuli, the donor, and the detection method and conditions.

Another cytokine under debate is CCL2 (MCP-1) that has first been reported not to be inducible in neutrophils by LPS solely (Strieter *et al.*, 1990). Our CBA results show that neutrophil preparations also released CCL2 up to 300 pg/mL upon 12 h of LPS treatment. This might be explained by the fact that an activation event upon longer incubation periods leads to CCL2 production in neutrophils (Burn *et al.*, 1994), probably due to a second induction by TNFα (Yamashiro *et al.*, 1999). Also, Yoshimura *et al.* found that CCL2 was dramatically upregulated by the synergistic effect of IFNγ (Yoshimura and Takahashi, 2007). In addition, reports claimed that LPS-activated c-Jun N-terminal and Tec kinase activity play a critical role in CCL2 expression in neutrophils (Malcolm and Worthen, 2003; Zemans and Arndt, 2009). Similar to IL-6, also CCL2 might require several activation events to be synthesized by neutrophils.

III Intracellular localization of cytokines in neutrophils

Emerging evidence shows that cytokines can be packaged in cell-specific cytoplasmic granules before extracellular release. Immunohistological examination of neutrophils suggests that TNFα (Beil *et al.*, 1995), TGFβ (Calafat *et al.*, 1997), IL-6 (Terebuh *et al.*, 1992; Denkers *et al.*, 2003), IL-12 (Bliss *et al.*, 2000), CXCL2 (Matzer *et al.*, 2001) and IL-8 (Brandt *et al.*, 2000) are stored within specific or gelatinase granules, or endosomal secretory vesicles. Also, Elbim *et al.* suggested that an intracellular pool of IL-10 receptors is stored in specific granules, and is mobilized upon pro-inflammatory conditions (Elbim *et al.*, 2001). Kinetics of fMLF- and TNFα-induced secretion of LTF and VEGF were found to be similar, suggesting the intracellular localization of this growth factor in the specific granule fraction (Gaudry *et al.*, 1997).

To date, investigations on the intracellular localization of cytokines in neutrophils are still challenging, most probably due to the low transcriptional and translational activity of these cells. Two methods have revolutionized the determination of the intracellular cytokine localization in blood cells. Firstly, Sander *et al.* detected cytokines microscopically in T cells and monocytes upon fixation with paraformaldehyde, permeabilization with saponin, immunofluorescence staining with monoclonal antibodies (Sander *et al.*, 1991). Secondly, Jung *et al.* then showed for the first time *via* flow cytometry how to accumulate cytokines in the Golgi by monensin treatment (Jung *et al.*, 1993). Although these methods are milestones to the research field, most studies were solely performed on T cells and monocytes due to their high intensity of cytokine generation (Mascher *et al.*, 1999). Additionally, limited availability of appropriate antibodies recognizing the different forms of cytokines existent in the cells (Schauer *et al.*, 1996) does not facilitate the investigations, and the number of alternative methods is restricted. For example, subcellular fractionation experiments show substantial limitations in resolution (Pasquali *et al.*, 1999).

In order to provide a robust guideline to investigate the spatial regulation of neutrophil cytokine release, we developed a linear fitting approach on 6 different stimulation time points ranging from 0 to 24h (see *Manuscript I*). Since neutrophils have been recognized to exert combinatorial effects of their functions during inflammation, the correlation between kinetics of LPS-induced cytokine secretion and degranulation represents an interesting tool for further investigations on the potential localization of cytokines in granules. As an example, many cytokines that are synthesized as precursor forms need to be cleaved to become biologically active. In this sense, MMP-9 from gelatinase granules leads to specific processing and release of cytokines, *e.g.* IL-1β, TNFα, IL-8 (Vaday *et al.*, 2001). Therefore, the release of granules and cytokines must be highly time-regulated in order to efficiently exert the combinatorial effects.

Traditionally, neutrophils have been seen as stores of preformed granules, and degranulation has been believed to mainly occur in a short-time range, meaning within maximally 2h upon stimulation (Bentwood and Henson, 1980). However, this assumption needs to be balanced since granule mobilization differs on the agonist used (*e.g.* fMLF, PMA) and its applied concentration. Our results show that degranulation in neutrophils behaves not only in a time- but also concentration-dependent manner and could thus account for the differential mobilization of the readily and slowly releasable granule pools towards the extracellular milieu (see *Manuscript I*). In line with our results, previous reports already showed that upon LPS stimulation, a concentration-dependency could be observed within neutrophil microbial activity. LTF release

from neutrophils varied with the concentration of LPS (1 to 1000 ng/mL) as well as with the duration of incubation (2 to 60 min) (Koivuranta-Vaara *et al.*, 1987). Our data demonstrates that upon LPS stimulation, the biggest release occurs far later than after 2h, and behaves similarly to the release of cytokines (see *Manuscript I*). Additionally, we observed that MMP-9 mRNA expression was significantly upregulated upon LPS stimulation of dHL-60 cells pointing out that neutrophil-specific granule proteins are also *de novo* synthesized (see *Supplementary Data Manuscript II*). Over the years, the concept of subdivided pools of granules has become largely accepted (Martin, 2002; Cassatella *et al.*, 2006; Pellme *et al.*, 2006) and overlaps with the recently described role of granule-specific proteins in the modulation of innate and adaptive immune responses (Wessels *et al.*, 2010).

In a similar fashion, time-course analysis with LPS resulted in early and late cytokine responses (Tsukahara *et al.*, 2003). On one hand, among the early responding agonists, we found that the secretion of TNFα, CCL3 and IL-1β is maximal at 6h. On the other hand, CCL2, CCL4, IL-6 and IL-8 secretion is obtained at later time points (see *Manuscript I*). This time-regulated cytokine secretion from neutrophils is largely dependent on a previous accumulation of the corresponding mRNA transcripts (Bazzoni *et al.*, 1991) and LPS-triggered protein synthesis that is under the control of MAP kinase-interacting serine/threonine-protein kinase 1 (MNK1) (Fortin *et al.*, 2011). The release of TNFα, CCL3 and IL-1β might be due to the direct rapid transcriptional and/or translational effect of LPS (DeForge and Remick, 1991). A second delayed phase involving CCL2, CCL4, IL-6 and IL-8 secretion, depending on protein synthesis, could derive from the endogenous production of neutrophil-derived agonists acting in an auto- or paracrine manner and promoting the sequential generation or/and simply the secretion of pro-inflammatory mediators at longer stimulation points (Cassatella, 1995). In accordance with our results, TNFα, and IL-1β have been described to increase IL-8 production and release by human neutrophils (Cassatella *et al.*, 1993; Ethuin *et al.*, 2001) and CCL2 has been found to be selectively expressed in the delayed phase by cytokine-stimulated neutrophils (Yamashiro *et al.*, 1999).

Upon analysis of LPS-induced kinetics of degranulation and cytokine release in neutrophils, we found that the IL-8 release is successfully fitted to the release of secretory vesicles, gelatinase granules and specific granules (see *Manuscript I*). This example underlines the importance of the time-regulated release of cytokines and granules, and thus the concurrent release into the inflammatory environment. Our linear fitting approach demonstrates that the exocytosis of

granules and cytokines is similarly regulated. The SNARE machinery present in our cells and its importance in the regulation of neutrophil functions is discussed in the next section.

IV SNAREs in the regulation of neutrophil cytokine secretion and degranulation

Over the last years, the involvement of SNAREs in immunological processes has been of great research interest due to its significant impact on the proper functioning of platelets (*e.g.* Flaumenhaft *et al.*, 1999; Chen *et al.*, 2000), mast cells (*e.g.* Paumet *et al.*, 2000; Vaidyanathan *et al.*, 2001), macrophages (*e.g.* Bajno *et al.*, 2000; Pagan *et al.*, 2003), eosinophils (*e.g.* Lacy *et al.*, 2001; Logan *et al.*, 2006), as well as plasma cells (Reales *et al.*, 2005). In neutrophil functions, SNAREs have also been implicated in the regulated secretory pathway during inflammation (*e.g.* Mollinedo *et al.*, 2006). While most of the studies focused on the role of SNAREs in degranulation, knowledge about their contribution to cytokine secretion is limited.

To facilitate investigations on neutrophil functions, we profiled SNARE expression in dHL-60 cells, and confirmed its similar profile expression in primary neutrophils. Our results showed that the transcripts of the Q_a-SNAREs STX2-12, 16-18, the $Q_{b,c}$-SNAREs SNAP23 and SNAP29; and the R-SNAREs VAMP1-5, 7-8 are expressed in HL-60 cells. These findings are largely overlapping with the report from Martín-Martín *et al.* in which STX1A, 3-7, 9-11, and 16 are expressed (Martin-Martin *et al.*, 1999). Further, only the upregulation of STX3 and STX11 was detected along HL-60 differentiation, in addition to VAMP1. Moreover, STX2, STX18 and VAMP5 transcripts were not expressed in human neutrophils and thus neglected in our investigations. In line with our results, Mollinedo observed that the secretory capacity of HL-60 cells was greatly increased during the differentiation process, and several exocytosis-related genes, *i.e.* SNAREs, were upregulated in parallel (Mollinedo *et al.*, 2008). Also, Xie *et al.* demonstrated that the level of STX3, 4, 6 and 11 was increased upon DMSO (Xie *et al.*, 2009).

However, mRNA expression levels of SNAREs do not necessarily reflect the relative protein expression levels. Even though mRNA levels of SNAP23 and SNAP29 were not upregulated upon DMSO differentiation of HL-60 cells, in agreement with Mollinedo *et al.* (Mollinedo and Lazo, 1997), we found that SNAP23 protein was upregulated upon DMSO differentiation in HL-60 cells.

Although previous studies reported that LPS could induce rapid down- or upregulation of SNARE expression in cells (*e.g.* Pagan *et al.*, 2003; Murray *et al.*, 2005), we did not observe

this dramatic change in expression upon LPS stimulation. In other words, LPS did not induce upregulation of STX3 expression (*data not shown*), suggesting that the trafficking machinery needs already to be set ready for action upon differentiation of HL-60 cells.

In dHL-60 cells, we could determine the role of the Q_a-SNARE STX3 in the release of the cytokines IL-1α, IL-1β, CCL4 and IL-12b, as well as the granule protein MMP-9. In accordance with our previous study, the linear fitting approach indicates that IL-1α belongs to secretory vesicles, gelatinase and specific granules, IL-1β to secretory vesicles, and CCL4 to specific granules. IL-12b however could not be fitted with high RSQ value (see *Manuscript I*). STX3 siRNA induced different inhibition levels on the release of the cytokines and MMP-9. These results are clarified by the fact that STX3, localized to gelatinase granules and secretory vesicles, could affect the release of differentially distributed cytokines in the granules. The fact that CCL4 secretion is also influenced by STX3, although specific granules are absent from dHL-60 cells, could be explained by a possible relocalization towards other granules in the cellular model. Taken together, these results could indicate the existence of a pathway in which cytokines are transferred to, or are located in neutrophil-specific granules.
The localization of STX3 in gelatinase granules and secretory vesicles under non-stimulated as well as LPS-stimulated conditions showed that STX3 was not translocated to another organelle. This statement does not collide with our previous results identifying the functional role of STX3 in cytokine release and degranulation, since STX3 belongs to the subfamily of Q_a- or also t-SNAREs. This means that neutrophil granules represent the resident compartment of STX3 (Brandhorst *et al.*, 2006), and that STX3 does not need to be relocalized to fulfill its role in neutrophil functions.

It is important to note that STX3 alone is not sufficient to mediate trafficking specificity. According to Bethani *et al.*, each fusion step of the secretory pathway requires a specific set of SNAREs (Bethani *et al.*, 2007). Therefore, SNARE partners that form complexes with STX3 and lead to the specific release of these pro-inflammatory mediators need to be uncovered (Martin-Martin *et al.*, 2000; Mollinedo *et al.*, 2006). Our results show that the only $Q_{b,c}$-SNAREs expressed in neutrophil-like HL-60 cells and neutrophils are SNAP23 and SNAP29. Since its discovery in neuronal cells, SNAP25 has always been regarded to be exclusively expressed in brain tissue (Brumell *et al.*, 1995). Although SNAP25 expression has been reported in neutrophils (Nabokina *et al.*, 1997), we could neither detect the expression of this $Q_{b,c}$-SNARE in our cell model nor in primary neutrophils (*data not shown*).

To uncover the identity of potential SNARE proteins that interact with STX3, we performed immunoprecipitation experiments and applied a proteomics approach using mass spectrometry. Although *in vitro* studies could provide evidence that STX3 interacts with SNAP29 (Steegmaier *et al.*, 1998), the difficulty of reproducibility to detect SNAP29 upon STX3 pull-down in dHL-60 and neutrophil total lysates can be clarified by the fact that the $Q_{b,c}$-SNARE SNAP29 is the lowest expressed SNARE detected in our cells (*data not shown*). Bethani *et al.* demonstrated that in case individual SNAREs are not present in stoichiometric amounts, co-immunoprecipitation of SNAREs might be inappropriate to be used as evidence for their functional interaction in a fusion event (Brandhorst *et al.*, 2006). Given the fact that quantitative precipitation of STX3 with monoclonal antibody brings down a little fraction of the SNAP29 pool, it is conceivable that this fraction can be difficult to detect and might also explain our unfruitful proteomics analysis.

While SNAP29 has already been localized to the Golgi (Hohenstein and Roche, 2001), plasma membrane and endocytic compartments (*e.g.* Wesolowski *et al.*, 2012), it was colocalized with STX3 on cytoplasmic granules in dHL-60 cells and neutrophils. Interestingly, SNAP29 is one of the SNAP25 homologues that lacks a membrane anchor (Steegmaier *et al.*, 1998) but is capable to bind to a variety of syntaxins. Since the intracellular localization of SNAP29, similar to the one of STX3, does not change upon LPS stimulation, we can conclude that the resident compartment of SNAP29 is also referred to gelatinase granules and secretory vesicles (Brandhorst *et al.*, 2006).

Colocalization studies showed that also $Q_{b,c}$-SNAREs SNAP23 shares common localization with STX3 in dHL-60 cells and human neutrophils (*data not shown*). On one hand, concerning the antibody-labeling of SNAP23, our results are in accordance with previous studies in which SNAP23 was primarily located in specific and gelatinase granules in neutrophils (Martin-Martin *et al.*, 2000). Uriarte *et al.* already investigated the role of this $Q_{b,c}$-SNARE by introducing a TAT-SNAP23 fusion protein into primary neutrophils (Uriarte *et al.*, 2011). By this treatment, they discovered inhibited exocytosis of granules in neutrophils. As we could not detect any effect of SNAP23 on the release of granules, and postulate that SNAP23 might form “non-cognate” SNARE complexes with no functional cross-talk (Bethani *et al.*, 2007). Observations from Uriarte *et al.* can be explained by the assumption that increased abundance of SNAP23 protein might result in unbalanced stoichiometric amounts and thus lead to the formation of artificial SNARE complexes preventing other SNAREs from doing their work. In contrast, according to our analyses, STX3 and SNAP29 act together in the release of the cytokine IL-12b, as well as

MMP-9. We assume that the formation of so-called “cognate” SNARE complexes implicating STX3 and SNAP29 occurs at the level of cytoplasmic granules.

The knockdown of the $Q_{b,c}$-SNARE SNAP29 alone impacted the release of the cytokines IL-8, IL-12b, and CCL2, as well as the granule protein MMP-9. Hence, we hypothesize that SNAP29 must act in other SNARE configurations without STX3 in the release of IL-8 and CCL2. In accordance with our previous study, the linear fitting approach indicates that IL-8 belongs to secretory vesicles, gelatinase and specific granules. However, IL-12b and CCL2 could not be fitted with high RSQ value to any granule marker (see *Manuscript I*). In this scenario, SNAP29 localized to gelatinase granules and secretory vesicles could affect the release of IL-8, fitted to the same granule types in addition to specific granules in human neutrophils. Similar to the case of STX3, these results could indicate the existence of a pathway in which cytokines are transferred to, or are located in neutrophil-specific granules.

In the last part of our study, we targeted VAMP3 to reveal if this R-SNARE is implicated in the STX3-SNAP29-mediated release of IL-12b and MMP-9. Our RNAi approach against VAMP3 led to the inhibited secretion of the cytokines CCL4 and IL-8, as well as the granule protein MMP-9. The intracellular localization of VAMP3 could be determined to be at the plasma membrane in dHL-60 cells. According to these results, we could assume that VAMP3 is interfering with cytokine release and degranulation at the “very last station” before exocytosis. Interestingly, as an R-SNARE generally localized at the vesicle membrane before fusion according to the SNARE hypothesis, it is quite surprising to find VAMP3 at the plasma membrane. This might be explained by the fact that VAMP3 has often been implicated in endocytic pathways (*e.g.* McMahon *et al.*, 1993) in the recycling of the transferrin receptor (Galli *et al.*, 1994; Daro *et al.*, 1996). Therefore, VAMP3 could be seen as having a dual role in the endocytic cycle and represent an interesting candidate for further investigations in neutrophil functions.

General Conclusion and Perspectives

General Conclusion and Perspectives

In the present study, we outlined the regulatory release mechanisms of neutrophil pro-inflammatory mediators, with emphasis on the role of SNARE fusion proteins. Our results consist of the following points:

- Generation of a linear fitting approach between similar kinetics of neutrophil cytokine secretion and degranulation, that can be used as a basis model for investigating spatial regulation of neutrophil cytokines,
- Determination of the requirement of
    - the Q_a-SNARE STX3 for the release of IL-1α, IL-1β, IL-12b, and CCL4,
    - the $Q_{b,c}$-SNARE SNAP29 for the release of CCL2, IL-12b, and IL-8,
    - the R-SNARE VAMP3 for the release of CCL4 and IL-8,
    - STX3, SNAP29 and VAMP3 in MMP-9 exocytosis from gelatinase granules,
- Identification of the intracellular localization of STX3, together with SNAP29, in gelatinase granules and secretory vesicles; and VAMP3 at the plasma membrane,
- Exclusion of the R-SNARE VAMP3 as a candidate for being part of the STX3-SNAP29 complex.

A summarized view of the hypothetical SNARE-mediated trafficking pathways in dHL-60 cells is depicted in *Figure 12*. To conclude, this study describes for the first time a functional role of SNARE proteins, notably STX3, SNAP29 and VAMP3, in cytokine release in a cellular model for neutrophils. Due to their additional implication in gelatinase degranulation, this study speculates on the intracellular localization of cytokines in granules before exocytosis.

Nevertheless, some questions on the regulatory mechanisms of neutrophil functions remain open.

How can the linear fitting approach be improved to investigate LPS-mediated degranulation and cytokine release? The linear fitting approach could be improved by adding additional parameters that respect the endocytosis rate of the granule-specific membrane receptors from the plasma membrane, the half-lives of the cytokines in the extracellular milieu, the intracellular content of cytokines as well as of granules.

Do STX3, SNAP29 and VAMP3 have the same functional role in primary neutrophils as in dHL-60 cells? Functional studies on primary neutrophils are still challenging due to purification steps, limited manipulation tools, short half-life, and low transcriptional activity. We performed transduction of primary neutrophils with lentiviral short-hairpin RNA (shRNA)-containing

constructs targeting STX3 but did not yield in sufficiently high percentages of positively transduced cells (3% of positively transduced neutrophils with lentiviral particles at MOI=3 upon 6h incubation with polybrene, *data not shown*). A new challenging approach could include the transduction of precursor cells, and the subsequent differentiation to mature neutrophils.

Do cytokines localize in, or are they translocated to neutrophil-specific granules for simultaneous exocytosis upon inflammation? To determine the localization of cytokines in neutrophils, the mediators could be stained intracellularly and subsequently analysed *via* flow cytometry. This method is also challenging due to the low content of cytokines but promising due to recent advances in this field (*e.g.* antibodies with high specificity and affinity, permeabilisation methods). Also, the localization of cytokines in different vesicle pools and the subsequent SNARE-regulated release upon stimulation could be visualized by total internal reflection fluorescence (TIRF) microscopy.

Are STX3, SNAP29 and VAMP3 post-translationally modified under pro-inflammatory conditions? Several SNAREs need to be post-translationally modified to successfully interact with other SNARE proteins or regulators (Craig *et al.*, 2003) and thus lead to vesicle trafficking. Mass spectrometry results have already predicted that *i)* STX3 possesses a phosphorylation site at serine 206, while *ii)* VAMP3 shows 7 different sites, and *iii)* SNAP29 even 23 different sites.

Do STX3, SNAP29 and VAMP3 depend on intracellular Ca^{2+} mobilization in order to be regulated? Intracellular Ca^{2+} concentrations have been implicated in the differential release of granule types, in the orientation of opposing bilayers before membrane fusion, as well as in the requirement for regulatory proteins (such as synaptotagmins, Munc proteins). Under the pro-inflammatory conditions used for our experiments, the induction of intracellular Ca^{2+} mobilization (*e.g.* by spectrofluorimetry, flow cytometry, or confocal microscopy), as well as the implication of regulatory proteins in this scenario could be investigated in more details.

Which are the still unknown and remaining SNARE interactors of STX3, SNAP29 and VAMP3 for specificity of trafficking? In a first attempt, we performed co-immunoprecipitation experiments and analysis by mass spectrometry to find SNARE interaction partners of STX3, and had many difficulties for detecting SNAP29 due to its low expression level and the huge amount of cells needed for investigations. Therefore, the proximity-ligation analysis provides an alternative approach with higher sensitivity, and specificity. The same approach can be applied for identifying SNARE partners of SNAP29 and VAMP3.

The molecular mechanisms underlying exocytotic pathways in neutrophils are complex and have been largely unknown so far. In this scientific work, we provide evidence that the SNAREs STX3, SNAP29 and VAMP3 are implicated in neutrophil degranulation and cytokine release. Further experiments will be necessary to identify the unknown SNARE players that act in configuration with the three candidates and specifically lead to trafficking during inflammation. A deeper understanding of the SNARE-regulated processes in neutrophils will help to determine new potential pharmacological targets in chronic inflammatory diseases that could prevent the release of mediators into the pro-inflammatory environment.

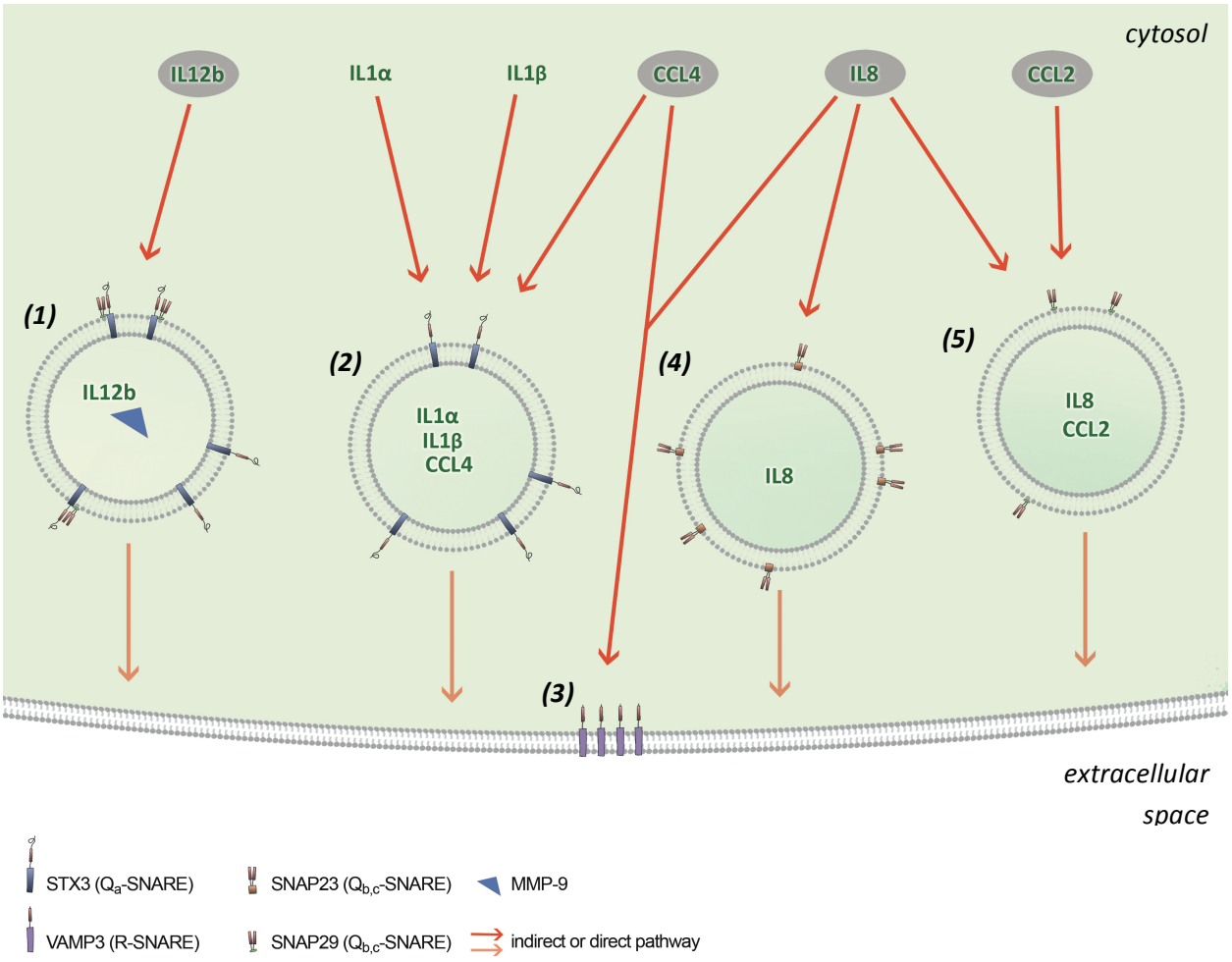


Figure 12 *Hypothetical SNARE-mediated trafficking in cytokine release and degranulation in dHL-60 cells. The scheme summarizes the implication of (1) STX3 and SNAP29 in the release of MMP-9 and IL-12b (with unknown R-SNARE), (2) STX3 in the release of IL-1α, IL-1β, and CCL4 (with unknown $Q_{b,c}$- and R-SNARE), (3) VAMP3 located at the plasma membrane in the release of CCL4 and IL-8 (with unknown Q_a- and $Q_{b,c}$-SNARE), (4) SNAP23 in the release of IL-8 (with unknown Q_a- and R-SNARE), and (5) SNAP29 in the release of IL-8 and CCL2 (with unknown Q_a- and R-SNARE).*

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Appendix

Appendix

Grants

PhD school grant, University of Luxembourg (Luxembourg) **Jun, 2012**
Bench fees of 8.000€ for proteomic analysis in collaboration with CRP Lippmann

Travel grant, Fonds National de la Recherche (Luxembourg) **Jun, 2012**
Grant of 2.000€ for participation at the meeting The Neutrophil in Immunity, Québec City (Canada)

Presentations

Life Sciences PhD Days, University of Luxembourg (Luxembourg) **Sep 9-10, 2013**
Oral: *Syntaxin 3 regulates the exocytosis of cytoplasmic granules and cytokines during inflammation in neutrophil-like HL-60 cells*
I. Naegelen, S. Plançon, A. Muller, T. Kaoma, N. Nicot, L. Vallar, E.J. Tschirhart and S. Bréchard

47th Scientific Meeting of the European Society for Clinical Investigation, Albufeira (Portugal) **Apr 17-20, 2013**
Poster: *Upregulation of pro-inflammatory functions by lipopolysaccharide in human neutrophils: Focus on cytokine secretion and degranulation*
I. Naegelen, S. Plançon, A. Muller, T. Kaoma, N. Nicot, L. Vallar, E.J. Tschirhart and S. Bréchard

Life Sciences PhD Days, University of Luxembourg (Luxembourg) **Sep 11-12, 2012**
Poster: *SNARE proteins in the regulation of matrix metalloproteinase 9 in human neutrophils under pro-inflammatory conditions*
I. Naegelen, S. Plançon, A. Manele, J. Hann, A. Muller, T. Kaoma, N. Nicot, L. Vallar, E.J. Tschirhart and S. Bréchard

The Neutrophil in Immunity, Québec City (Canada) **Jun 9-12, 2012**
Poster: *Analysis of lipopolysaccharide-mediated degranulation and cytokine secretion in neutrophils*
I. Naegelen, S. Plançon, A. Muller, T. Kaoma, N. Nicot, L. Vallar, E.J. Tschirhart and S. Bréchard

46th Scientific Meeting of the European Society for Clinical Investigation Budapest (Hungary) **Mar 22-24, 2012**
Poster: *Lipopolysaccharide-mediated cytokine regulation in neutrophil-like HL-60 cells and human neutrophils*
I. Naegelen, S. Plançon, A. Muller, T. Kaoma, N. Nicot, L. Vallar, E.J. Tschirhart and S. Bréchard

Life Sciences PhD Days, University of Luxembourg (Luxembourg) **Sep 12-13, 2011**
Oral: *Regulation of neutrophil functions during inflammation: Role of SNARE fusion proteins*
I. Naegelen, S. Plançon, A. Muller, T. Kaoma, N. Nicot, L. Vallar, E.J. Tschirhart and S. Bréchard

Courses & Workshops

Advanced Biostatistics, University of Luxembourg (Luxembourg) **Dec 2-6, 2013**
Doctoral School in Molecular Biomedicine, Dr. Petr V. Nazarov

Good Scientific Practice, University of Luxembourg (Luxembourg) **Oct 7-8, 2013**
Doctoral School in Molecular Biomedicine, Dr. Michael Gommel

Profiling Cellular Metabolism, University of Luxembourg (Luxembourg) **Jun 17-21, 2013**
Doctoral School in Molecular Biomedicine, Dr. Karsten Hiller

Late Summer Practical Proteomics Seminar, IMP/IMBA Vienna (Austria) **Aug 27-28, 2012**

Advanced Proteomics, University of Luxembourg (Luxembourg) **Jul, 2012**
Doctoral School in Molecular Biomedicine, Dr. Bruno Domon

Microinjection Training, University of Luxembourg (Luxembourg) **Jun, 2012**
Dr. Andreas Girod

Project Management, Fonds National de la Recherche (Luxembourg) **Mar, 2012**
fast training agency

Scientific Writing, University of Luxembourg (Luxembourg) **Feb-May, 2012**
Doctoral School in Molecular Biomedicine, Dr. Maja Altarac

Computational Systems Biology, University of Luxembourg (Luxembourg) **Feb, 2012**
Doctoral School in Molecular Biomedicine, Dr. Antonio Del Sol Mesa

Boolean Modeling of Signaling and Gene Regulatory Networks, **Dec, 2011**
Lucilinx Dr. Thomas Sauter

Gilson Pipetting Training, University of Luxembourg (Luxembourg) **Nov, 2011**

Bio-Rad qPCR Seminar Tour, University of Luxembourg (Luxembourg) **Oct, 2011**

Statistical Data Analysis in Excel, Lucilinx **Oct, 2011**
Dr. Petr V. Nazarov

Lentiviral vectors, University of Luxembourg (Luxembourg) **Nov, 2010**
Theoretical course, Xibios Biosafety Consulting

Teaching

Teaching Assistant, University of Luxembourg (Luxembourg) **Oct, 2010-2014**
1st year Bachelor Practical Courses in Biochemistry

Teaching Assistant, University of Vienna (Austria) **Jan, 2014**
3rd year Bachelor Practical Courses in Biochemistry