NEUTROPHIL SECRETION OF THE PROINFLAMMATORY CYTOKINE TNF *VIA* RECYCLING ENDOSOMES

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Abstract

Neutrophils are highly abundant innate immune cells that are important in immediate responses to injury and infection, and secrete the proinflammatory cytokine tumor necrosis factor (TNF). Inflammatory cytokines have many potent effects and their excess or deficiency may have many clinical consequences. However, cytokine trafficking and secretion in neutrophils has not been well characterized. Recycling endosomes (REs) are specialized secretory compartments that perform multiple functions including trafficking of cytokines to cell surfaces, although these are not characterized in neutrophils. Our objective is to identify trafficking components in neutrophils that may contribute to cytokine secretion. This study presents data that shows that LPS induced 30-40% TNF secretion from stored sources, with the remainder newly synthesized. We also found that neutrophils possess REs as determined by transferrin uptake and VAMP-3 labeling. TNF also colocalized with REs, primary and secondary granules as well as early and late endosomes, suggesting multiple sites of TNF storage and trafficking in neutrophils. TNF colocalized with VAMP-3 around periphery of cells after 1 h stimulation with LPS, suggesting TLR4-induced TNF trafficking *via* REs. The present study provides evidence that movement of $TNF^{+}VAMP\text{-}3^{+}$ vesicles towards the cell periphery in response to LPS. This suggests that neutrophils utilize REs for trafficking of TNF to the cell surface in response to TLR4 signaling. These findings contribute to our understanding of how neutrophils package, transport, and release cytokines.

<u>This Thesis is dedicated to my very supporting husband, Keshav and my sweet daughter, Nandini</u>

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Nutan Srivastava

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Abbreviations

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CCD	Charged-coupled device
CCV	Clathrin-coated vesicle
C3b	Complement component 3b
CR	Complement receptor
CHX	Cycloheximide
DNA	Deoxyribonucleic acid
DV	Deltavision
DAPI	4', 6-diamidino-2-phenylindole
Fc	Fragment crystallizable
EDTA	Ethylene diamine tetra acetic acid
EE	Early endosomes
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
GTP	Guanosine-5'-triphosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
IFN-γ	Interferon gamma
IgG	Immunoglobulin gamma
IL	Interleukin
LE	Late endosomes
LPS	Lipopolysaccharide
mTNF	Membrane tumor necrosis factor
MPO	Myeloperoxidase
M6PR	Mannose-6-phosphate receptor
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular traps
NF-κB	Nuclear factor-κB
NK	Natural killer
NLR	Nucleotide-binding oligomerization domain-like receptor
NOD	Nucleotide-binding oligomerization domain
NO	Nitric acid
O_2^-	Superoxide
PAMP	Pathogen-associated molecular pattern
PCC	Pearson's correlation coefficients
PBS	Phosphate buffered saline
PBST	Phosphate buffer saline Tween-20
PRR	Pattern recognition receptor

PSF	Point-spread function
RA	Rheumatoid arthritis
RBC	Red blood cells
RE	Recycling endosomes
RLR	Retinoid acid-inducible gene I-like receptor
ROS	Reactive oxygen species
ROI	Reactive oxygen intermediate
ROI	Region of interest
SNAP	Soluble *N*-ethylmaleimide-sensitive fusion protein attachment Protein
SNARE receptor	Soluble *N*-ethylmaleimide-sensitive fusion protein attachment Protein
sTNF	Soluble tumor necrosis factor
Stx	Syntaxin
TACE	TNF-α converting enzyme
Tfn	Transferrin
TfnR	Transferrin receptor
TGN	Trans-Golgi network
TGFR	Transforming growth factor receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TX	Triton-X
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain
2D	Two-dimension
VAMP	Vesicle associated membrane protein

Chapter 1

Introduction

1.1.1 Role of neutrophils in inflammation and immunity

Inflammation is defined as a complex sequence of events triggered by injuries and infections. This process normally leads to healing and/or recovery from a lesion (Nathan *et al.,* 2002). Neutrophils are key cells during the initial inflammatory response, as they are among the first line of cellular defense and are rapidly recruited to the sites of injuries or infections. Efficient neutrophil activation and migration are essential for a protective innate immune response (Cheretakis *et al.,* 2006 and Nathan *et al.,* 2006). Activated neutrophils undergo major cytoskeletal changes and differential gene expression (Kobayashi *et al.,* 2005) that mediate their directional migration to sites of injury (chemotaxis) and ultimately trigger bactericidal reactions. The efficiency of these processes depends on highly regulated signaling pathways responsible for actin polymerization, motility, phagocytosis and bacterial killing (Cicchetti *et al.,* 2002; Fenteany *et al.,* 2004 and Dale *et al.,* 2008).

1.1.2 Differentiation

Neutrophils derive from the bone marrow following stimulation by IL-3 and GM-CSF of stem cells differentiation (Bainton *et al.,* 1971). Differentiation is a multi-step process that involves consecutive cellular divisions and transformations regulated by hematopoietic growth factors and cytokines. Growth stimulating factors are synthesized by a variety of cells and tissues and have fundamental roles in neutrophil hematopoiesis. These cytokines determines the initial differentiation of these cells into myeloblasts, which are the first recognizable granulopoietic progenitor cells. A combination of cytokines, including GM-CSF and IL-3, will continue to differentiate myeloblasts into promyelocytes, which accumulate peroxidase-positive primary granules. In the next

differentiation step, myelocytes start to appear, with a prominent accumulation of peroxidase-negative granules (secondary granules), and less abundant azurophilic granules (Lieschke *et al.,* 1994). In the final stages of this process, myelocytes differentiate into metamyelocytes, which are non-dividing cells.

1.1.3 Structure of neutrophils

Blood neutrophils are relatively short-lived cells (<6 days) and are the most abundant leukocytes as they represent 50-70% of total white blood cells (Simmons *et al.,* 1974). Every day, nearly 10^{11} neutrophils are generated in the bone marrow and released into the blood circulation. Under electron microscopy, they have a very distinct appearance, with a multilobed nucleus and numerous electron-dense vesicles in the cytoplasm (Figure 1). A key structural characteristic of neutrophils is the abundance of cytoplasmic granules. These granules contain a variety of enzymes, membrane and matrix proteins. They are divided in three major groups, primary (azurophilic), secondary (specific) and tertiary (gelatinase) granules (Faurschou *et al.,* 2003). Azurophilic granules are characterized by the presence of hydrolytic enzymes, myeloperoxidase and other potent bactericidal enzymes. Secondary granules are peroxidase-negative and are twice as abundant as primary granules. The key components of secondary granules are lactoferrin and lysozyme. High levels of gelatinase characterize tertiary granules. The combination of antimicrobial proteins, receptors, and other membrane proteins in individually packed granules represent a powerful mechanism to eliminate bacteria and provide a fast activation/degranulation mechanism during inflammation.

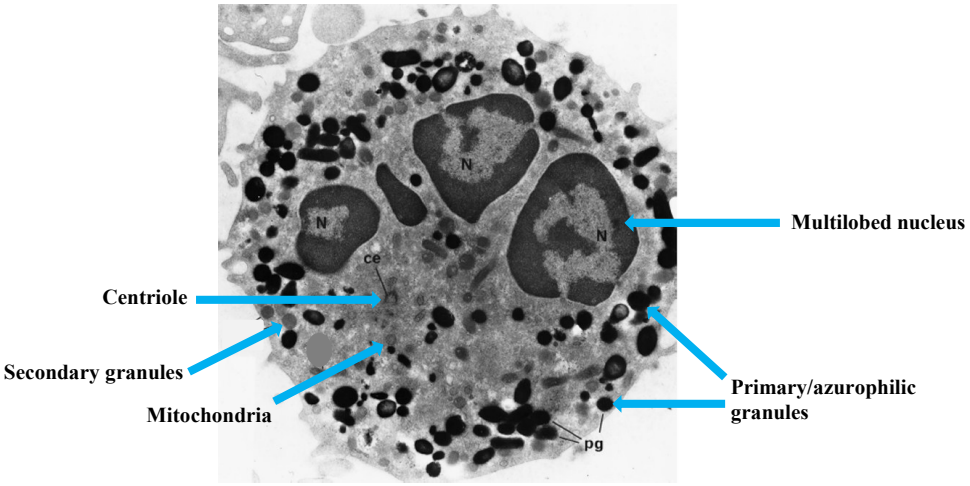


Figure 1: Ultrastructure of neutrophils. Image from transmission electron microscopy showing the various cytoplasmic granules of a resting neutrophil. Resting neutrophils were first fixed in 1.25% glutaraldehyde and peroxidase-positive granules were visualized with diaminobenzidine. Azurophilic/primary granules are peroxidase-positive granules, which appear as dark (electron-dense) granules. Specific/secondary granules are electron-lucent and smaller in size (Source: Modified from Witko-Sarsat *et al.,* 2000).

By electron microscopy, neutrophils are characterized by a multitude of granules and vesicles varying in density and randomly allocated in the cytoplasm with very few mitochondria (Shinagawa *et al.,* 1966).

1.1.4 Transmigration of neutrophils towards inflammatory stimuli

Recruitment of neutrophils from the blood circulation to sites of infection or injury is a key innate immune response against invading pathogens and tissue injury. They migrate to the tissues in large numbers by molecular interaction between the cell surfaces of the neutrophil and endothelial cells of blood vessels (Nathan *et al.,* 2006). Circulating neutrophils undergo a series of activation steps and respond to inflammation through three classes of surface proteins: selectins, integrins, and chemokines. To resist shear forces, a neutrophil initially binds the blood vessel wall through selectins and undergoes a transient, rolling adhesion. Integrins expressed by neutrophils forcing them to decelerate from rolling to a stable arrest. In addition, neutrophils rely on different chemoattractants to perform efficient transmigration, such as interleukin 8 (IL-8), tumor necrosis factor (TNF), and IL-1. Neutrophils roll along the blood vessel walls, then adhere and migrate through a process called diapedesis.

1.1.5 Neutrophil killing mechanisms

1.1.5.1 Phagocytosis

Neutrophils use different killing mechanisms to eliminate ingested pathogens in complex processes. They have potent antimicrobial means including different types of intracellular granules by which they can kill and degrade targets (Nauseef *et al.*, 2007). Neutrophils also express different sets of cell surface receptors, including complement and antibody

recognition receptors (CR and Fc respectively) and toll like receptors (TLRs). Through these receptors, neutrophils recognize opsonins, proteins that bind to bacteria and enhance phagocytosis in a process called opsonization. Opsonins include the heavy chain of immunoglobulin G (IgG-Fc) and complement components, such as C3b. These important molecules are involved in the initiation of neutrophil phagocytosis and intracellular signaling processes (Akira *et al.,* 2008).

1.1.5.2 Neutrophil Respiratory Burst

Within neutrophils, in a metabolic process termed the respiratory burst, microbes are killed as a result of the production of highly reactive free radicals called reactive oxygen species (ROS) or reactive oxygen intermediates (ROI). Specifically, upon stimulation of its receptors, the neutrophil becomes activated and starts engulfing the microbes *via* folding its surface membrane around the pathogen forming a vacuole called a phagosome. In the cytoplasm of the neutrophils, secretory granules, also known as lysosome related organelles, containing various degenerative enzymes, fuse with the phagosome to form a phagolysosome; inside the phagolysosome, killing and degradation takes place (Nathan *et al.*, 2006). In respiratory burst, various bactericidal proteins, reactive oxygen species and hydrolytic enzymes are produced including; superoxide (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), hypochlorite, myeloperoxidase, lactoferrin, proteases, nucleases and lipases etc. (Segal *et al.,* 2006; Yokoyama *et al.,* 2005).

1.1.5.3 Degranulation

Neutrophil-derived microbicidal molecules are packed in granules and released upon cell activation (Elsbach *et al.,* 1998). This release from neutrophils occurs by a tightly

controlled receptor-coupled mechanism known as regulated exocytosis and is a four-step process (Toonen *et al.,* 2003). In the first step of exocytosis, granules translocate to the target membrane, which dependents on actin cytoskeleton remodeling and microtubule assembly (Burgoyne *et al.,* 2003). The second step is granule tethering and docking, leading to contact of the outer surface of the granule lipid bilayer membrane with the inner surface of the target membrane (Stow *et al.*, 2006). Granule priming is followed by granule fusion in third step. In the fourth and final step fusion pore expansion allows release of granule contents outside of the cell.

1.1.5.4 Neutrophil Extracellular Traps (NETs)

One of the mechanisms of neutrophil action is the formation of neutrophil extracellular traps (NETs). The process of NET generation, called netosis, is a specific type of cell death but different from necrosis and apoptosis. NETs arise from the release of the nuclear contents into the extracellular space of neutrophils. NETs are formed by neutrophils upon contact with various bacteria, fungi, and activated platelets or under the influence of numerous inflammatory stimuli (Brinkmann *et al.,* 2004). The main components of NETs are DNA and granular antimicrobial proteins. The pathogens trapped in NETs are killed by oxidative and non-oxidative mechanisms. During activation, neutrophils produce large amounts of ROS through the action of NADPH oxidase (Nishinaka *et al.,* 2011) and neutrophil chromatin undergoes decondensation (Fuchs *et al.,* 2007). This process is mediated by neutrophil elastase (NE) and myeloperoxidase (MPO), enzymes stored in the azurophilic granules. First, NE degrades histones, leading to chromatin decondensation, which is enhanced by MPO (Papayannopoulos *et al.,* 2010). Subsequently, the nuclear membrane is damaged;

chromatin expands inside the cell and is mixed with granular antimicrobial factors. Finally, the cell membrane breaks releasing NETs (Brinkmann *et al.,* 2012).

1.1.6 Bacterial lipopolysaccharide and Toll-like receptors

The host recognition of small microbial molecules, called pathogen-associated molecular patterns (PAMPs), initiates the inflammatory response characteristic of innate immunity. The most extensively studied PAMP is lipopolysaccharide (LPS), also called endotoxin (Mogensen *et al.*, 2009). LPS represents an essential component of the outer membrane of various Gram-negative bacteria such as *Escherichia coli, Neisseria meningitidis, Haemophilus influenzae, Klebsiella pneumoniae, Chlamydia trachomatis* and *Yersinia pestis.* These endotoxins lack protein and are composed solely of carbohydrates, fatty acids, and phosphorus-containing compounds. LPS consists of three characteristic structural building blocks: lipid A, a predominantly lipophilic component, a core oligosaccharide region, and a terminal *O*-specific chain (*O*-antigen) (Luderitz *et al.*, 1966). Cellular signaling cascades are initiated by recognition of PAMPs by a class of germ-line receptors, pattern recognition receptors (PRRs). PRRs are expressed on the surface of cells of the innate immune system and are comprised of several classes, including Toll-like receptors (TLRs) (O'Neill *et al.*, 2008), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) (Yoneyama *et al.*, 2008) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Shaw *et al.*, 2008). TLRs are a family of receptors classified on the basis of homology to the cytoplasmic domain of the interleukin-1 receptor (IL-1R) family, known as the Toll/IL-1R (TIR) domain (Gay *et al.*, 1991). There have been 13 TLRs identified in mice (Lauw *et al.*, 2005), and 11 TLRs found in humans (O'Neill *et al.*, 2008). The exact cellular location of human TLRs

remains under study but the family can be segregated into receptors that are found on the plasma membrane such as TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10, and that are present in the endosomal compartment including TLR3, TLR7, TLR8 and TLR9 (Wu *et al.*, 2008). The intracellular location of TLRs that recognize bacterial and viral DNA/RNA is important as it has ability to distinguish between foreign and self nucleic acids (Barton *et al.*, 2006).

1.1.7 Cytokine synthesis by neutrophils

Neutrophils, either constitutively or in an inducible manner, can synthesize or release a wide range of cytokines, chemokines, and growth factors (Cassatella *et al.*, 1999). The production of cytokines is largely influenced by vast variety of stimulating agents. Cytokines such as IFNγ, TNF, IL-4, IL-10, and IL-13 are known to influence cytokine production in neutrophils (Romagnani *et al.*, 1994). The pattern of cytokines produced by neutrophils greatly differs depending on the agonist. Bacterial endotoxins such as lipopolysaccharide (LPS) are among the most potent cytokine inducers. For some cytokines, co-stimulation by at least two agonists are required, eg, IFNγ + LPS in the case of IL-12 production (Cassatella *et al.*, 1995).

1.1.8 Tumor necrosis factor-α (TNF)

TNF is a serum factor that induces necrosis of sarcomas when passively transferred to tumor-bearing mice (Carswell *et al.,* 1975) and it can also kill tumor cells *in vitro* (Helson *et al.,* 1975). It is also referred to as cachectin as it has the ability to induce cachexia. TNF is a proinflammatory cytokine, which is primarily produced by macrophages, neutrophils and monocytes. Other cells are capable of producing TNF, although in smaller amounts; include T cells, fibroblasts, smooth muscle cells,

granulocytes, keratinocytes, neurons, mast cells, and natural killer cells.

TNF is synthesized as a membrane-bound homotrimer (mTNF) consisting of 26-kDa monomers. TACE (TNF-α converting enzyme) is also found in the membrane and cleaves the cytokine for release from the cell membrane as a soluble trimer (sTNF) of 17 kDa. TNF can signal both in the membrane-bound as well as in soluble form (Smookler *et al.,* 2006). Both mTNF and sTNF can bind either one of the two trimer TNF receptors known as TNFR1 (p55) and TNFR2 (p75). Although both TNFRs bind TNF, they differ in affinity for ligand, cytoplasmic tail arrangement, and signaling mechanisms (Figure 2) For instance, TNFR1 is constitutively expressed on all cell types, whereas TNFR2 is mostly inducible and characteristically expressed on endothelial and hematopoietic cells. Additionally, soluble TNF preferentially binds to TNFR1 over TNFR2 due to a 30-fold faster dissociation rate from TNFR2 than from TNFR1 (Grell *et al.*, 1998). At physiological levels (1 ng/mL), TNF enhances wound closure and stimulates proliferation through TNFR2, while pathological levels of TNF (100 ng/mL) inhibit migration and proliferation though TNFR1 (Kaiser *et al.,* 1997; Corredor *et al.,* 2003).

1.1.8.1 TNF signaling and secretion

TNF is a principal cytokine in mediating inflammatory responses and innate immunity. Regulated secretion of TNF is very important, as signaling of TNF is a double-edged sword. TNF is implicated in the pathogenesis of several diseases, including cancer, sepsis, rheumatoid arthritis, diabetes and inflammatory bowel disease (Wong *et al.,* 2008). Major pathways activated by TNF include caspases, NF-κB, and mitogen-activated protein kinases (MAP kinases). TNF signaling is mediated by adaptor proteins that bind to the cytoplasmic domain of the TNFRs. TNFR1 contains a death domain in its

cytoplasmic region that binds TNFR-associated death domain (TRADD) and couples the receptor either to the nuclear factor-κB (NF-**κ**B) activating pathway or to an apoptosis-inducing pathway. NF-**κ**B belongs to a family of transcription factors that regulate the transcription of a large number of inflammatory genes. Normally, NF-**κ**B activation suppresses the apoptosis pathway. Thus, if NF-**κ**B activation is compromised, apoptotic signaling through caspase-8 and caspase-3 becomes dominant through the TNFR1-mediated pathway (Van Antwerp *et al.,* 1998). Each innate immune cell appears to have a distinct mechanism for packaging and secretion of TNF (Stanley and Lacy, 2011).

1.1.8.2 TNF secretion from macrophages

Macrophages lack secretory granules that may be used for pre-storage of cytokines. Macrophages synthesize and transport newly made cytokines to the cell surface after activation (Murray *et al.,* 2005). In macrophages, TNF is trafficked to the cell surface by constitutive exocytosis (Low *et al.,* 2010). LPS induction increases the expression and synthesis of specific SNARE proteins and other trafficking regulators functioning throughout the TNF secretory pathway (Pagan *et al.,* 2003). In the *trans*-Golgi network (TGN), proteins are selectively packaged into distinct tubules for post-Golgi transport (Lock *et al.,* 2005). After transit through the Golgi stack, TNF-loaded carriers subsequently bud off the TGN and fuse with recycling endosomes (REs). The subsets of tubular carriers that transport TNF contain the Golgi carriers p230/golgin245. LPS activation significantly and selectively increases the number of p230-labeled tubules/vesicles emerging from the TGN (Lieu *et al.,* 2008).

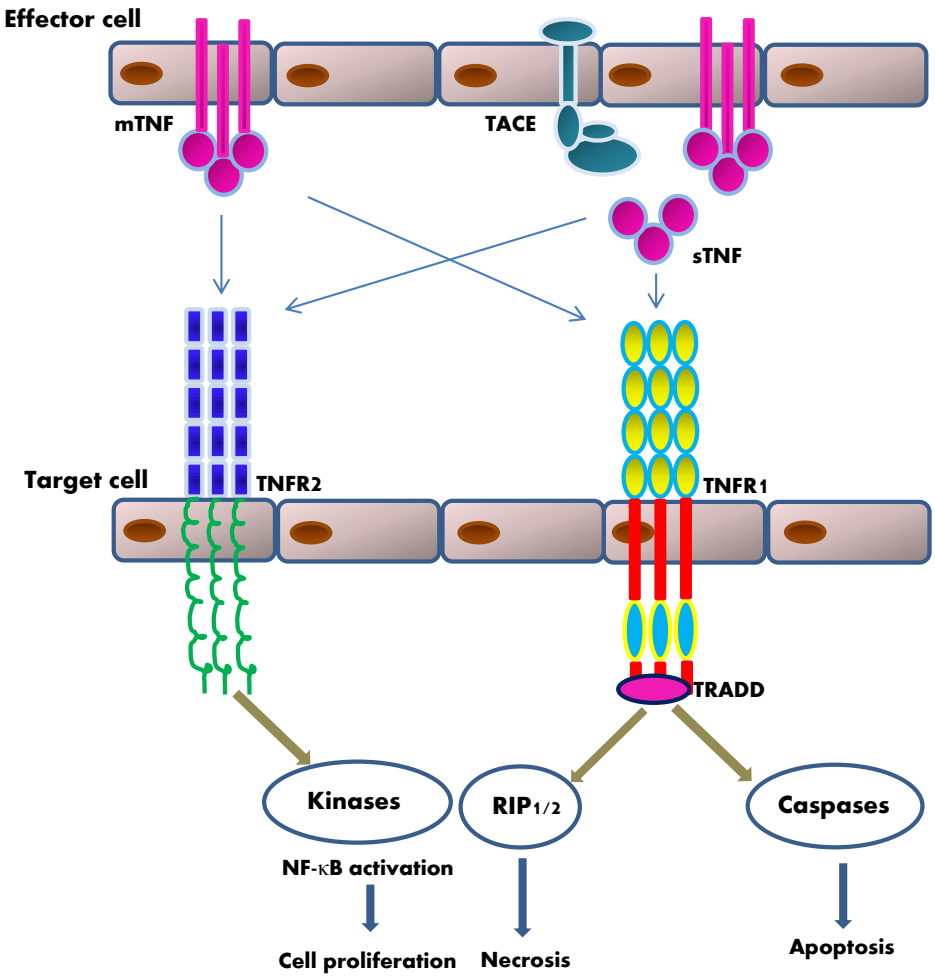


Figure 2: TNF and its receptors. TNF is produced as a 26 kDa membrane-associated protein (mTNF), which is cleaved and released as a soluble 17 kDa protein (sTNF) by membrane-bound TNF-converting enzyme (TACE). Both bound and soluble TNF signal as trimers through TNFR1 and TNFR2 to mediate cell apoptosis, activation, and/or proliferation involving nuclear factor-kappa B (NF-κB) activation (Source: Modified from Probert *et al.,* 2000).

The SNARE complex, Stx6/Stx7/Vti1b, is also present in the TGN-derived TNF transport carriers, which fuse with the R-SNARE VAMP-3 on REs (Murray *et al.,* 2005). Within REs, TNF is sorted into clusters by AP-1 and clathrin. Following AP-1 cleavage and dissociation from clathrin, TNF is transported to the cell surface for secretion in an ARF1-dependent manner (Braun *et al.,* 2007). The final fusion step is regulated by the R-SNARE, VAMP-3 on TNF-containing REs and the Q-SNARE complex, Stx4/ SNAP23, on plasma membrane (Murray *et al.,* 2005).

1.1.8.3 TNF secretion from eosinophils

Mature eosinophils contain specific or crystalloid granules, which store many cytokines as well as cationic proteins such as major basic protein (Lacy *et al.,* 2005). The proinflammatory cytokine TNF is found in these granules (Beil *et al.,* 1993). Cargo secretion from eosinophil granules involves piecemeal degranulation (Melo *et al.,* 2005) and the R-SNAREs, VAMP7 and VAMP8 are associated with these granules (Logan *et al.,* 2006). TNF release in eosinophils remains to be studied in terms of vesicle fusion at the cell surface.

1.1.8.4 TNF secretion from mast cells

Mast cells are known for their role as central effectors in allergic responses. They secrete proinflammatory mediators and cytokines in response to activation *via* high affinity IgE receptors (Amin *et al.,* 2012). Mast cells exhibit an immediate degranulation in response to activation and large numbers of granules may be discharged through compound exocytosis (Puri *et al.,* 2008). TNF is secreted by mast cells through different pathways, *via* secretory granules and by constitutive vesicle trafficking (Gordon *et al.,* 1991). Newly synthesized TNF is secreted *via* the constitutive pathway and occurs through

VAMP-3-mediated surface fusion (Tiwari *et al.,* 2008). TNF is also present in subsets of granules in mast cells as a preformed mediator, and stored TNF can be released from these secretory granules upon stimulation (Gordon *et al.,* 1991).

1.1.8.5 TNF secretion from T-lymphocytes and natural killer (NK) cells

T-lymphocytes and natural killer (NK) cells secrete newly synthesized TNF *via* constitutive pathways (Huse *et al.,* 2006). In T-lymphocytes, TNF colocalizes with some of the same trafficking machinery identified in macrophages, such as the Q-SNAREs Stx6 and Vti1b (Mori *et al.,* 2011). In NK cells, TNF is transported *via* Rab11 and transferrin receptor (TfnR)-positive REs that are regulated by VAMP-3 (Reefman *et al.,* 2010). Thus, TNF trafficking pathways from the Golgi complex to the cell surface *via* the RE are share common features across distinct immune cells.

1.1.8.6 TNF secretion from neutrophils

Neutrophils express and secrete a wide range of pro-inflammatory cytokines including including interleukin (IL)-6, IL-8, IL-12, transforming growth factor-α (TGF-α) and tumor necrosis factor-α (TNF), which are hypothesized to traffic through different types of secretory organelles (Stanley *et al.,* 2010). Earlier studies using transmission electron microscopy of human neutrophils suggest that neutrophils store TNF in their intracellular secretory organelles, with localization to electron-lucent granules (Beil *et al.,* 1995). TNF has been reported to localize to the nucleus and cytoplasm of human neutrophils, and cellular release of TNF from stimulated neutrophils was shown to be mediated by the nuclear export receptor CRM1-dependent nuclear export mechanism by using an inhibitor of CRMI (Miskolci *et al.,* 2006). However, the specific intracellular sites of TNF storage and how TNF is trafficked to the cell surface have not been determined in

neutrophils.

1.1.9 Pathogenesis and clinical importance of neutrophils

Neutrophils are key players in fighting invading pathogens and microorganisms, which can cause serious infections. The loss of neutrophils in number or of aspects of their function leaves individuals more susceptible to infections. Conditions such as inherited neutropenias, acquired autoimmune neutropenias, and neutropenias resulting from chemotherapy leave vulnerable patients at risk of serious life-threatening infections (Nathan *et al.,* 2006).

Neutrophils are also involved in chronic inflammatory diseases. Rheumatoid arthritis (RA) is known to be a predominantly T cell/macrophage driven process in early stages of the disease, but the cell with the greatest capacity to inflict damage within joints is the neutrophil (Edwards *et al.*, 1997). Neutrophils are numerous in the synovial fluid and joint tissues during the early stages of rheumatoid arthritis and during acute exacerbation of the disease (Mohr *et al.,* 1984). They contribute directly to cartilage damage, through serine and metalloproteases (Chatham *et al.,* 1993), as well as through production of ROS and chlorinated oxidants (Edwards *et al.,* 1997). Neutrophil-derived cytokines (IL-6, TNF, TGF-β and IL-8) are found in large quantities in synovial fluids (Kowanko *et al.,* 1990). A major role of TNF in the pathogenesis of RA has been demonstrated by a successful clinical trial using anti-TNF antibodies (Elliott *et al.,* 1994).

1.2 Cytokine secretion pathways

Cytokines and chemokines are soluble messengers that help to coordinate innate and adaptive immunity. The production and release of cytokines from innate immune cells is a fundamental response to injury and infection in the body. These cells collectively fulfill

an essential role in immunity by controlling the invasion of pathogens and recognize these through a diverse array of receptors, such as TLRs (Iwasaki *et al.,* 2010). LPS is a highly potent trigger of cytokine secretion through TLR4. Some of the most potent pro-inflammatory cytokines and chemokines released by innate immune cells include TNF, interleukin (IL)-6, IL-1β, and CCL5 (Fields *et al.,* 2007). For the immune system to function appropriately, the synthesis and release of cytokines must be highly regulated. Dysregulated cytokine secretion can give rise to many autoimmune diseases. It is thus increasingly important to understand how these chemical messengers are synthesized, trafficked and secreted. Multiple secretory pathways for cytokines have been characterized in innate immune cells.

1.2.1 Classical secretory pathways

1.2.1.1 Constitutive secretory pathway

Almost every cell in the body has a constitutive secretory pathway for the trafficking of extracellular proteins that contain signal peptide sequences (Dancourt *et al.,* 2010). Constitutive secretion involves the continuous release of newly made protein from a continuous stream of small volume carriers, whether in response to receptor stimulation or under basal conditions. Secretory proteins are transported in vesicles from the ER to the *cis*-Golgi and through successive cisternae of the Golgi stack for posttranslational modifications, such as glycosylation (Derby *et al.,* 2007). Post-Golgi transport to the cell surface can occur through direct vesicular transport from the TGN to the plasma membrane or *via* tubulo-vesicular carriers (De Matteis *et al.,* 2008). REs are transit hubs

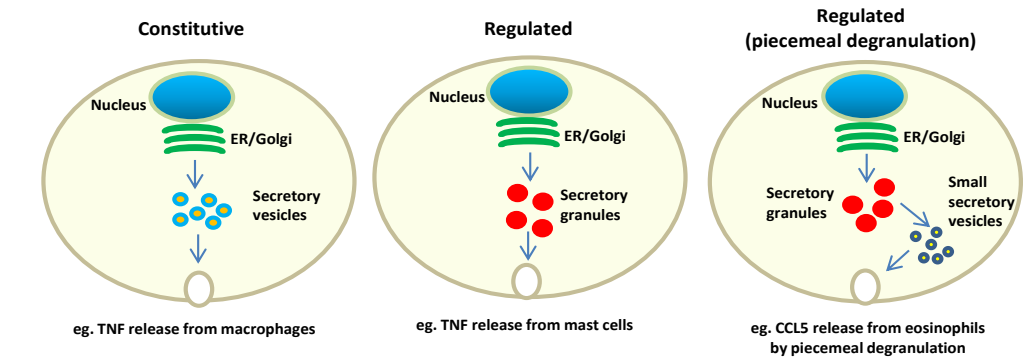


Figure 3: Different secretory pathways for release of cytokines The panels depicted classic secretory pathways. All cells have constitutive pathways, while specialized cell types (e.g. mast cells) additionally have regulated (granule-mediated) pathways and some cells have a variation of this process (e.g. piecemeal degranulation in eosinophils). (Source: Modified from Lacy *et al.,* 2011).

for endocytic and exocytic traffic moving to and from the cell surface and are an important compartment for the release of various cytokines (Murray *et al.,* 2005). Another exit pathway at the TGN is by the budding of clathrin-coated vesicles, which deliver cargo into the late endosomal/lysosomal network (De Matteis *et al.,* 2008) (Figures 3 and 4). Many cytokines are made as soluble precursors or mature proteins, while TNF is a transmembrane cytokine precursor. TNF is cleaved at the cell surface by TACE for the external release.

1.2.1.2 Regulated secretory pathway

In specialized secretory cells, such as granulocytes, there are additional, regulated secretory pathways that release cytokines (Burgoyne *et al.,* 2003). In regulated secretion, the cell has the ability to rapidly deliver a larger bolus of cytokine from storage sources to a specific cell site, in response to a specific signal. The combination of newly made and pre-packaged cytokines could be released to help to increase an inflammatory response. There are several modes by which cytokines may be released during regulated secretion (Burgoyne *et al.,* 2003). These are as follows: (i) in the direct-fusion model, granules fuse with the plasma membrane and release their contents outside the cells; (ii) kiss-and-run events involving the transient opening of a fusion pore for the escape of some or all of the granule contents without loss of granule integrity (Nemoto *et al.,* 2001); (iii) compound exocytosis, in which a population of granules fuse with each other prior to fusion with the cell membrane, and only one fuses with the plasma membrane to provide more focal secretion of mediators, for example, to kill parasites (Hafez *et al.,* 2003); and (iv) "piecemeal degranulation", a distinctive form of regulated exocytosis, where tubules bud off from the large granules to ferry their contents to the plasma membrane for release,

as seen in eosinophils (Melo *et al.,* 2010) and mast cells (Crivellato *et al.,* 2002).

1.2.2 Unconventional secretory pathways

Proteins lacking a signal peptide sequence are translated on free ribosomes in the cytoplasm and cannot be targeted to the endoplasmic reticulum (ER). Cytokines such as IL-1β, IL-15 and IL-18 lack the N-terminal signal sequence, required for ER entry, and thus may not be released by either constitutive or regulated exocytosis. A number of mechanistically distinct exit portals are known for trafficking of such proteins to the plasma membrane. These include the shuttling of soluble proteins directly from the cytoplasm into the extracellular milieu *via* membrane transporters; such as the ABC transporters (Zhou *et al.,* 2002). Cytokines can also be packaged in microvesicles derived from the plasma membrane and can be secreted as membrane-bound parcels (Bianco *et al.,* 2005). Moreover, autophagy, necrosis and cell lysis have also been implicated in unconventional secretion of some cytokines such as IL-1β. IL-1β can be secreted by exosomes, which are released when multivesicular bodies fuse with the plasma membrane (MacKenzie *et al.,* 2001). In addition, a cytolytic pathway of nonclassical granule release has been described in eosinophils that generate "cell-free" granules that can be detected in tissues of persons with allergies. Cytolysis is responsible for 33% of degranulation events during allergic reactions, aside from the predominant form of release through piecemeal degranulation (Erjefalt *et al.,* 1999).

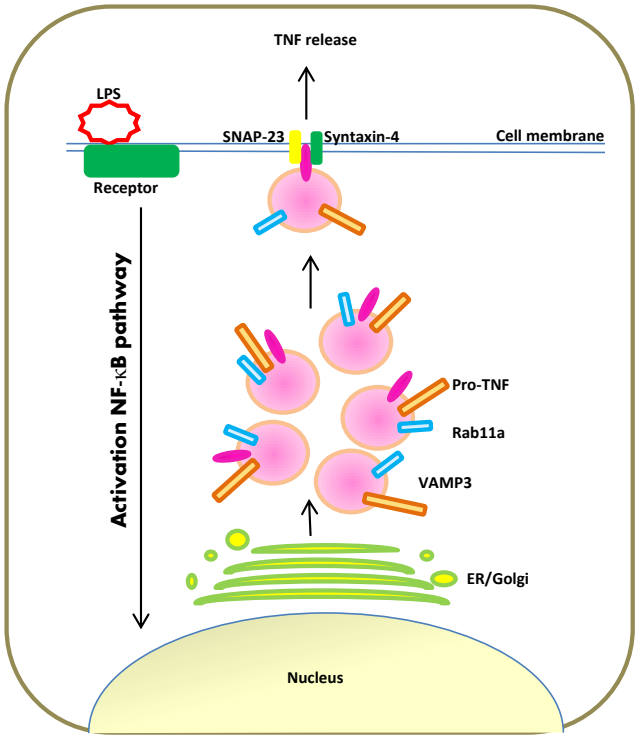


Figure 4. Constitutive exocytosis of cytokines. Representative pathway showing SNARE-mediated constitutive release of TNF in macrophages. Constitutive exocytosis occurs during stimulation by LPS, which leads to continuous trafficking of newly synthesized pro-TNF from the ER through the Golgi to recycling endosomes, and from there to the cell membrane. The R-SNARE, VAMP-3, is present on recycling endosomes and binds to the Q-SNAREs, SNAP-23 and syntaxin-4, in the cell membrane to fuse with recycling endosomes for subsequent TNF cleavage and release at the cell exterior. Rab11a is involved in directing SNARE binding at the cell membrane. (Source: Modified from Lacy *et al.,* 2011).

1.3 Traffic machinery

Movement of cargos through the trafficking pathways requires a complex array of cellular machinery, including membrane proteins and lipids as well as cytoplasmic proteins. There are three trafficking machineries, which are mainly involved in cytokine trafficking.

1.3.1 Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins

SNAREs are protein machinery that govern fusion between distinct membranes. They provide directionality to the fusion process (Risselada *et al.,* 2012). There are 38 members in the mammalian SNARE family. Most are ubiquitously expressed in many different cells (Hong *et al.,* 2005 and Stow *et al.,* 2006). Different types of vesicles and organelles possess distinct SNARE proteins capable of binding only their cognate SNARE partners on opposing membranes. Each SNARE has a region of amino acids that make up the SNARE motif. The SNARE motif contains a central arginine (R) or glutamine (Q) residue; on the basis of this amino acid difference, SNAREs are separated into R-SNAREs and Q-SNAREs, respectively (Südhof *et al.,* 2009). In exocytosis, R-SNAREs are typically found on granular or vesicular membranes, while Q-SNAREs are predominantly associated with plasma membranes, although there is some overlap between these groups.

1.3.2 Rab GTPase proteins

Members of the Rab family of small GTPases are distributed on membranes throughout eukaryotic cells where they participate in multiple stages of trafficking (Mizuno-Yamasaki *et al.,* 2012). Rabs interact with a variety of effectors to mediate vesicle

transport, tethering, docking and fusion, in their active GTP bound forms. Multiple Rabs are required for sequential transport steps along secretory pathways (Hutagalung *et al.,* 2011). Some Rabs are standard for trafficking of specific organelles, such as Rab5a on early endosomes, Rab7 on late endosomes, Rab6 on Golgi membranes and Rab11a on REs. Rapid REs are characterized by their expression of Rab5 and Rab35, while slow REs contain Rab10, Rab11, Rab14, and Rab22A (Grant *et al.,* 2009). Neutrophils have been shown to endocytose bacteria (*Anaplasma phagocytophilum*) containing vacuoles that have Rab10, Rab11a and Rab14 which are characteristic of slow REs (Huang *et al.,* 2010). Because of this specification, such Rabs are experimentally used as organelle markers and also denote specific trafficking steps (Zeigerer *et al.,* 2012). In immune cells, the expression and functions of individual Rabs can be up or down-regulated in response to different stimuli (Pei *et al.,* 2012).

1.3.3 Golgins

Golgins are a family of membrane associated, coiled-coil proteins that are typically found on sub-compartments of the Golgi complex. Their filament-like structures help them functions as membrane tethers or scaffolds (Goud *et al.,* 2010). GRIP-golgin, p230/golgin-245 and golgin-97 are found on the *trans*-Golgi network (TGN), while GRASP55 and golgin-45 are present in the middle Golgi, and GM130, p115 and GRASP65 are the *cis*-Golgi associated family members. Golgins interact with many proteins including microtubules and Rabs, to mediate trafficking through the Golgi complex (Short *et al.,* 2005). Golgins also play a fundamental role in maintaining the structure of the Golgi itself (Radulescu *et al.,* 2011) and depletion of the TGN GRIP-golgin, causes fragmentation of the Golgi ribbon (Derby *et al.,* 2007). TGN-based GRIP-

golgins and p230/golgin-245 have established roles in forming distinct tubule-vesicular carriers for TNF, IL-6 (Stanley *et al.,* 2012) and IL-10 (Lieu *et al.,* 2008) in LPS-activated macrophages.

1.4 Adaptor protein complexes

Adaptor protein (APs 1-5) complexes are major coat proteins in endocytic pathways. These belong to a family of five closely related protein complexes with key functions in vesicle trafficking, found in all five eukaryotic groups (Hirst *et al.,* 2011). These complexes participate both in the recognition of cargo and the formation of a carrier vesicle. They are positioned between clathrin and membranes (Unanue *et al.,* 1981, Vigers *et al.,* 1986) and provide a bridge between clathrin and transmembrane cargo proteins, hence called adaptor proteins (Pearse *et al.,* 1981). APs are heterotetramers, comprising two large subunits (~100 kD), one medium (~50 kD) and one small (~20 kD) subunit. The medium subunits of the AP complexes are involved in sorting signal recognition and the small one stabilizes the trunk structurally, while the two appendage domains are responsible for recruitment of accessory proteins. All APs have distinct subcellular localizations, and mediate different transport steps (Hirst *et al.,* 2012). AP-1 carries out transport between the TGN and endosomes, while AP-2 mediates rapid uptake from the plasma membrane so called clathrin-mediated endocytosis (Robinson *et al.,* 2001). AP-3 traffics cargo from tubular endosomes (REs) to late endosomes, lysosomes, and related organelles, while AP-4 traffic protein from the TGN to endosomes (Burgos *et al.,* 2010). AP-1 and AP-2, sort cargo proteins into clathrin-coated vesicles (CCVs) however, other APs are able to work without clathrin (Boehm *et al.,* 2001). AP-5 is the most recently identified AP complex, and it does not associate with clathrin (Hirst *et al.,*

2011). Transmembrane proteins often contain a sorting signal, which is a short amino acid stretch usually present in the cytoplasmic end of proteins that defines their localization in cells. Sorting signals for cargo selection into coated vesicles are usually in the form of short linear motifs. Three motifs for clathrin-mediated endocytosis have been identified: YXXΦ (where Φ is a bulky hydrophobic residue), [D/E]XXXL[L/I], DXXLL and FXNPXY, which interact with components of clathrin coats such as GGAs (Golgi-localized, γ-adaptin ear-containing, ARF-binding) or adaptor protein complexes. The most common motif is the tyrosine-based motif with the consensus sequence YxxΦ (Canfield *et al.,* 2001). This motif binds directly to the μ2 subunit of AP-2 and is found in proteins such as the epidermal growth factor (EGF) receptor, mannose 6-phosphate receptor and transferrin receptor. This motif can be also found in cytoplasmic region of type I, type II and multi-spanning membrane proteins. Moreover, it mediates targeting to the plasma membrane, lysosomes, lysosomes-related organelles such as melanosomes and antigen processing compartments (Mellman *et al.,* 2006). D/EXXXLL/I (where D, E, L, I and X are aspartate, glutamate, leucine, isoleucine and any amino acids respectively) (Bonificano *et al.,* 2003) this signal is recognized by AP complexes while the DXXLL signal is recognized by GGAs (Owen *et al.*, 2004). Di-leucine signals are found in the cytoplasmic tails of CD3 subunit of T cell receptor and also found in cytosolic proteins such as ubiquitin (Nakatsu *et al.,* 2000). FXNPXY signal present in the LDL receptor family and is recognized by ARH (autosomal recessive hypercholesterolemia) and Dab2 (Robinson *et al.,* 2004)

1.5 The endocytic pathway

The endosomal system contains a network of organelles that includes early endosomes,

REs, late endosomes and lysosomes. They are abundant in the cell and the number ranging from a few hundred and up to thousand (Steinman et al., 1976). These organelles differ in biochemical composition, cellular location and morphology (Perret *et al.,* 2005) and they interact with the cytoskeleton and microtubule motors (Soldati *et al.,* 2006). The endocytic pathway starts with the internalization of membrane proteins and lipids from the plasma membrane into primary endocytic vesicles, which fuse with each other or preexisting organelles to form early endosomes (EEs). From EEs, proteins are either sorted towards late endosomes (LEs)/lysosomes, returned to the plasma membrane or are transferred to REs.

1.5.1 Endosomal compartments

Internalized molecules will first appear in early endosomes that are mostly located at the cell periphery. Upon reaching the early endosomes, the molecule can be sorted into a pathway of recycling back to the cell surface, or it can be directed toward later endocytic compartments for degradation (Gruenberg *et al.,* 1995). Early endosomes have a pH around 6.0 and contain material directed for late endosomes, for example internalized epidermal growth factor receptor (Renfrew *et al.,* 1991). REs have a pH around 6.5 and contain proteins that are recycled to the cell surface, for example the transferrin receptor (Sonnichsen *et al.,* 2000). In late endosomes, the concentration of membrane bound ATP-driven proton pumps is higher than in early endosomes with more acidic environment of pH 5.0-6.0 (Tycko *et al.,* 1983). Lysosomes are the end point for many internalized components where they are finally degraded. Late endosomes and lysosomes are similar in many ways. They both have lysosomal glycoproteins for example lysosome-associated membrane proteins and lysosome integral membrane proteins. They both are

concentrated in perinuclear regions (Eskelinen *et al.,* 2003). However, there are differences that make late endosomes and lysosomes distinguishable. Late endosomes have Rab7 and Rab9 on the membranes (Feng *et al.,* 1995; Lombardi *et al.,* 1993), and have a high concentration of the mannose-6-phosphate receptor (M6PR) (Griffiths *et al.,* 1988) but lysosomes do not contain any of these molecules and are also slightly more acidic with pH 5.0-5.5 (Perret *et al.,* 2005).

1.5.2 Recycling endosomes

The transferrin receptor (TfR) serves as a major marker for REs. It is endocytosed upon binding of transferrin from the plasma membrane (PM) and reaches early endosomes (EEs) from where it is efficiently recycled either directly or *via* REs (Sheff *et al.,* 1999). Direct sorting from EEs to the plasma membrane reflects a fast method of recycling (Hao *et al.,* 2000), whereas sorting through REs is much slower (Mayor *et al.,* 1993). REs comprise a collection of vesiculo-tubular membrane structures that are often associated with microtubules (Yamashiro *et al.,* 1984). The subcellular localization of REs varies between different cell types. Most frequently, REs are found in a perinuclear location opposing elements of the Golgi complex TGN (Hopkins *et al.,* 1994). However, in some cells REs are distributed more widely throughout the cytoplasm (Lin *et al.,* 2002). REs can sort molecules to several distinct destinations and most of them are returned to the plasma membrane. The trafficking of molecules from REs depends on the formation of transport intermediates and is regulated by the small GTPase Rab11. Interestingly small GTPase are required for transport towards both the TGN and the plasma membrane (Wilcke *et al.,* 2000).

1.6 Vesicular transport

Vesicular transport is important for eukaryotic cells to carry soluble macromolecules, proteins as well as membrane components from one chemically distinct, membrane-enclosed compartment to another. Thereby, selective and precise cargos are maintained in the cells (Jamieson *et al.,* 1967). The movement of cargo between compartments occurs by small, membrane-enclosed carrier vesicles, which bud from a donor compartment (Palade *et al.,* 1975). The budding process is initiated by recruitment of vesicle coat proteins, which assemble around the donor membrane in the cytoplasm (Bonifacino *et al.,* 2004). Assembly of coat proteins is regulated by GTPases (Palmer *et al.,* 1993). During the transport to the target compartment, the coat proteins are removed from the vesicles and recycled. After reaching the target organelle, the uncoated vesicles tether and fuse with the target membrane and cargo is released into the acceptor compartment (Bonifacino *et al.,* 2004). Three major vesicle coats have been identified so far, COPI (Letourneur *et al.,* 1994), COPII (Barlowe *et al.,* 1994) and clathrin (Pearse *et al.,* 1975).

Proteins destined for the cell surface, lysosomes and transmembrane proteins, are assembled in the endoplasmic reticulum (ER) membrane (Redman *et al.,* 1966). The proteins exit the ER in COPII-coated carrier vesicles, budding at special sites for transport, called ER exit sites (ERES) (Hughes *et al.,* 2008). These vesicles are delivered either to interconnected ER-Golgi intermediate compartments (ERGIC) also known as vesicular tubular cluster (VTCs) (Ladinsky *et al.,* 1999) or directly to the cis-face of the Golgi, where membrane fusion takes place (Barlowe *et al.,* 1994). These ERGICs (or VTCs) are cargo-rich compartments mediating the transport of secretory cargo between the ER and the Golgi (Presley *et al.,* 1997). COPI-coated transport vesicles recycle

proteins from the Golgi to the ER (Griffiths *et al.,* 1995). These vesicles are also responsible for transport within the Golgi (Malhotra *et al.,* 1989). Clathrin-coated vesicles are involved in two major routes of the vesicular transport system: the transfer of molecules between the trans-Golgi network (TGN) and the endosome, as well as the transport between the plasma membrane and the early endosome (Friend *et al.,* 1967). Very little has been done on neutrophils and their release of cytokines.

1.7 Rationale of the study

Neutrophils are key effector cells during the initial inflammatory response, as they are among the first line of cellular defense and are rapidly recruited to the sites of injuries or infections. Despite the presence and secretion of TNF in neutrophils being well established, the specific details of TNF trafficking and release are still largely unstudied. Other cells including macrophages use REs to traffic TNF to the cell surface for release (Stanley et al., 2012). An important aim is to elucidate the trafficking of TNF in order to shed light on mechanisms that may be targets to prevent the release of TNF from inflammatory cells.

Neutrophils are packed with many secretory granules that may store and transport TNF. It is also anticipated that neutrophil possess REs but these secretory organelles have never been identified in neutrophils. Other studies suggest that macrophage utilizes REs for trafficking of TNF.

My study is therefore focused on determining intracellular sites of TNF storage, and role of REs in the trafficking of TNF in human neutrophils.

1.8 Working hypothesis

Recycling endosomes (REs) are specialized secretory compartments that perform multiple functions including receptor-mediated endocytosis and trafficking of cytokines to cell surfaces (Stanley et al., 2012). In light of this background knowledge and rationale, we hypothesize that:

- **Neutrophils traffic intracellular TNF to the cell surface upon LPS induction *via* recycling endosomes.**

1.9 Aims and objectives

1. To assess the release of newly synthesized and/or pre-stored TNF from secretory organelles upon LPS stimulation.

2. To determine whether TNF is stored as a preformed mediator in secretory organelles in resting neutrophils.

3. To assess the pattern and localization of TNF within neutrophil secretory organelles, and if it is released following *de novo* synthesis, upon LPS induction.

4. To determine (*in silico*) the potential sorting pathway of TNF and other granule cargo protein stored in granules of neutrophils.

1.10 Experimental design to achieve objectives

1. Treatment of neutrophils with LPS with or without the protein synthesis inhibitor cycloheximide.
2. Staining of unstimulated neutrophils with specific markers of different compartments (early endosomes and recycling endosomes) in neutrophils.

3. Staining of unstimulated neutrophils with specific markers of different compartments (early endosomes, recycling endosomes, primary and secondary granules) along with TNF.
4. Staining of LPS-stimulated neutrophils with specific markers of different compartments along with TNF.
5. Colocalization analysis between TNF and markers of different compartments for comparative analysis after LPS treatment in neutrophils.
6. Bioinformatic analysis of adaptor motifs in granule and cytokine protein sequences to determine the involvement of adaptors in sorting of TNF and other cargos to the granules.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Blood collection set

Blood was drawn by peripheral venipuncture using phlebotomy on healthy volunteers. Safety-lok butterfly needles with attached holders were used to collect blood in 10 ml glass tubes containing sodium heparin (BD, Vacutainer, Mississauga, ON, Canada).

2.1.2 Materials for neutrophil isolation

Dextran (cat. # 31389, Sigma-Aldrich, St. Louis, USA), 6% solution was prepared for sedimentation of blood contents in 50 ml conical centrifuge tubes (BD Falcon, Mississauga, ON, Canada**).** Ficoll-Paque (GE Healthcare, Uppsala, Sweden) was used for density gradient separation of neutrophils. Sterile water from Baxter Corporation (Mississauga, ON, Canada) was used for the lysis of RBC to obtain a pure neutrophil population. Lysis was terminated using culture media with 5 mM EDTA (Sigma-Aldrich).

2.1.3 Media, supplements and other chemical reagents

RPMI 1640 (cat. # 11875), fetal bovine serum (cat. # 16000-044), penicillin-streptomycin (cat. # 15140-122, 100X, 10000 U/ml) and L-glutamine (cat. # 25030-081, 100X, 200mM) were purchased from Gibco Life Technologies (Burlington, ON, Canada). Cycloheximide (cat. # C7698), lipopolysaccharide (cat. # L3024) of *E. coli*, bovine serum albumin (BSA, fraction V, cat. # A2934), Triton X-100 (cat. # T9284) and paraformaldehyde (cat. # P6148) were of highest quality purchased from Sigma-Aldrich. Tween-20 (cat. # P337) and sulfuric acid (cat. # SA212-4) were bought from Fisher Scientific (New Jersey, USA). Phosphate-buffered saline (cat. # SH30258-01, 10X) was bought from Hyclone (Thermo Scientific, South Logan, Utah).

2.1.4 Kits

Human TNF alpha ELISA ready-set-go kit (cat. # 88-7346) from eBioscience (San Diego, USA) was used to analyze peripheral blood neutrophils culture supernatants.

2.1.5 Glassware and plastics

Plastic pipettes (5, 10, 50 ml) as well as 96 well plates were purchased from corning costar (Sigma-Aldrich). Glass slides, round glass coverslips (0.13-0.16 mm thickness, #1 German borosilicate) and centrifuge tubes (15 and 50 ml) were bought from Fisher Scientific. For cell counting, C-Chip disposable hemocytometers were purchased from Fisher Scientific.

2.1.6 Antibodies and other fluorescent markers

Transferrin-Alexa 488 (cat. # T13342) and dextran-Alexa 488 (cat. # D7171) were purchased from Invitrogen (Oregon, USA). Rabbit polyclonal anti-rat VAMP-3 (cat. # 104103) and mouse monoclonal anti-human VAMP-3 (cat. # sc-136162) was obtained from Synaptic Systems (Goettingen, Germany) and Santa Cruz Biotechnology, Inc. (Dallas, Texas) respectively. Mouse monoclonal anti-human TNF (cat. # GF31) was purchased from Calbiochem (Ontario, Canada). Mouse monoclonal anti-human CD63-FITC (cat. # 312003) was purchased from AbD Serotec (Oxford, UK) and mouse monoclonal anti-human CD66b-FITC (cat. # 60086FI.1) was bought from StemCell Technologies (Vancouver, BC, Canada). Rabbit polyclonal anti-human Rab5 (cell signaling, cat. # 2143) and rabbit monoclonal anti-human Rab7 (abcam, cat. # a6137029) were generously provided by Dr. Thomas Simmen, Department of Cell Biology, University of Alberta. Secondary antibodies Alexa 488 conjugated to donkey anti-mouse IgG (cat. # 715-545-151), Cy3 conjugated to donkey anti-mouse IgG (cat. # 715-165-

151) and Cy3 conjugated to donkey anti-rabbit IgG (cat. # 711-165-152) were purchased from Jackson Immunoresearch Laboratories (Baltimore, USA). For nuclei counterstaining DAPI-containing mounting media, Prolong Gold (cat. # P36935) was purchased from Invitrogen.

2.1.7 Scientific Instruments

To capture images, a Deltavision OMX super resolution (Applied Precision, Mississauga, ON, Canada) microscope was used at University of Alberta. To read ELISA plates, a Powerwave HT microplate spectrophotometer (BioTek, Burlington, USA) was used. ELISA data were analyzed on GraphPad Prism, version 6. Images were processed by Softworx Suite 2.0 (Applied precision) and Fiji-ImageJ, version 1.49b (downloaded from http://fiji.sc/Fiji).

2.2 Methods

2.2.1 Neutrophil isolation from human peripheral blood

Human neutrophils were isolated from the peripheral blood of healthy donors in accordance with the University of Alberta Health Research Ethics Board requirements for the use of human samples **(**Mitchell *et al.,* 2008**).** Briefly, 60-80 ml of blood was collected from healthy volunteers in 10 ml glass vacutainer tubes containing sodium heparin. Blood was transferred to a 50 ml centrifuge tube containing 10 ml of 6% dextran diluted in RPMI and incubated for 30 min at room temperature for sedimentation. Out of two distinct and visible layers, red blood cells (RBCs) were in the lower layer and plasma-rich white blood cells were on the top layer. The upper leukocyte-rich suspension was collected and layered on 50 ml centrifuge tube containing 15 ml Ficoll for density gradient separation. The tube was then spun for 30 min at 400 *g* at room temperature in a

centrifuge with the brake off. After centrifugation, the top layer containing plasma, lymphocytes and monocytes was removed by using an aspiration pipette. The cell pellet, containing granulocytes and RBCs, was resuspended in 1.5 ml of cold sterile water for 20-30 sec to induce hypotonic lysis of red blood cells. Lysis was terminated by adding 12 ml buffer A (RPMI 1640 with 5 mM EDTA) and then the suspension was centrifuged at 300 *g* for 10 min at 4ºC. The supernatant was discarded and pellet-containing neutrophils was re-suspended immediately in 10 ml Buffer B (1640 RPMI with 2% fetal bovine serum). Cells were allowed to rest on ice for 1 h before experiments and counted using Kimura's stain. Typically, >97% pure and >95% viable neutrophil populations were obtained from normal donors.

2.2.2 LPS stimulation of neutrophil for TNF secretion

To examine the secretion of TNF, neutrophils (0.5 x 10^6 cells/ml) were treated with LPS (100 ng/ml) for 0, 2, 4, 6, 8 or 24 h with/without the protein translation inhibitor cycloheximide (10 μg/ml) in complete culture media (RPMI 1640, L-glutamine and penicillin-streptomycin). The cells were cultured at 37°C with 5% CO_2 in a humidified incubator. Supernatants from culture were harvested at a different time points as described above and spun at 300 *g* for 5 min at 4°C. Collected supernatants were snap frozen in liquid nitrogen and stored at -80°C until analyzed for TNF.

2.2.3 Multiplex analysis of cytokines

Stored supernatants (-80 °C) of stimulated neutrophils culture were sent to Eve Technologies, Inc. (Calgary, AB). Undiluted samples were used to quantify the production of 64 different human cytokines. The 64-plex assay allows the simultaneous measurement of the following proteins: 6Ckine, BCA-1, CTACK, EGF, ENA-78,

Eotaxin-2, Eotaxin-3, FGF-2, Fit-3L, Fractalkine, G-CSF, GM-CSF, GRO, I-309, IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17A, IL-20, IL-21, IL-23, IL-28a, IL-33, IP-10, LIF, MCP-1, MCP-2, MCP-3, MCP-4, MDC, MIP-1α, MIP-1β, MIP-1d, PDGF-AA, PDGF-AB/BB, RANTES, SDF-1 α+β, sCD40L, SCF, TARC, TGFα, TNFα, TNFβ, TPO, TRAIL, TSLP, VEGF. The multiplexing technology used by Eve Technology is based on color-coded polystyrene beads and analyzed by Bio-Plex 200, which includes a dual-laser and a flow-cytometry system.

2.2.4 Quantification of secreted soluble TNF from neutrophils by enzyme linked immunosorbent assay (ELISA)

To analyze secreted TNF from peripheral blood neutrophils culture supernatants, a human TNF ELISA kit (eBioscience) was used as per manufacturer's instructions. Briefly, 96-well plates were coated with 100 µl/well of capture antibody (diluted in Coating Buffer). Plates were sealed and incubated overnight at 4°C. Wells were washed three times with wash buffer (250 µl/well) containing 1X PBS+0.05% Tween-20 (PBST). Non-specific sites were blocked by 200 µl/well of Assay Diluent with incubation at room temperature for 1 h. Serial dilutions of TNF standards were prepared in Assay Diluent and 100 µl/well were added in appropriate wells (triplicates). The culture supernatants (100 µl/well) were used without any dilution to the appropriate wells and sealed plates were incubated overnight at 4°C for maximal sensitivity. Wells were thoroughly washed with PBST. Detection antibody (100 µl/well) was added and plates were incubated at room temperature for an hour. The washing step was repeated with PBST. For the enzymatic reaction, 100 µl/well of avidin-HRP (diluted in Assay Diluent) was added and

plate was incubated for 30 min at room temperature. Wells were thoroughly washed with PBST and then 100 µl/well substrate solution was added to the wells and plates were incubated for 15 min at room temperature. The reaction was terminated by the addition of 2N H_2SO_4 (50 µl/well) and the plate was read at 450 nm on a BioTek microplate spectrophotometer using KC4 software from the manufacturer.

2.2.5 Statistical analysis for ELISA

ELISA data were analyzed on GraphPad Prism software (versions 5 and 6). Data shown were the mean values of triplicates from three independent experiments. Two-way statistical analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test was used to evaluate the statistical significance between multiple groups. Results are presented as standard error of the mean (±SEM) and p value < 0.05 was considered statistically significant.

2.2.6 Slide preparation and immunolabeling

Human peripheral blood neutrophils (>97% pure and >95% viable) were resuspended in the serum-free RPMI media and 2 x 10^6 cells/well added into a Nunc 6-well plate containing ethanol-washed glass coverslips. Neutrophil-containing plates were incubated at 37 ºC for 30 min to allow cells to adhere to the coverslips. Adherent neutrophils on coverslips were then stimulated with 10 ng/ml lipopolysaccharide (LPS) for 1 h at 37 ºC. Cells were fixed with 4% paraformaldehyde for 40 min and then permeabilized by 0.1% Triton X-100 for 2 min. Non-specific protein binding sites were blocked by adding 0.5% bovine serum albumin (BSA) with PBS for 10 min at room temperature. Fixed neutrophils were stained with different primary and secondary antibodies, and nuclei were counterstained with DAPI (Figure 5).

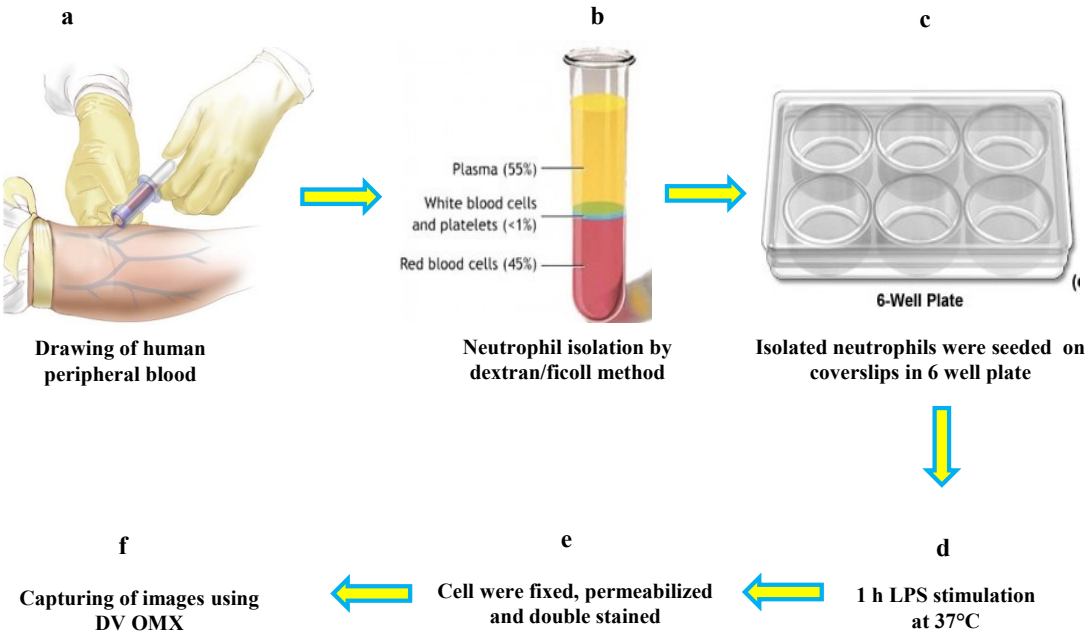


Figure 5: Scheme of sample preparation for image analysis on Deltavision OMX. Human peripheral blood was drawn by phlebotomy (a). Neutrophils were isolated from blood by the dextran/ficoll method (b). Purified neutrophils were adhered on a 6-well plate containing glass coverslips (c). Cells were stimulated with LPS for 1 h at 37°C (d). Adhered neutrophils were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (e). Images were captured on a DV OMX super resolution microscope (f).

Firstly, colocalization of TNF with transferrin and VAMP-3 (markers for REs) was performed and neutrophils were double-stained with anti-human TNF (0.5µg/ml) along with transferrin-Alexa 488 (10µg/ml) and anti-rat VAMP-3 (0.25µg/ml). Alexa 488 conjugated to anti-mouse IgG (0.5µg/ml), and Cy3 conjugated to anti-rabbit IgG (0.5µg/ml) were used as secondary antibodies to detect TNF and VAMP-3, respectively.

Further, colocalization of TNF was also performed with CD63 and CD66b, the specific membrane markers for primary and secondary granules, respectively. Neutrophils were double stained with anti-human CD63-FITC (0.25µg/ml) and anti-human CD66b-FITC (0.5µg/ml) respectively along with anti-human TNF. An additional secondary antibody, Cy3 conjugated to anti-mouse IgG (0.5µg/ml), was used to detect TNF when necessary.

We also determined the colocalization of Rab5 and Rab7 (markers for early and late endosomes, respectively) with TNF. The double immunolabeling of neutrophils was done with anti-human Rab5 (0.5µg/ml) and anti-human Rab7 (0.5µg/ml) along with anti-TNF. The secondary antibodies to detect TNF and Rabs were Alexa 488 conjugated to anti-mouse IgG, and Cy3 conjugated to anti-rabbit IgG respectively. Coverslips were thoroughly washed between successive incubations with 0.5% BSA in PBS. Nuclei were counterstained with DAPI containing mounting media.

2.2.7 Staining of neutrophils with endosomal compartment markers

To label REs and early endosomes, neutrophils were serum-starved in RPMI for 30 min and incubated with 10 µg/ml of fluorescently labelled, transferrin-Alexa 488 and dextran-Alexa 488 for 20 min, followed by a 10 min chase. Cells were then fixed with paraformaldehyde (4%) and nuclei were stained with DAPI.

2.2.8 Microscopic parameters

Images were captured by Deltavision OMX super-resolution microscope. The system was incorporated with multiple charged-coupled device (CCD) cameras (Applied Precision), one for each fluorescent dye bandwidth, and an inverted, widefield deconvolution fluorescence microscope (Olympus). This custom-built microscope was equipped with a plan-apochromatic oil objective lens (Olympus) with 60X magnification and 1.42 numerical aperture.

Slides containing coverslips were placed over the objective, and cells were selected for imaging on the basis of their quality of staining, intact cellular membranes, nuclear morphology with standard multilobular shapes. All images were collected in z-stacks of varying numbers of slices (each slice 0.125 μm apart) of the entire cell, acquired in a unilateral direction and xyz plane. Images were acquired using the following settings: 60X lens, with image size 512-512 pixels, 16-bit image depth with an intensity range of 0 to 65535. The exposure time range from 0.02 to 0.08 sec for three different channels (DAPI, FITC and Cy3).

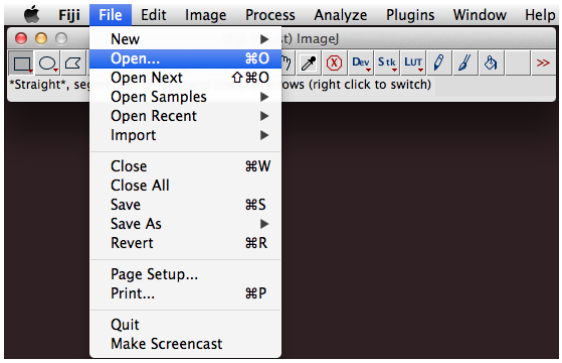
Optical sections were acquired using the attached Applied Precision motorized stage. Images were deconvolved by Softworx Suite 2.0 (Applied Precision) to remove out-of-focus fluorescence. TIFF files of images were compiled for figures using Adobe Photoshop CS5 (version 12.0.4).

2.2.9 Colocalization analysis on Fiji

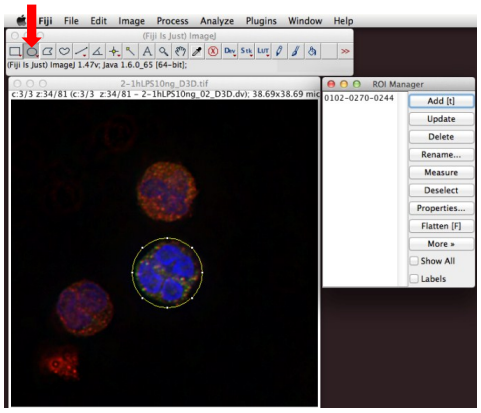
Fiji-ImageJ (version 1.49b) was used for colocalization analysis of the images collected from the Deltavision OMX. The following multistep process was used in order to achieve Pearson's correlation coefficient values for double-stained specimens. After opening Fiji,

clicking “File-Open” opened the image data, and cells were brought into focus by scrolling through z sections. An appropriate cell along with a z section was selected and focused. The circle button from Fiji-toolbar was selected to mark a region of interest (ROI) over the cell. To nudge the selection over the cell, arrow keys on the keyboard were used. The number assigned to the selection was saved for further analysis to the ROI Manager, which appeared by pushing Ctrl-T button from the keyboard. Next, the "Image-Color-Split Channels" menu command was used to get a separate z stack for the three dyes, BGR (blue, green and red). The DAPI (blue) channel window was closed, as colocalization analysis was needed for red and green channels. This was a very important step as we were only interested in the correlation between two channels (green and red), where the proteins of interest are located. The colocalization analysis was done by going to the "Analyze-Colocalization-Coloc 2" in menu tab. In the Coloc 2 window, the channels were checked (to show C2 and C3 for red and green, respectively), ROI Manager selected under “ROI or mask”, and all options were checked. In addition, the Costes’ Significance Test was checked for showing a point-spread function (PSF) of 3.0 and 10 randomisations before clicking OK. The progress may be monitored at the top of the window in the Fiji toolbar. Results were saved as PDF files, which show 2D intensity histograms for correlation of pixel intensities as a way of visualizing correlations. The results also contained different correlation values: Pearson’s R values (no, below, and above threshold), Manders M1 and M2 (no threshold/threshold), and other values. We chose Pearson’s values (R) above the threshold for all analyses, as the use of Manders generated very high correlation coefficients that did not allow comparisons with treatment effects (Figure 6).

a



b



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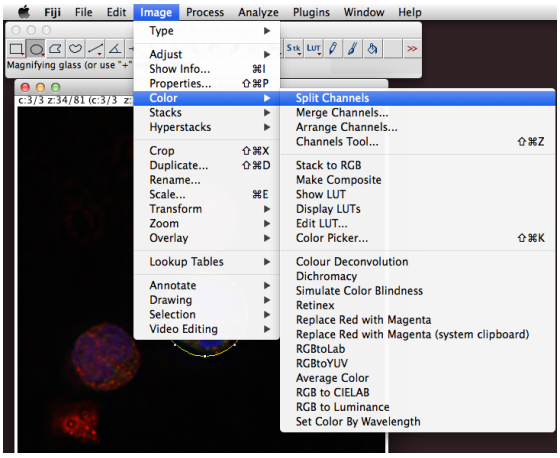
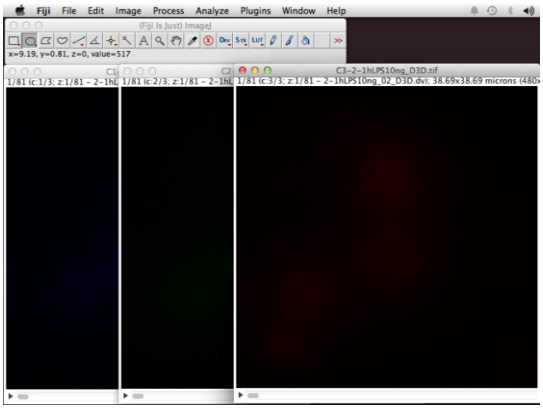


Figure 6 (a-c): Schematic of colocalization analysis using Fiji-ImageJ. Images were opened by using “File-open” plugins in Fiji (a). To analyze only region of interest (ROI) in the images, selection tools on the ImageJ was selected (red arrow) and ROI was marked (b). To get a separate z stack for the 3 dyes the "Image-Color-Split Channels" menu command was used (c).

d



e

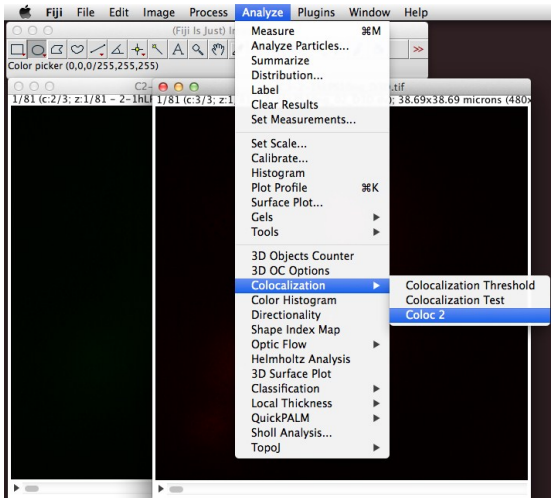


Figure 6 (d-e): Schematic of colocalization analysis using Fiji-ImageJ. File C1, z stack for blue dye (nuclei staining) was closed (d). Coloc 2 plugin from the menu item was launched by using Analyze-Colocalization Analysis-Coloc 2 (e).

2.2.10 Statistical Analysis for colocalization

All data was analyzed on GraphPad Prism software, and shown as mean Pearson's correlations values from ten cells. The Student's *t* test was used to evaluate statistical significance between two groups, and a *p* value of less than 0.05 was considered statistical significant.

Chapter 3

Results

3.1 Neutrophils produce and secrete a variety of cytokines upon LPS stimulation

Circulating peripheral blood polymorphonuclear neutrophils were once considered to be terminally differentiated cells that cannot synthesize newly translated proteins as mature cells. In 1988, however Jack *et al.* showed that circulating mature neutrophils are capable of *de novo* synthesis of mRNA and proteins, and since then, many other studies have shown that neutrophils upon stimulation can synthesize and secrete various cytokines such as IL-1β (Tiku, *et al.,* 1986), TNF (Dubravec *et al.,* 1990), IFN-γ, IL-4, IL-10, IL-13 (Romagnani *et al.*, 1994), IL-6, IL-8, IL-12, TGF-β, IFN-α, MIP-1α/β and VEGF (Jaillon *et al.,* 2013). It is not understood which cytokines are stored and which are *de novo* synthesized upon stimulation. Thus, it is very important to know which cytokines, including Th1 (pro-inflammatory), and Th2 (anti-inflammatory) cytokines, chemokines (MCP1-3), and growth factors (erythropoietin, EGF) stored and/or synthesized in neutrophils and secreted by LPS stimulation. LPS activates the NF-κB pathway through TLR4 and induces the synthesis of cytokines in neutrophils, which can be detected in culture supernatants.

To investigate this, we determined the effect of LPS stimulation on cytokine release from neutrophils. Human peripheral blood neutrophils (> 95% pure, > 98% viable) were divided in two groups. In the first group, neutrophils were treated with LPS at multiple time points (0, 2, 4, 6, 8 and 24 h) and their supernatants examined for cytokines that were secreted. The second group was treated for 15 min with cycloheximide (10 μg/ml), an inhibitor of *de novo* protein synthesis and subsequently with 100 ng/ml LPS (CHX+LPS). Cycloheximide blocks the synthesis of new proteins, and upon LPS stimulation, only stored cytokines may be secreted from the cells. Culture

supernatants from three independent experiments (singlet sample) were sent for multiplex cytokine analysis at the Eve Technology Corporation, Calgary, Canada. Color-coded polystyrene beads were used in multiplex human cytokine array to perform 64-plex-cytokine assays on culture supernatants.

We observed that out of 64 cytokines analyzed, 26 cytokines (BCA, 6Ckine, CTACK, ENA-78, Eotaxin-2, GM-CSF, GRO, IL-5, IL-8, IL-9, IL-10, IL-15, IL-17A, IL-18, MCP-1, MCP-2, MCP-3, TGF-α, TNF, TRAIL, PDGF-AA, PDGF-BB, RANTES, sCD40L and VEGF) were released into culture supernatants upon LPS stimulation in neutrophils. On the basis of secretion levels, we divided these cytokines into four secretion groups: high (30-2800 pg/ml), moderate (7-30 pg/ml), low (0.5-10 pg/ml), and negligible (0-1.5 pg/ml).

Figure 7a shows highly secreted cytokines shown in order of most abundant to least amount of release (Table 1). Among these cytokines, the secretion levels of IL-8 (2583 pg/ml) and MCP-1 (186 pg/ml) at 24 h were significantly higher ($p < 0.01$) in LPS-stimulated cells than in CHX+LPS treated cells. The levels of RANTES (CCL5) and eotaxin-2 (2356 pg/ml and 84 pg/ml, respectively) were also significantly higher ($p < 0.05$) in LPS- stimulated samples compared to CHX+LPS at 24 h. On the other hand, the levels of other highly secreted cytokines such as GRO, PDGF-BB, IL-16 and sCD40L were not significantly different between LPS and CHX+LPS treatments.

Highly secreted (30-2800 pg/ml)	Moderately secreted (10-30 pg/ml)	Low secreted (0.5-10 pg/ml)	Negligibly secreted (0-1.5 pg/ml)	Out of range (<OOR)
Eotaxin-2 GRO IL-8 IL-15 MCP-1 PDGF-BB RANTES sCD40L	ENA-78 IL-20 TNF TRAIL PDGF-AA VEGF	6Ckine Flt-3L GM-CSF IL-10 MCP-2 MCP-3 TGF-β	BCA CTACK IL-5 IL-7 IL-9 IL-17A	EGF Eotaxin-3 FGF-2 Fractalkine G-CSF I-309 IFNα2 IFNγ IL-1α IL-1β IL-1ra IL-2 IL-3 IL-4 IL-6 IL-12 (p40+p70) IL-13 IL-16 IL-21 IL-23 IL-28a IL-33 IP-10 LIF MCP-4 MDC MIP-1α MIP-1β MIP-1d SDF-1 α+β SCF TARC TGFα TPO TSLP

Table 1. Multiplex detection of cytokines in supernatants of LPS-stimulated neutrophils. Out of range (OOR) indicates cytokines that were too low to be detected by multiplex analysis.

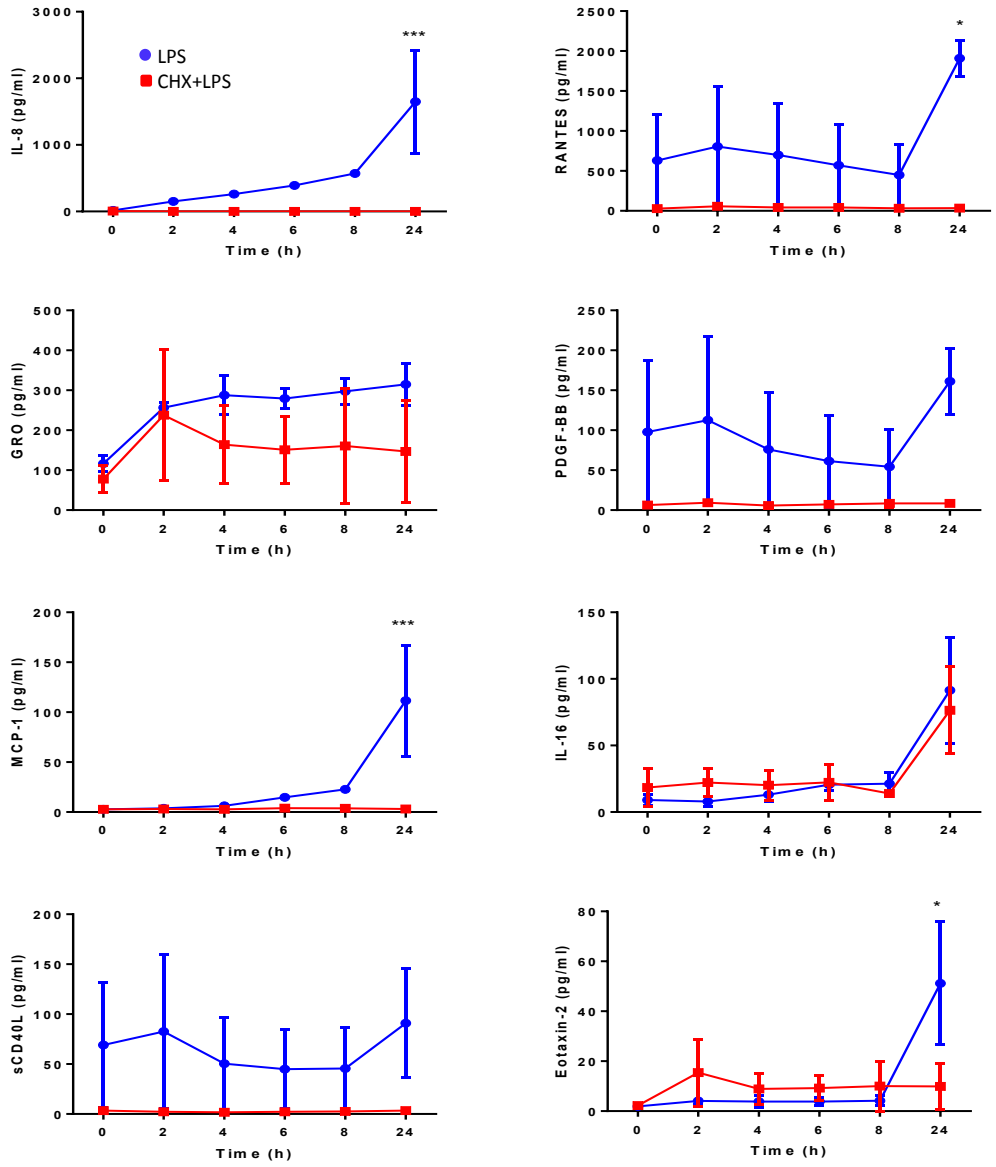


Figure 7a: Multiplex cytokine analysis of culture supernatants from LPS-treated neutrophils showing highly secreted cytokines. Neutrophils (0.5 x 10^6 cells/ml) were treated with 100 ng/ml LPS and/or the protein synthesis inhibitor, cycloheximide (10 µg/ml, pretreatment for 15 min and then LPS added) for 0, 2, 4, 6, 8 or 24 h. Eight highly secreted cytokines were detected in culture supernatants. Data were analyzed and compared by two-way ANOVA followed by Bonferroni's post-hoc analyses. * $p < 0.05$, *** $p < 0.01$ between LPS and CHX+LPS values at the same time point, $n = 3$.

Figure 7b shows the moderately secreted cytokines (with the levels ranging from 7-30 pg/ml): ENA-78, IL-20, TNF, TRAIL, PDGF-AA, and VEGF. Out of six moderately secreted cytokines, TNF was significantly higher in LPS-treated samples than in CHX+LPS stimulated cells and at 2 h significance was $p < 0.005$ and at 4, 6, 8 and 24 h the significantly difference was higher ($p < 0.001$). Additionally, the level of PDGF-AA was significantly higher at 24 h ($p < 0.05$) in LPS-induced samples compared with LPS+CHX. Conversely, levels of other moderately secreted cytokines, such as ENA-78, VGEF, IL-20 and TRAIL, were equivalent in both treatments.

Cytokines secreted at low levels, such as 6Ckine, Flt-3L, GM-CSF, MCP-2, MCP-3 and TGF-β were secreted at very small amounts (ranging from 0.5-10 pg/ml) with no significant differences between the two treatments (Figure 7c). However, IL-10 was significantly higher in LPS-treated samples at 8 and 24 h ($p < 0.05$) compared with CHX+LPS treatment.

The final group of cytokines detected was secreted at negligible levels, such as BCA, CTACK, IL-5, IL-7, IL-9 and IL-17A ranging from 0.20-1.5 pg/ml over the same period of time, with no significant differences found between the two treatments (Figure 7d).

The remaining 39 cytokines tested in this assay (EGF, Eotaxin-3, FGF-2, Fractalkine, G-CSF, I-309, IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-16, IL-20, IL-21, IL-23, IL-28a, IL-33, IP-10, LIF, MCP-4, MDC, MIP-1α, MIP-1β, MIP-1d, SDF-1 α+β, sCD40L, SCF, TARC, TGFα, TPO and TSLP) were below the detection limit of the assay and therefore were not included in the analysis (data not shown). However, many of these cytokines have

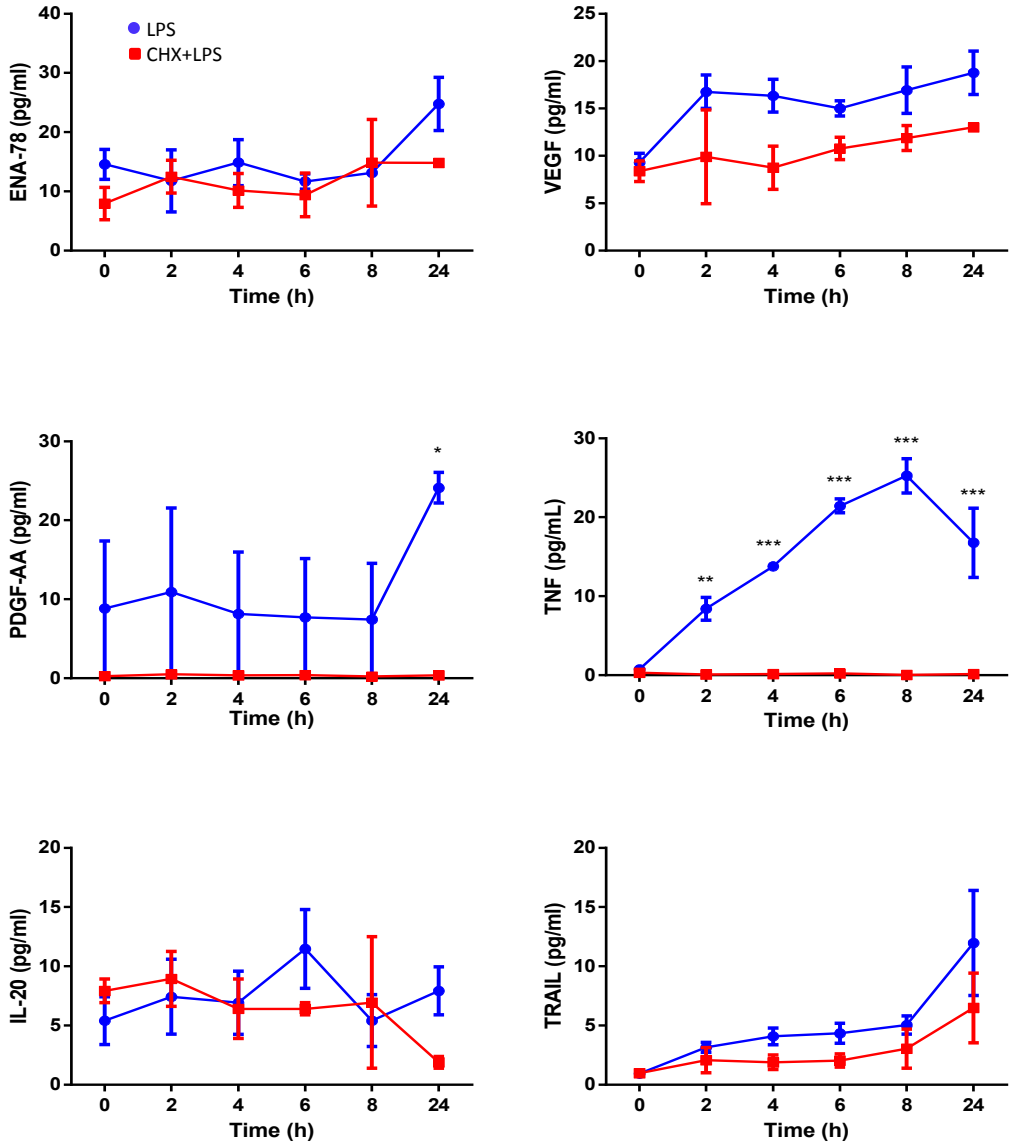


Figure 7b: Multiplex cytokine analysis of culture supernatants from LPS-treated neutrophils showing moderately secreted cytokines. Cytokines were detected by multiplex analysis. Data were analyzed and compared by two-way ANOVA followed by Bonferroni's post-hoc analyses. * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.001$ between LPS and CHX+LPS values at the same time point, $n = 3$.

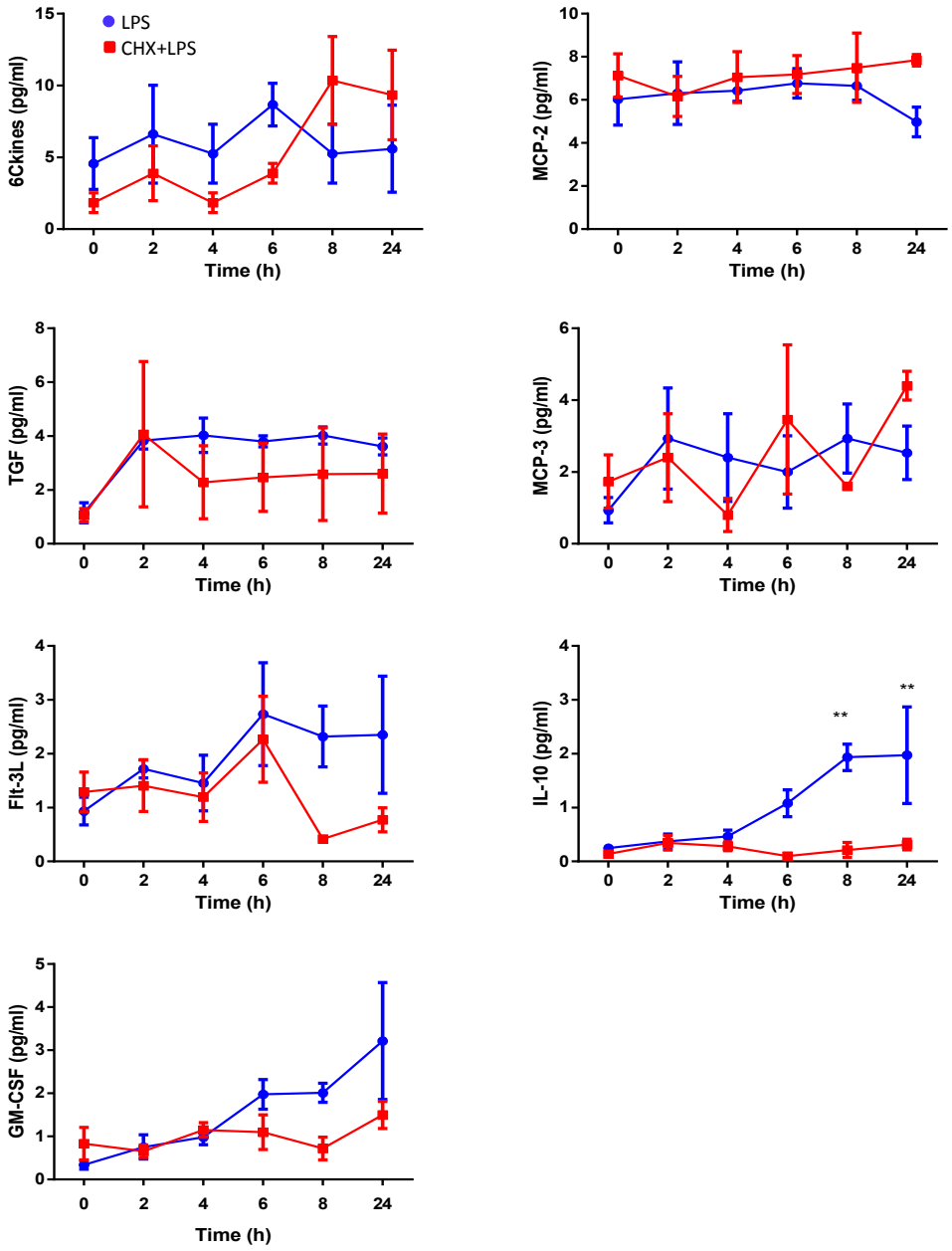


Figure 7c: Multiplex cytokine analysis of culture supernatants from LPS-treated neutrophils showing low cytokine secretion. Cytokines were detected by multiplex analysis. Data were analyzed and compared by two-way ANOVA followed by Bonferroni's post-hoc analyses. ** $p < 0.005$ between LPS and CHX+LPS values at the same time point, $n = 3$.

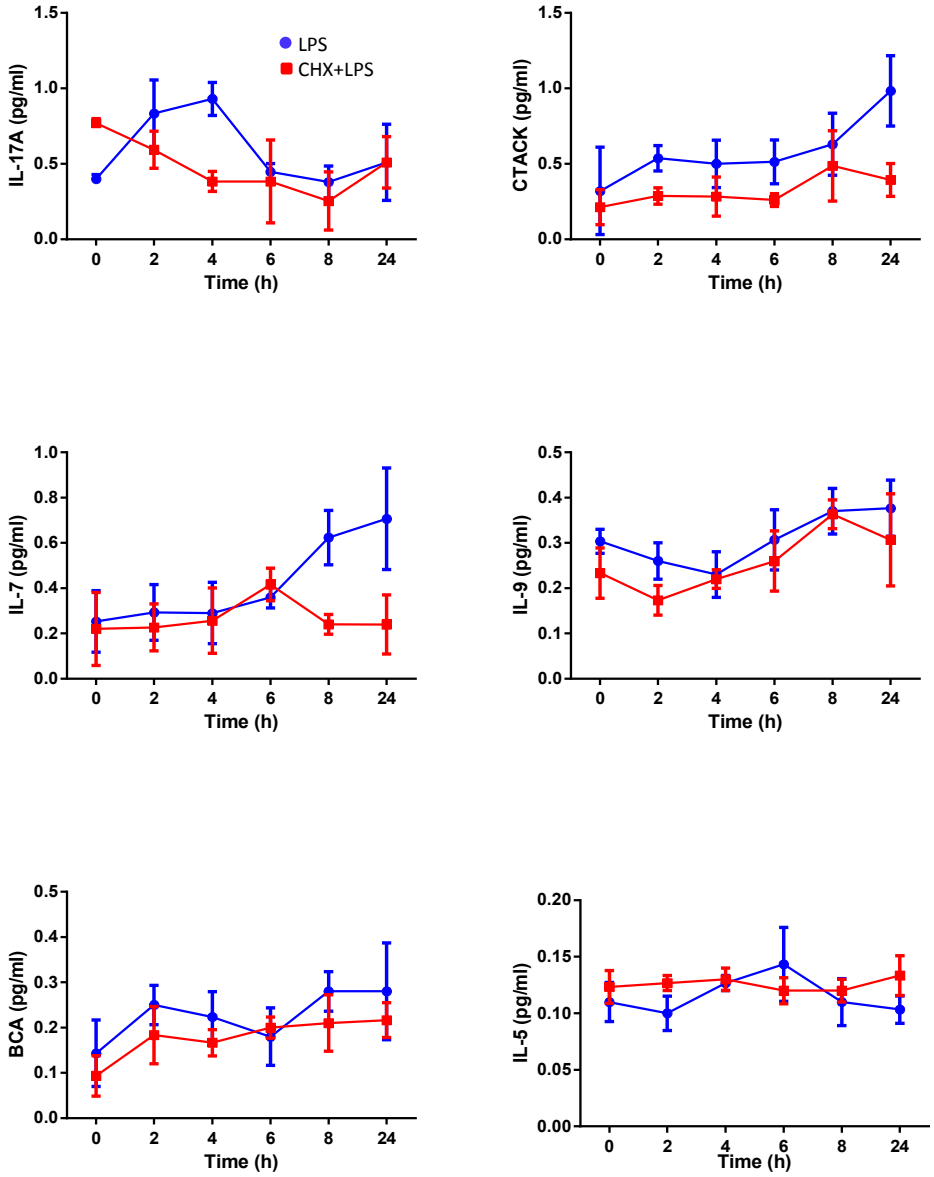


Figure 7d: Multiplex cytokine analysis of culture supernatants from LPS-treated neutrophils showing negligible cytokine secretion. Cytokines were detected by multiplex analysis. Data were analyzed and compared by two-way ANOVA followed by Bonferroni's post-hoc analyses. No significant differences were observed between LPS and CHX+LPS treatments, $n = 3$.

been shown to release by neutrophils in earlier studies, for example, IL-1β. The reasons for why these cytokines were not detectable in culture supernatants may be because they were below the limit of detection by this multiplex assay, which appear to be less sensitive than specific ELISA-based assays at least for TNF as discussed below.

The multiplex analysis data suggests that terminally differentiated neutrophils can express and secrete a variety of cytokines upon LPS stimulation. Some cytokine, such as eotaxin-2, IL-8 and TNF appeared to be stored as preformed mediator in intracellular compartments for immediate release upon LPS stimulation, as these were secreted into culture supernatants after *de novo* synthesis inhibited by cycloheximide.

3.2 Secretion of newly synthesized and stored TNF from neutrophils upon LPS stimulation

The multiplex cytokine analysis showed that TNF secretion was significantly higher in LPS-stimulated neutrophils compared to that of CHX+LPS treatment. TNF is one of the most important products released by neutrophils, and its dysregulated secretion may give rise to many acute and chronic inflammatory conditions, such as those that occur in rheumatoid arthritis and cancer. Therefore, it is important to understand how TNF is stored, trafficked and secreted in neutrophils.

To further investigate the pattern of TNF release from neutrophils upon LPS-stimulation, more detailed kinetics were analyzed using a specific human TNF ELISA. Normal neutrophils were cultured with LPS for 0, 2, 4, 6, 8 and 24 h at 37°C for TNF levels assessment. Figure 8a shows that within the first 2 h of LPS stimulation, neutrophils released detectable levels of TNF. The level of secreted TNF increased over

the course of time, and peak induction was observed at 24 h. The secretion levels of TNF ranged from 10 to 300 pg/ml.

To investigate whether TNF may be stored in neutrophils and released upon LPS induction, or released only as a *de novo* synthesized protein, cells were first treated with a protein synthesis inhibitor, cycloheximide, to inhibit *de novo* synthesis of proteins, and were then stimulated with LPS. As Figure 8a, reveals that protein synthesis was inhibited and secretion decreased significantly with cycloheximide treatment at 8 h ($p < 0.01$) and 24 h ($p < 0.001$).

To determine the proportion of newly synthesized and stored TNF that is released from neutrophils, we calculated the percentage of TNF released by dividing the secreted amount by total amount of TNF present in an equivalent number of lysed cells. Figure 8b shows that the secretion of TNF was reduced by 60% with cycloheximide treatment, suggesting that at least ~ 40% of TNF was secreted from stored sources. We concluded that it was possible that LPS induced the release of both newly synthesized as well as stored TNF from neutrophils.

The levels of TNF secretion determined by Eve Technologies (Figure 7b) were much lower (~10 fold) than analysis performed using the specific TNF ELISA kit (Figure 8) in both the treatments (LPS and CHX+LPS). This discrepancy could be due to the lower sensitivity of the multiplex cytokine analysis compared to individual cytokine-specific ELISAs.

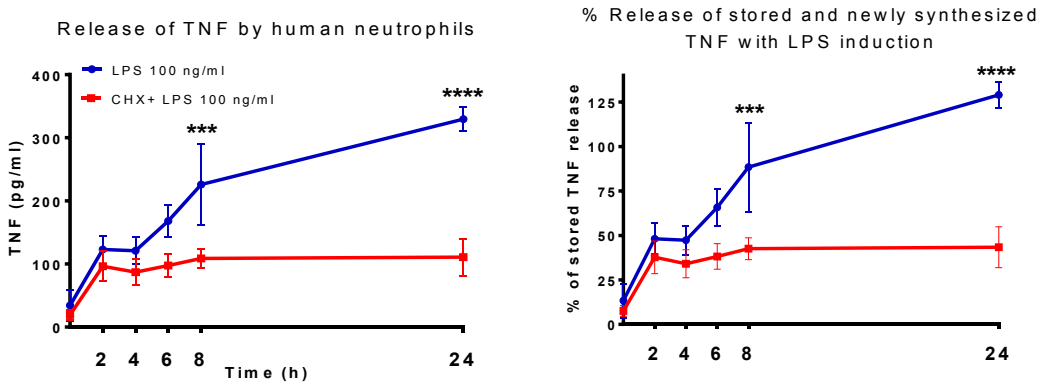


Figure 8: LPS-induced TNF release from human neutrophils. Neutrophils (0.5 x 10^6 cells/ml) were purified from human peripheral blood and treated with LPS (100 ng/ml) for 0, 2, 4, 6, 8 or 24 h in the presence or absence of the protein synthesis inhibitor cycloheximide (10 µg/ml). The graph at the right indicates the percentage of stored TNF that was determined by dividing the supernatant concentration into the lysed cell concentration of TNF at the start of incubation (time = 0). LPS induced TNF secretion, with 30-40% from stored sources, and the remainder newly synthesized (n = 3), ***p<0.001, **** p<0.0001.

3.3 Endosomal compartments, recycling endosomes and early endosomes localized within neutrophils

We know that LPS-activated macrophages traffic *de novo* synthesized TNF to the cell surface by transportation *via* REs, a process that has not been studied in neutrophils. Since neutrophils secrete *de novo* synthesized TNF based on our experiment using cycloheximide, we hypothesized that REs are also involved in trafficking of TNF in neutrophils. Although it is anticipated that neutrophils have endosomal compartments such as early endosomes (EEs) and REs (REs), the presence of these compartments and their role in TNF trafficking in neutrophils have never been studied. Thus, it is very important to identify and characterize these compartments in neutrophils.

To investigate this, we first located endosomal compartments (REs and EEs) in neutrophils by performing transferrin and dextran uptake as markers for REs and EEs, respectively. Human neutrophils were adhered to glass coverslips and incubated with fluorescently labeled transferrin, which binds to transferrin receptors that are present on the cell surface and endocytosed by early endosomes. As Figure 9a shows, incubation of neutrophils with transferrin-Alexa 488 for 20 min resulted punctate staining of transferrin in the cytoplasm of cells, indicative of endocytosis of transferrin and presence of REs. To further confirm the presence of REs, immunostaining of neutrophils was done with anti-VAMP-3, another marker for REs. We obtained a similar pattern of labeling as we found in transferrin (Figure 9b).

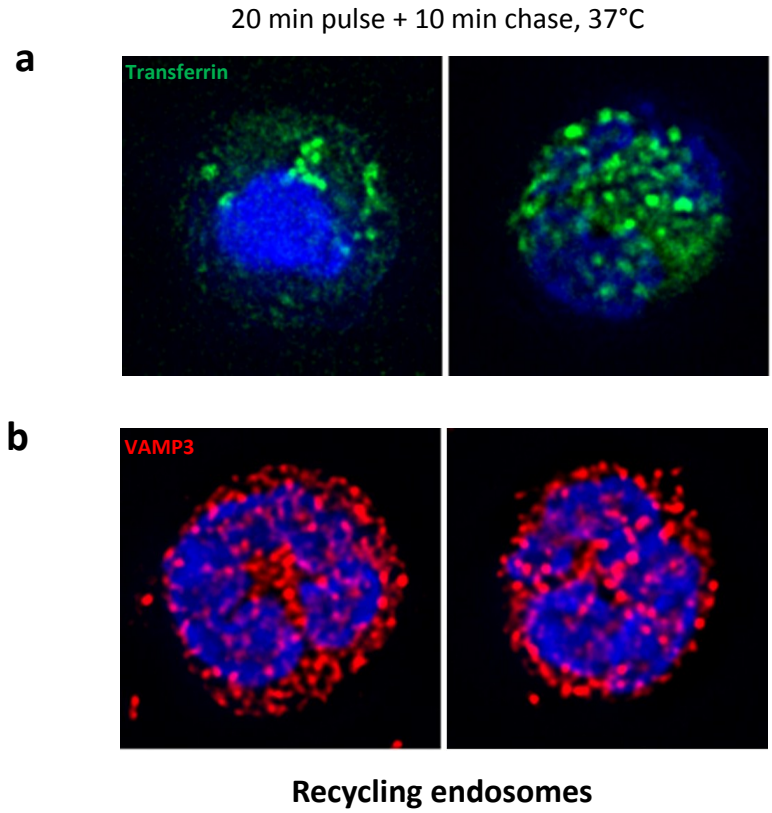


Figure 9: Detection of recycling endosomes in neutrophils. Neutrophils were incubated with transferrin-Alexa 488 for 20 min and chased for 10 min with fresh media, resulting in cells exhibiting a punctate staining pattern throughout the cytoplasm (a). A similar pattern was obtained with anti-VAMP3, another marker for REs (b). Blue stain indicates nuclei labeled with DAPI nuclear stain in this and all following images.

Concurrently, the localization of EEs in neutrophils was also evaluated on the basis of the uptake of dextran, a marker for EEs. Cells were pulsed with dextran-Alexa 488 (10 μg/ml) for 20 min at 37°C. It was observed that the staining pattern obtained with dextran-Alexa 488 was quite distinct from that of transferrin-Alexa 488 and that it predominantly accumulated in organelles near the cell membrane (Figure 10a). The localization of EEs in neutrophils was further confirmed by obtaining a similar pattern of labeling with anti-Rab5, another marker for EEs (Figure 10b).

We concluded that human peripheral blood neutrophils contain different endosomal compartments such as REs and EEs.

3.4 TNF colocalizes with different intracellular compartments in resting neutrophils

To determine whether TNF is stored in granules and/or other compartments of resting neutrophils, cells were immunolabeled with TNF along with markers of granules and endosomal organelles. We used CD63 as a marker for primary granules (Cham *et al.,* 1994) that are enriched in myeloperoxidase and elastase, and CD66b, which is marker for secondary/ tertiary granules containing lactoferrin and MMP-9 (reviewed by Faurschou *et al.,* 2003). In addition we examined Rab5 and Rab7 distribution as markers of EEs and LEs, respectively. Colocalization of TNF was performed with transferrin-Alexa 488 and anti-VAMP-3 (markers for RE); anti-CD63 and anti-CD66b and Rab5 and Rab7. We found that TNF colocalized with transferrin (Figure 11a), VAMP-3 (Figure 11b), CD63 (Figure 11c), CD66b (Figure 12a), Rab5 (Figure 12b), and Rab7 (Figure 12c), in unstimulated neutrophils. Staining pattern for TNF revealed a punctate pattern of distribution. This was indicated by the combination of red and green fluorescence resulting in yellow color.

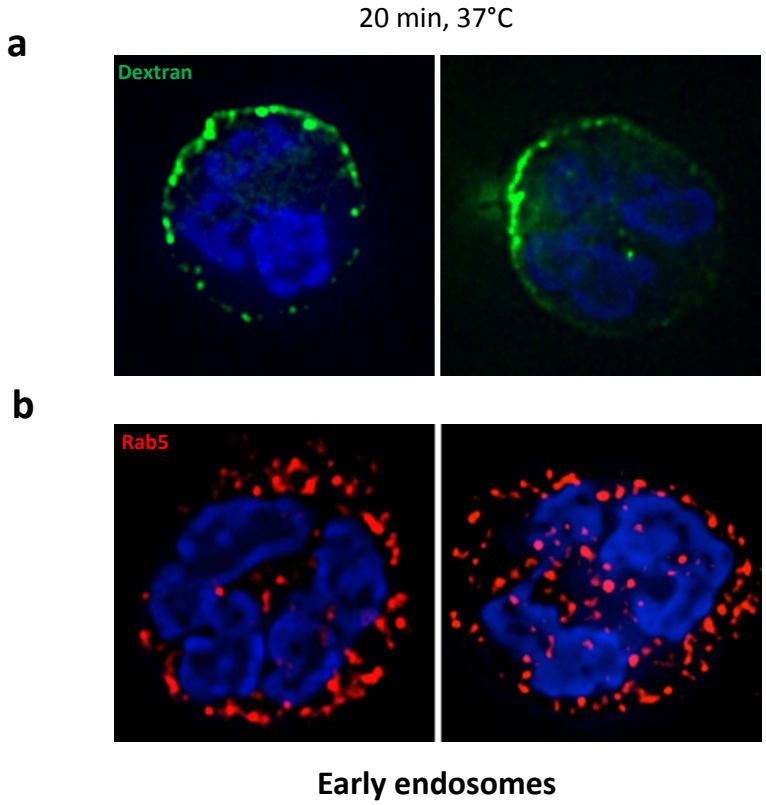


Figure 10: Detection of early endosomes in neutrophils. Neutrophils were incubated with dextran-Alexa 488 for 20 min and chased for 10 min with fresh media, resulting in cells exhibiting a punctate staining pattern near the cell periphery (a). A similar pattern was obtained with anti-Rab5, another marker for EEs (b). The staining patterns obtained with dextran-Alexa 488 and anti-Rab5 showed organelle accumulation around the cell periphery.

Quantification of colocalization was done using Fiji-ImageJ software, and Pearson's correlation coefficient (PCC) of at least 10 entire cells per staining condition was obtained. Colocalization was determined from 20-30 section for each z-stack obtained. PCC (mean of 10 cells) for colocalization of RE markers, transferrin and VAMP-3, with TNF were 0.56 and 0.70, respectively. TNF was also present in the primary and secondary granules of resting neutrophils, and PCCs were 0.77 and 0.80 for TNF with CD63 and CD66b, respectively. Early and late endosomes also contained TNF with PCCs of TNF with Rab5 and Rab7 being 0.63 and 0.57, respectively. This shows that unstimulated neutrophils indeed store TNF at intracellular sites, suggested that TNF is packaged as a preformed mediator that may be released immediately upon LPS stimulation, in confirmation of my previous finding using cycloheximide.

These observations suggest that neutrophils express TNF and store this as a pre-formed mediator in granules as well as other intracellular organelles including recycling endosomes.

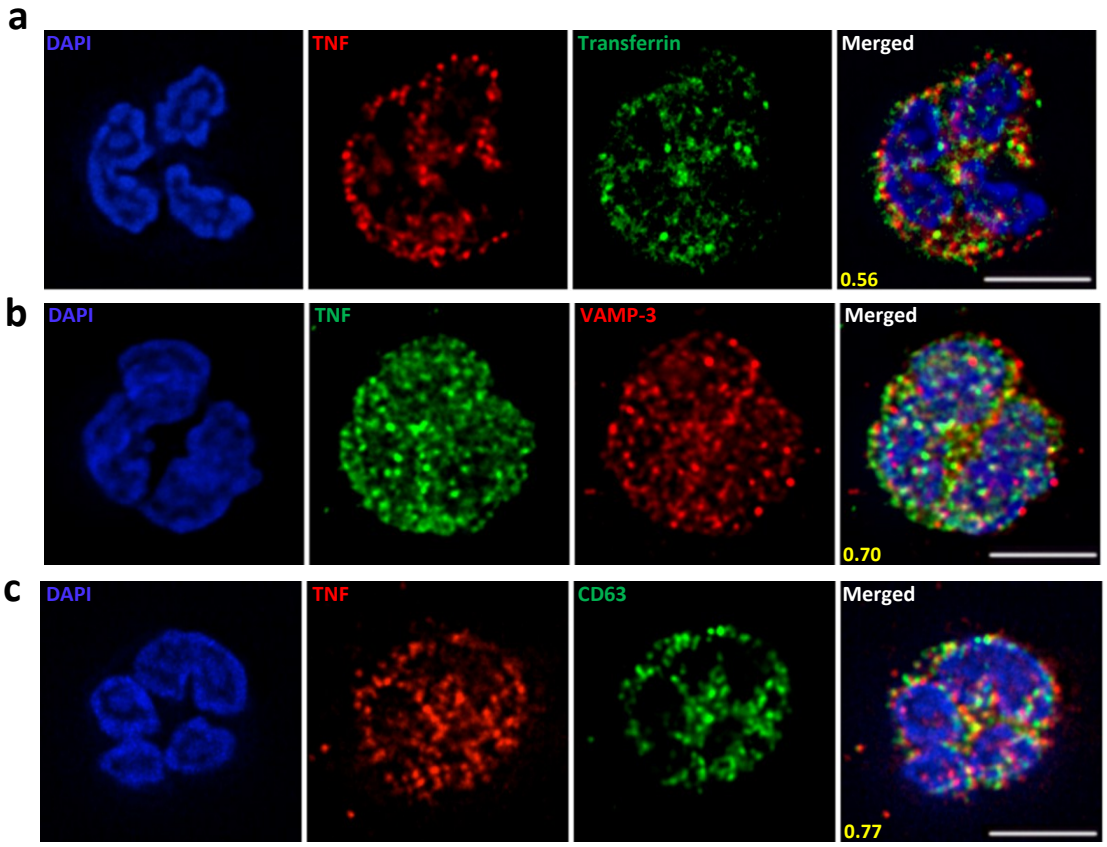


Figure 11: Distribution of TNF in REs and primary granules in resting neutrophils. Human peripheral blood neutrophils were fixed with 4% paraformaldehyde and labeled with the RE marker transferrin (conjugated with Alexa-488), or (a) antibody to VAMP-3, which also labels REs, or (b) antibody to the primary granule membrane marker, CD63 (c). In unstimulated neutrophils partial colocalization was observed as yellow punctate staining of TNF with transferrin, VAMP-3, and CD63 in merged images. Scale bar, 5 μm.

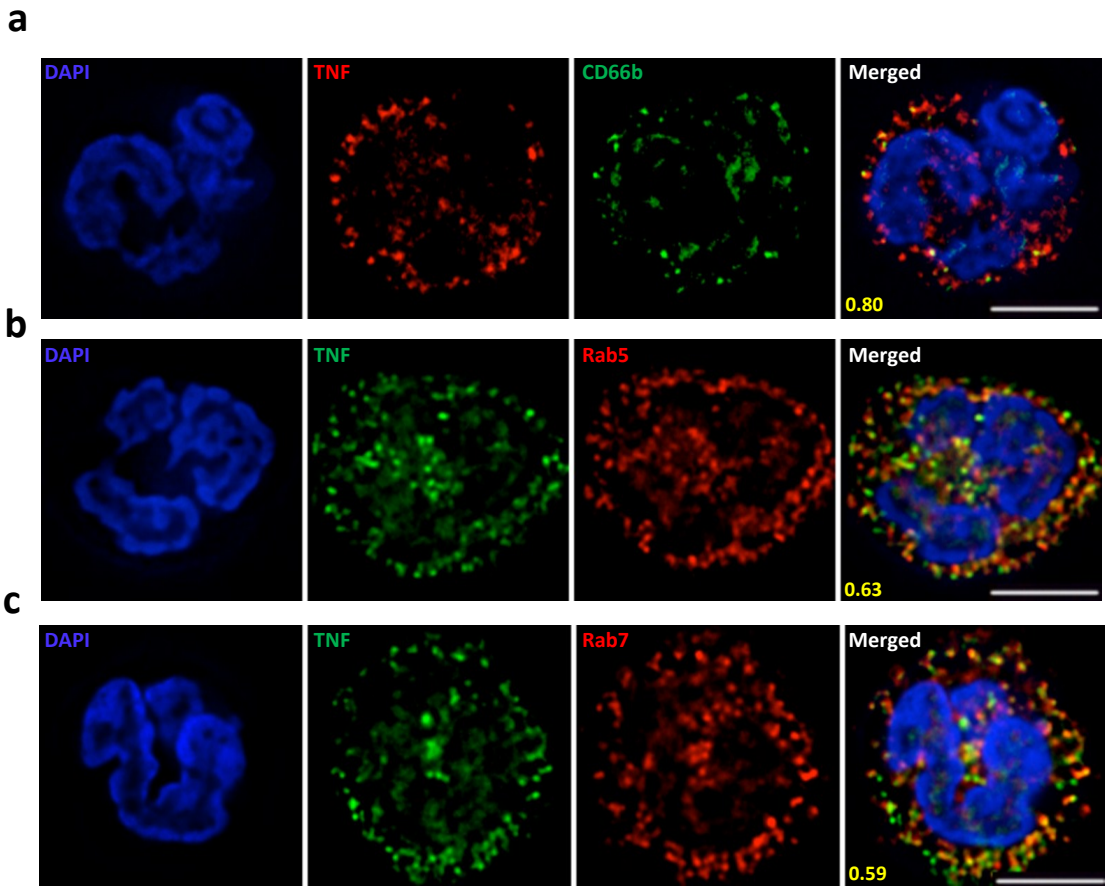


Figure 12: Distribution of TNF in secondary granules and early endosomes in resting neutrophils. Human peripheral blood neutrophils were fixed with 4% paraformaldehyde and labeled with antibodies against the secondary granule membrane marker, CD66b (a), the early endosome marker, Rab5 (b), or the late endosome marker Rab7 (c). Partial colocalization of TNF with CD66b, Rab5, and Rab7 was observed as regions of yellow. Scale bar, 5 μm.

3.5 Colocalization analysis of markers of two different intracellular compartments in resting neutrophils

Neutrophils contain three types of granules (approximately 200 in number) along with secretory vesicles and various endosomal compartments (e.g. early, late and recycling endosomes, among others) (Zucker-Franklin *et al.,* 1988). In our results, we provide evidence that TNF is present in various compartments of human neutrophils. TNF colocalized to varying degrees with VAMP-3 and transferrin uptake (markers of recycling endosomes), Rab5 and Rab7 (markers of early and late endosomes), and also with CD63 and CD66b (markers of primary and secondary granules). It may be possible that colocalization between these specific markers for different compartments is because of overlapping of these compartments in the tightly packed cytoplasm of neutrophil. Thus, it would be very interesting to know whether colocalization patterns of each pair of specific markers is actual or because of overlapping due to excessive crowding inside the neutrophil. To investigate this possibility, colocalization patterns of markers of two different compartments were assessed. Cells were double immunolabeled with VAMP-3 and a validated marker of early endosomes, Rab5. Staining patterns for these two markers revealed a punctate pattern of distribution. Quantification of colocalization was done using Fiji-ImageJ software, and PCC (mean of 10 cells) for colocalization of RE marker VAMP-3 and EE marker Rab5 was 0.27 (Figure 13a). These observations suggest that the baseline PCC is 0.27 for two compartments that do not normally colocalize in cells.

3.6 Colocalization analysis of markers of the same intracellular compartment in resting neutrophils

It is also interesting to determine the PCC value of two markers from same predicted intracellular compartment in neutrophil. To investigate this, neutrophils were adhered to glass coverslips and incubated with fluorescently labeled transferrin for 20 min, resulting in a punctate cytoplasmic stain, which indicates the presence of REs. The same cells were further immunostained with anti-VAMP-3, a second marker for REs.

In contrast to the findings in the previous experiment, there was comparatively higher colocalization of VAMP-3 and transferrin uptake. The degree of colocalization was quantified by Fiji-ImageJ software, and the resulting PCC was 0.64 (Figure 13b). This suggests that VAMP-3 and transferrin colocalize to a similar compartment with a higher PCC than that of VAMP-3 and Rab5. In addition, this observation suggests that transferrin uptake in neutrophils may not be a reliable indicator of REs, as transferrin may be present in multiple compartments after being endocytosed *via* early endosomes. This may explain the lower than expected PCC value (0.64) for two markers of the same compartment.

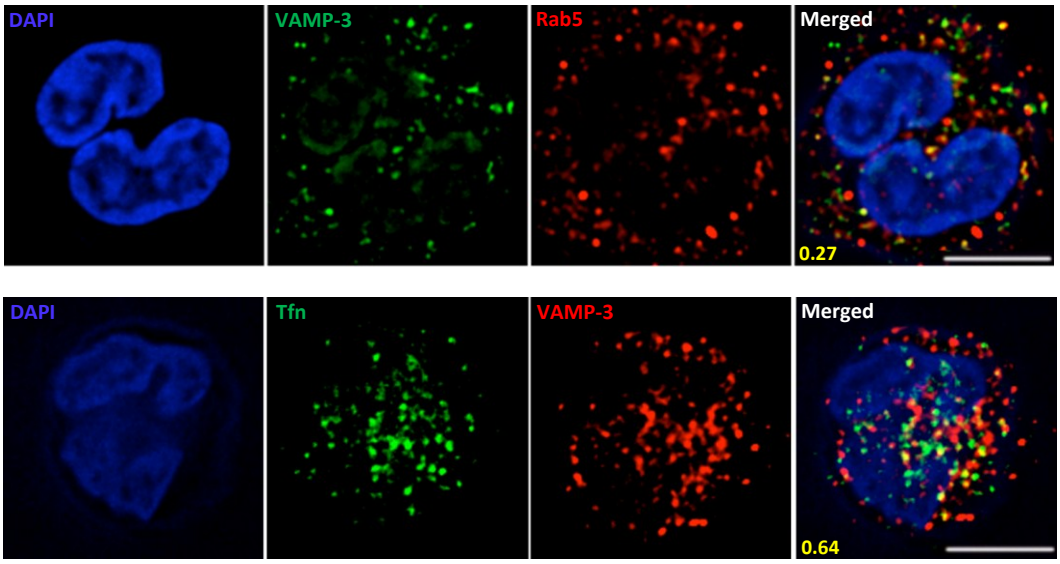


Figure 13: Colocalization study between markers of intracellular compartments in resting neutrophils. Human peripheral blood neutrophils were fixed with 4% paraformaldehyde and double labeled with antibodies against the recycling endosomes marker, VAMP-3 and the early endosome marker, Rab5 (a). The study was also performed between two recycling endosome, markers, transferrin and VAMP-3 (b). Partial colocalization was observed as regions of yellow. Scale bar, 5 μm.

3.7 LPS induces trafficking of TNF to the plasma membrane of neutrophils *via* recycling endosomes

Activated neutrophils secrete an array of proinflammatory cytokines, including TNF, which are temporally secreted for sequential roles in inflammation. In LPS-stimulated macrophages, newly synthesized TNF is transported to an intermediate compartment, the recycling endosome, before delivery to the plasma membrane for secretion (Pagan *et al.,* 2003); however, to our knowledge, nothing is known about intracellular trafficking of TNF in neutrophils. Therefore, it is important to determine whether TNF in neutrophils is also secreted *via* the same pathway, and if it relies similar intracellular carriers to REs. In our previous experiments, we noted that REs possessed TNF in unstimulated neutrophils. To further determine whether REs may be associated with LPS-stimulated trafficking of TNF in neutrophils, cells were stimulated with 10 ng/ml LPS for 1 h, and then fixed with 4% paraformaldehyde. LPS-stimulated cells were stained for intracellular TNF along with transferrin (conjugated to Alexa 488), a marker of REs. In Figure 13a, the left panel shows double-stained unstimulated neutrophils, while the right panel of the figure depicts LPS-stimulated neutrophils. The extensive yellow punctate staining reveals that there is partial colocalization between TNF and transferrin-Alexa488 in unstimulated cells, which decreased significantly after 1 h LPS stimulation. Quantitative assessment of changes in colocalization of TNF with transferrin-Alexa 488 upon LPS stimulation was performed using PCC analysis on 10 cells/condition. This analysis also revealed that PCC was significantly decreased ($p < 0.01$) between control conditions (PCC = 0.56) and LPS stimulation (0.35). However, we also observed overall

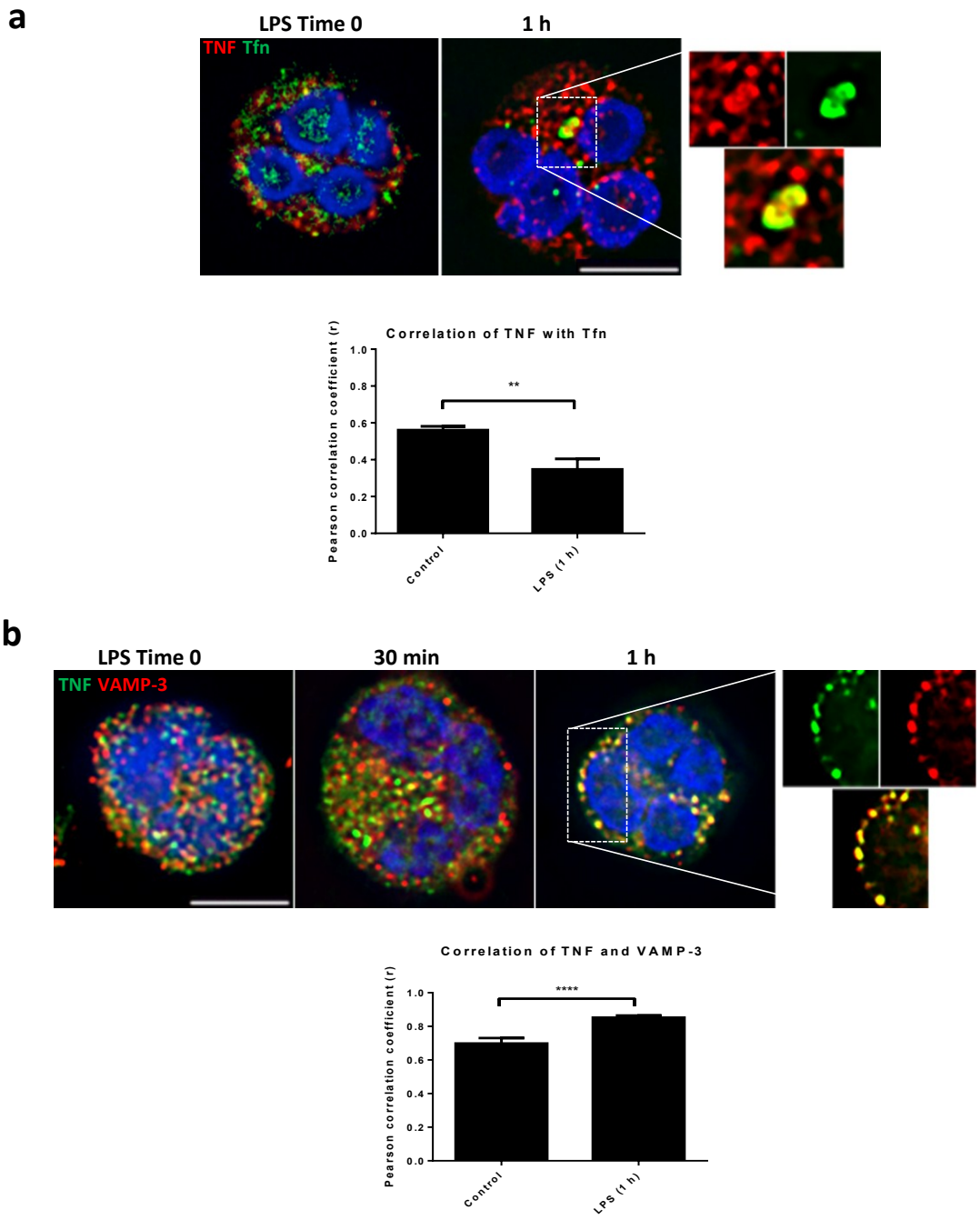


Figure 14: Effects of LPS stimulation on TNF trafficking via REs in neutrophils. TNF localized with transferrin (Tfn) (a) and VAMP3 (b) in neutrophils stimulated with LPS (10 ng/ml). Graphs showing Pearson's correlation coefficient values for 10 z-stacks of individual cells are shown beneath each panel. $n = 10$, $^{**}p < 0.01$, $^{****}p < 0.0001$. Scale bar, 5 µm.

reduction of transferrin fluorescence upon LPS stimulation, which was responsible for diminished colocalization values. These findings indicate that transferrin was not a suitable marker for REs in LPS-stimulated neutrophils (Figure 14a).

Furthermore, a colocalization study was also performed to determine intracellular TNF and VAMP-3 (another marker of REs) under similar conditions. Figure 14b shows that yellow punctate staining indicating colocalization was more prominent in LPS-stimulated cells than in control cells. It was also observed that TNF^+ $VAMP\text{-}3^+$ vesicles had moved towards the cell periphery after 1h LPS stimulation. Quantitative assessment of changes in colocalization of TNF with VAMP-3 upon LPS stimulation was also performed. We found that colocalization of VAMP-3 with TNF increased significantly (p <0.001), from PCC 0.70 to 0.85, when cells were stimulated with LPS, suggesting that TNF traffics through REs upon LPS stimulation.

We also double-stained and quantified the colocalization of intracellular TNF with CD63-Alexa 488 and CD66b-Alexa 488, markers of primary and secondary granules, respectively. Figure 15a shows that TNF colocalized with $CD63^+$ primary granules, and this decreased significantly (p <0.01) after LPS stimulation. PCCs of control cells decreased from 0.77 to 0.67 in LPS-stimulated cells, indicating that TNF may be depleted from primary granules upon LPS stimulation. Interestingly, the marker for secondary granules (CD66b) showed instead a significant increase in colocalization with TNF (p <0.05) upon LPS stimulation (Figure 15b), and PCCs were 0.80 and 0.86 in control and LPS-stimulated cells, respectively. This suggests that secondary granules may also play an important role in the trafficking of TNF.

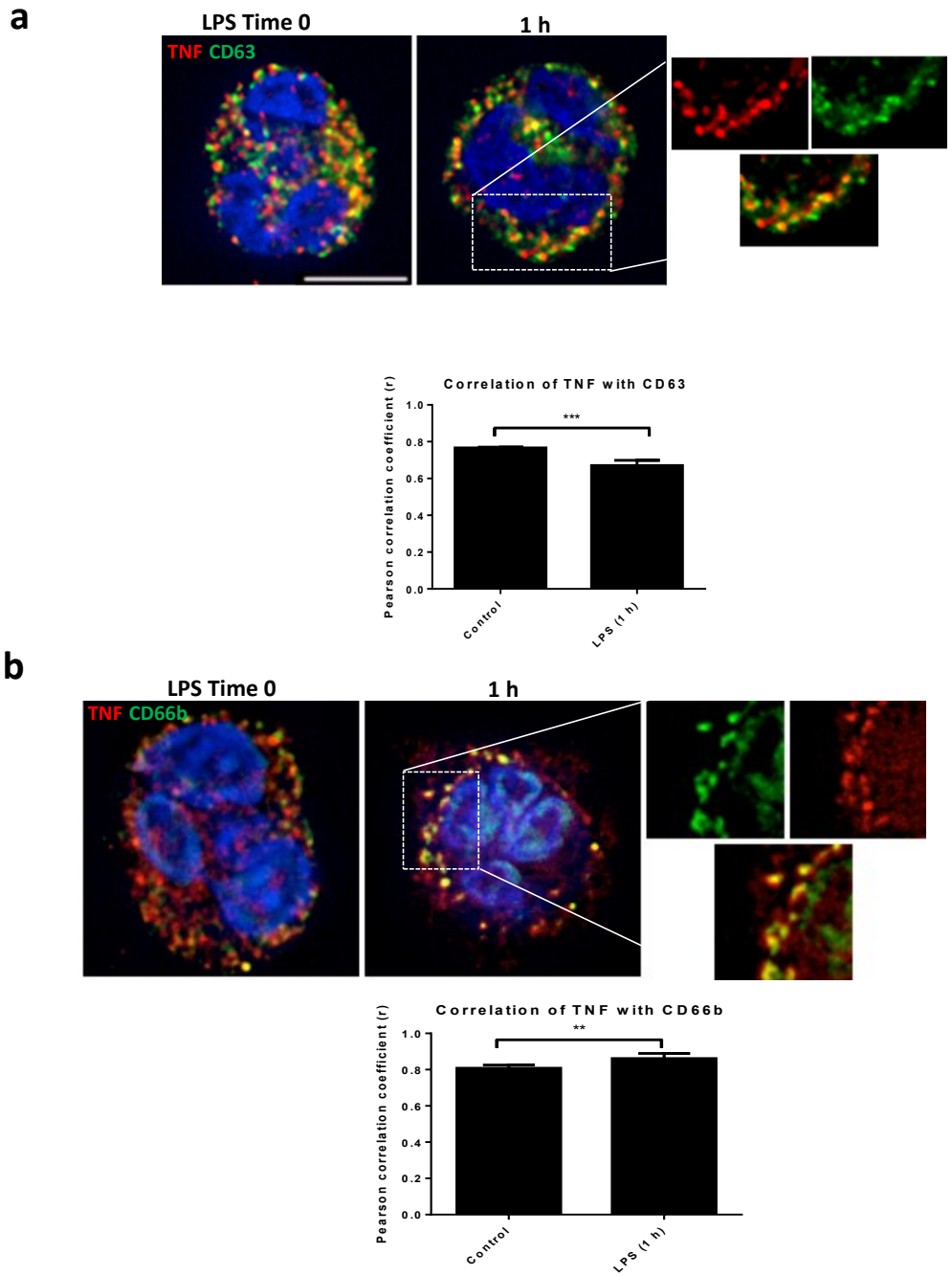


Figure 15: Effects of LPS stimulation on TNF trafficking via secretory granules in neutrophils. TNF localized with CD63 (a) and CD66b (b) in neutrophils stimulated with LPS (10 ng/ml). Graphs showing Pearson's correlation coefficient values for 10 z-stacks of individual cells are shown beneath each panel. $n = 10$, $^{**}p < 0.01$, $^{***}p < 0.001$. Scale bar, 5 µm.

3.8 Colocalization of TNF with endosomal compartments in LPS-stimulated neutrophils

We also determined the colocalization of TNF using markers of early and late endosomes upon LPS-stimulation, and compared these with control cells. Figure 14a shows that there was no significant difference in colocalization of Rab5 (a marker of early endosomes) with TNF after LPS stimulation; the PCC for control cells was 0.63, which was not significantly different from 0.50 in LPS-stimulated cells. This indicates that early endosomes are not involved in the trafficking of TNF to the cell periphery. Interestingly, we found a similar pattern using the late endosomes marker, Rab7, with a PCC of 0.66 in resting neutrophils, which was not significantly altered to 0.57 after LPS stimulation (Figure 16a and b).

These results suggest that upon LPS stimulation, TNF transits to the cell surface through REs in neutrophils. Moreover, secondary granules may also play a role in the trafficking of TNF. Primary granules contain stored TNF, and may release this upon stimulation with LPS. However, early endosomes and late endosomes do not appear to contribute to extracellular trafficking of TNF upon LPS stimulation.

3.9 Tyrosine-based adaptor motifs are present in granular proteins of neutrophils

Transmembrane proteins often contain a short amino acid stretch at the cytoplasmic end, which drives their sorting to different cellular compartments. Sorting signals for the selection of cargo into clathrin-coated vesicles are usually in the form of short linear motifs such as YXXΦ (where Φ is a bulky hydrophobic residue), [D/E]XXXL[L/I] and

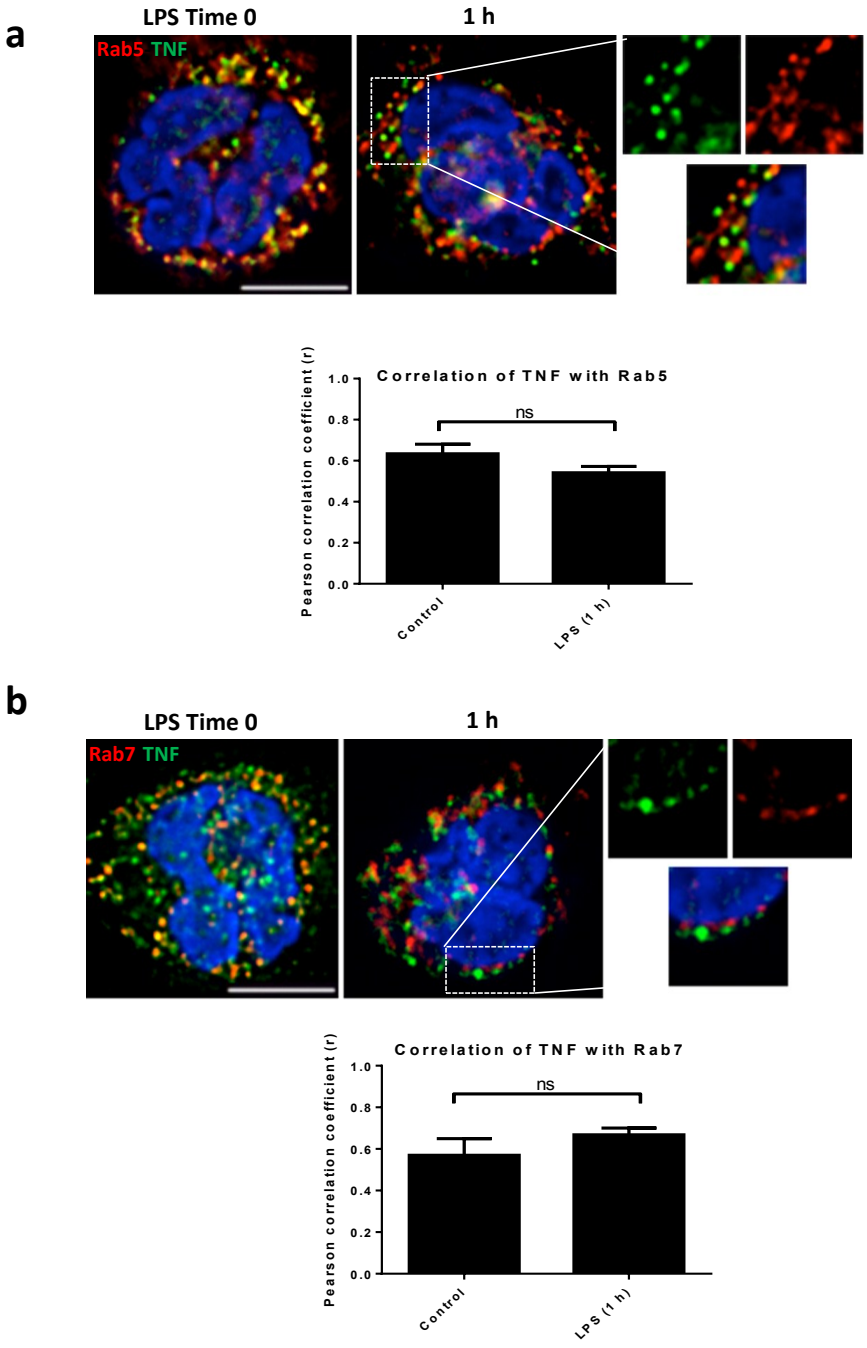


Figure 16: Effects of LPS stimulation on TNF colocalization with early endosomes in neutrophils. TNF localized with Rab5 (a) and Rab7 (b) in neutrophils stimulated with LPS (10 ng/ml). Graphs showing Pearson's correlation coefficient values for 10 z-stacks of individual cells are shown beneath each panel. *n* = 10, ns, not significant. Scale bar, 5 µm.

FXNPXY (Nakatsu *et al.*, 2003). Adaptor protein (AP1-3) function in vesicle trafficking by positioning between clathrin coated proteins and membranes whereas, AP-4 and AP-5 are act independently of clathrin (Hirst *et al.,* 2011). These adaptor proteins sort cargos either from endosomes or directly from the *trans*-Golgi network (TGN) (Nakatsu *et al.*, 2003). AP-3 traffics cargo from tubular endosomes such as REs to late endosomes, lysosomes, and related organelles, while both AP-1 (as part of clathrin-coated vesicles) and AP-4 traffics protein from the TGN to endosomes or directly to lysosomes (Braulke *et al.*, 2009). AP-2 mediates uptake from the plasma membrane (Robinson *et al.,* 2001).

Previously, we have noticed that TNF is stored in various compartments of neutrophils including primary and secondary granules. It would be interesting to know the sorting pathway of TNF as well as other granule proteins. There are two main trafficking pathways for granule proteins: AP-1/GGA-dileucine-based sorting and AP-3/AP-4-tyrosine-based sorting (Sheshachalam *et al.,* 2014). To investigate whether neutrophil granule proteins possess any of these adaptor motifs, the amino acid sequences of these proteins were examined. The amino acid sequences of neutrophil granule proteins were obtained from the Uniport database (www.uniprot.org), and C-terminal sequences were examined for the presence of adaptor motifs. Figure 17 illustrates CD63, a membrane protein that is abundant in primary granules; possesses the YXXΦ motif at the C-terminal end, which interacts with AP-3/AP-4 complexes. This indicates that CD63 may be sorted to primary *via* REs and late endosomes.

Interestingly, other proteins present in the matrix of neutrophil granules, such as cathepsin L1, sialidase-1, cysteine-rich secretory protein 3, and amiloride-sensitive amine oxidase, also possess the YXXΦ motif (Figure 17), indicating that this motif is not

exclusive to membrane-bound molecules. Conversely, cytokines, which are secreted by neutrophils, such as IL-1α/β, IL-6, IL-8, IL-12, TNF, IFNγ, CXCL2, TGFβ, MIP-1α/β, and VEGF, do not possess any of the above adaptor motifs, suggesting that they do not directly bind to adaptors in order to enter sorting pathways for their trafficking. Based on the absence of adaptor motifs in cytokines, it is likely that cytokine trafficking is mainly determined by adaptor motifs present on membrane proteins, such as CD63, which may direct cytokine cargo to appropriate granular and vesicular compartments in cells (Figure 18).

CD63 (238aa), accession number: C9JV86
MAVEGGLVKSIRSG……**YEVM C-terminal**

Cathepsin L1 (333aa), accession number: P07711
MNPTLILAAFCLGIA……..**YPTV C-terminal**

Sialidase-1 (415aa), accession number: Q99519
MTGERPSTALPDRR……**YGTL C-terminal**

Cysteine-rich secretory protein 3, (191aa) accession number: I3L0A1
MKQILHPALETTDPC……..**YYYV C-terminal**

Amiloride-sensitive amine oxidase, (751aa) accession number: P19801
MPALGWAVAAILML……..**YRPV C-terminal**

Figure 17: Membrane and matrix granular proteins of neutrophil contain tyrosine-based motifs. Amino acid sequences of human neutrophil proteins were obtained from the Uniport database available online, and the C-terminal ends of amino acid sequences were analyzed for the presence of adaptor motifs. CD63, membrane protein of primary granules of neutrophils, possesses the YXXΦ motif at the C-terminal ends of its amino acid sequence. Other neutrophil granule matrix proteins such as cathepsin L1, sialidase-1, cysteine-rich secretory protein-3, and amiloride-sensitive amine oxidase also contain specific tyrosine-based motifs which may allow their sorting to specific compartments. On the other hand, CD66b, a membrane protein in secondary granules does not contain a tyrosine-based motif. The N-terminal end of the proteins is shown at left and C-terminal at right, showing the tyrosine based motif as red.

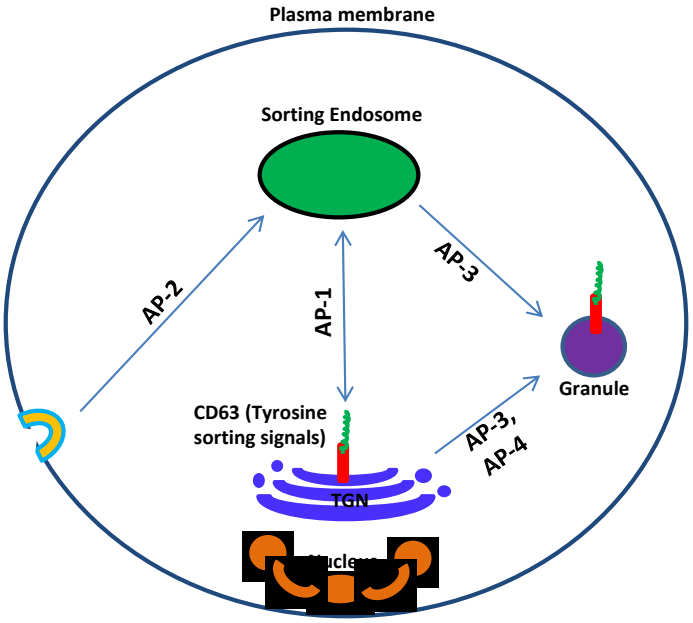


Figure 18: Schematic of neutrophil granular protein sorting pathway. Transmembrane proteins (such as CD63) contain tyrosine motifs that may allow sorting of cytokines directly to granules via AP-3/AP-4 adaptor complexes from the *trans*-Golgi network (TGN), or from endosomes via AP-3. (Source: modified from Sheshachalam *et al.,* 2014).

Chapter 4

Discussion

4.1 Multiplex cytokine analysis of culture supernatants of LPS-stimulated human neutrophils

In the context of inflammatory responses, the first line of cellular responses in host defense consists of the neutrophil (Hafeman *et al.,* 1979). Although neutrophils were once viewed as terminally differentiated leukocytes with a limited capacity to produce newly synthesized proteins, investigations by several groups demonstrated that neutrophils generate a number of polypeptides through *de novo* protein synthesis, including heat-shock and cytoskeletal proteins (Granelli-Pipemo *et al.,* 1979). In addition, activated neutrophils have the ability to generate many cytokines, such as G-CSF, IFN-α, IL-1, IL-6, IL-8, GM-CSF and TNF, (Shirafuji *et al.,* 1990). The production of these cytokines by neutrophils at sites of inflammation play significant roles in the subsequent evolution of the inflammatory response *via* their autocrine or paracrine stimulation of other immune or non-immune cells.

In this study, supernatants from LPS-treated human peripheral blood neutrophils were screened with a 64-cytokine multiplex assay. We were able to demonstrate that the levels of IL-8, RANTES, GRO, PDGF-BB, MCP-1, IL-15, sCD40L and eotaxin-2 were high (up to 2800 pg/ml) after LPS induction. The secretion levels of several other cytokines, such as ENA-78, IL-20, VEGF, TNF, TRAIL and PDGF-AA were moderate (up to 30 pg/ml). For 6Ckine, Flt-3L, GM-CSF, MCP-2, MCP-3 and TGF secretion levels were very low (up to 10 pg/ml). Finally, secretion levels of BCA, CTACK, IL-5, IL-7, IL-9 and IL-17A were almost negligible (up to 1.5 pg/ml), and the levels of release of the remaining cytokines (37) were below the detection limit.

Some chemokines, such as BCA (CXCL13), 6Ckine, CTACK (CCL27), eotaxin-2, GRO, IL-5, IL-8 (CXCL8), IL-17, MCP-2 (CCL8), MCP-3 (CCL7), PDGF and RANTES cause neutrophils and other innate immune cells to migrate toward sites of infection and inflammation. The multiplex cytokine analysis showed that neutrophils have the ability to produce these cytokines upon LPS stimulation, indicating that they can enhance their own recruitment along with that of other cells (e.g. eosinophils, B cells, dendritic cells, T cells and NK cells). Therefore, these cells can induce antimicrobial defense through the local production of these cytokines at the sites of infection.

Several pro-inflammatory cytokines, IL-17, IL-20 and TNF were secreted by LPS-induced neutrophils, as revealed in the multiplex cytokine analysis. Interestingly, the secretion of TNF, a cytotoxic and pyrogenic cytokine, was significantly higher in LPS-treated samples than in CHX+LPS stimulated cells. CHX is an inhibitor of protein biosynthesis, which binds ribosomes and inhibits eEF2-mediated translocation (the movement of two tRNA molecules and a single mRNA strand in relation to the ribosome), thus blocking elongation of the polypeptide chain (Obrig *et al.,* 1971). In addition, IL-8, RANTES, MCP-1, and eotaxin-2 appeared to be released as newly synthesized mediators from neutrophils, since after cycloheximide treatment, their secretion was significantly inhibited at 24 h of LPS stimulation.

4.2 Secretion of preformed and newly synthesized TNF from circulating human peripheral blood neutrophils induced by LPS

Neutrophil-derived TNF may have an autocrine or paracrine role in various neutrophil-mediated diseases. Although TNF production by neutrophils has previously been identified, and CHX has been shown to inhibit spontaneous cytokine production from

sputum neutrophils (Pang *et al.*, 1997), the effects of cycloheximide on LPS-induced cytokine secretion from neutrophils have never been studied to our knowledge.

In the present study, we demonstrated that neutrophils produce and release TNF in response to stimulation with LPS. Multiplex cytokine analysis showed that after CHX+LPS treatment, the TNF secretion is almost negligible, but ELISA data indicated that the secretion of TNF was reduced by 60% upon CHX treatment, and at least ~ 30% of TNF was secreted from stored sources. TNF appeared to be stored in intracellular compartments, as its *de novo* synthesis was inhibited by CHX. This discrepancy in CHX+LPS samples may be due to the lower sensitivity of the multiplex cytokine analysis compared to individual cytokine-specific ELISAs (personal communication with Dr. Dean Befus, University of Alberta, 2013 and Elshal *et al.,* 2006).

Our study showed that CHX treatment inhibited the secretion of TNF. It is possible or even likely that CHX inhibits the *de novo* synthesis of other proteins that may be important in trafficking of TNF, and that this may explain the loss of TNF release during CHX treatment. However, several other cytokines were released in the presence of CHX, suggesting that the trafficking and exocytotic machinery were intact in these cells.

4.3 Transferrin and dextran uptake in endosomal compartments of neutrophils

A study by Maneva and Taleva in 2009 found that neutrophils possess transferrin receptors on the plasma membrane, suggesting that neutrophils may bind and use transferrin. However, they did not establish whether neutrophils bind to transferrin for it to undergo receptor-mediated endocytosis. The result of transferrin uptake in our experiment shows that transferrin appears as a punctate granular staining in neutrophils. This suggests that neutrophils may require iron for metabolism, as transferrin is an iron-

binding protein. In addition, REs are characteristically found near the microtubule-organizing center, which is usually located near the nucleus (Perret *et al.,* 2005). Our observations regarding the location of transferrin suggest that transferrin-positive endosomes are REs like compartment. REs were also present at the membrane, which matches the appearance of transferrin at the membrane of macrophages and NK cells (Stanley *et al.,* 2010).

The identification of REs is important because of their role in constitutive cytokine release, in which cytokines traffic through the ER and Golgi to the plasma membrane. It has been suggested that REs are responsible for the movement of post-Golgi cargos in certain innate immune cells (Stanley *et al.,* 2012). In addition, the roles of REs have already been described in macrophages and NK cells (Stanley *et al.,* 2010). Therefore, confirming the presence of REs in neutrophils is an important step to establish its role in constitutive cytokine release.

Furthermore, intracellular dextran also appeared in individual organelles in neutrophils, suggesting that dextran is present within a separate endosomal compartment. Our findings indicated that the localization of dextran was near the cell periphery, which agrees with previous studies describing the location of early endosomes normally being near the cell periphery (Stanley *et al.,* 2010). These observations suggest that neutrophils displayed intracellular fluorescence corresponding to dextran within early endosomes.

Endosomes also have a significant role in cytokine secretion. A study has found that the accumulation of activated Toll-like receptor 4 (TLR4) within endosomes induces constitutive cytokine secretion in cells (Suzuki *et al*., 2007). The presence of REs suggests that constitutive cytokine secretion may also be present in neutrophils. Thus,

early endosomes and REs may be a novel component involved constitutive cytokine release and therefore merit investigation.

4.4 Storage of TNF in different intracellular compartments of resting neutrophils

Several studies have found that neutrophils store cytokines as pre-formed mediators in various intracellular organelles. For example, Calafat *et al.,* in 1997 showed that neutrophils stored transforming growth factor-α (TGFα) as a pre-formed mediator in secretory granules. In addition, Denkers *et al.* (2003) showed that peripheral blood neutrophils possess pre-formed IL-6, IL-12, and CXCL2 in their secretory vesicles or tertiary granules. However, the mechanisms of cytokine secretion have not been investigated in neutrophils, even though it is evident that SNAREs and other trafficking machinery are essential for exocytosis of cytokine-carrying granules (Logan *et al.,* 2006; Mollinedo *et al.,* 2003 and 2006). We found that TNF colocalized with transferrin^{+} and VAMP-3^{+} compartments, which are characteristics of REs. REs have a role in recycling proteins back to the plasma membrane (PM) to maintain the integrity of the membrane. These recycling pathways are essential for maintaining the proper composition of proteins in various organelles that carry out specific functions (Mellman *et al.,* 2006).

Neutrophils have three distinct granule subsets: (i) primary or azurophilic granules, which contain elastase and myeloperoxidases (MPO), (ii) secondary or specific granules, which contain high levels of lactoferrin, and (iii) tertiary or gelatinase granules, which contain matrix metalloproteinases. Our observations suggest that TNF colocalized with CD63 and CD66b, markers of primary and secondary granules, respectively. Since these are abundant membrane proteins found in only within their respective granule types, they have been identified as specific markers of these compartments.

Our study also showed that early and late endosomes contain TNF, as it colocalized with Rab5 and Rab7, the markers of early and late endosomes, respectively. A study in 1993 by Beil *et al.,* showed that neutrophils contain TNF in subcellular organelles included lipid bodies, Golgi structures and vesicle membranes. This study supported that observation that neutrophils may store TNF in multiple intracellular compartments.

Figure 13a showed the PCC value for markers of two different compartments is 0.27. This value could be considered as baseline PCC as it shows colocalization of two markers due to overlapping. Additionally, markers of similar compartment (VAMP-3 and transferrin for REs) have shown PCC value 0.64 (Figure 13b). Although, the value (0.64) is higher than baseline PCC (0.27), it should be higher as it represents colocalization of the markers of same compartment. It seems that transferrin is not a perfect marker for REs. Therefore, for future study it would be interesting to evaluate other reside markers of REs, for instance Rab11 and VAMP-3 to perform colocalization analysis. Similarly, it would be interesting to evaluate other markers rather relying on single marker for different compartments (VAMP-3, marker of recycling endosomes and Rab7, marker of late endosomes) to get clear idea about the colocalization pattern.

Since both studies were done on single events, thus it would be very interesting to analyze the colocalization of other compartment's marker to get the conclusive results.

4.5 Trafficking of TNF to the plasma membrane *via* recycling endosomes upon LPS stimulation of neutrophils

Our study suggested that TNF traffics through VAMP-3^{+} REs at the cell periphery upon LPS stimulation. Previously it was shown that in RAW 264.7 cells (macrophage-like cell

line derived from Balb/c mice), TNF was trafficked from Golgi to REs positive for VAMP-3, then to the plasma membrane (Murray *et al.,* 2005). Importantly, knockdown of VAMP-3 impaired the delivery of TNF to plasma membrane (Murray *et al.,* 2005). Another study also suggested that REs were involved in the trafficking of E-cadherin in HeLa cells. E-cadherin exited the Golgi in tubule-vesicular carriers, which, instead of moving directly to the plasma membrane, most frequently fused with REs positive for Rab11 (Lock *et al.,* 2005).

Our study also showed that TNF was depleted from primary granules upon LPS stimulation, indicating that they were stored as pre-formed mediators and released upon stimulation. By contrast, in secondary granules, the colocalization of TNF is increased with CD66b (a membrane marker of secondary granules) upon LPS stimulation, indicating that secondary granules may also play an important role in the trafficking of TNF, similarly as we have shown with REs. This suggests a novel observation that has not been previously described. Early endosomes and late endosomes appeared to store TNF, but upon stimulation with LPS, the colocalization of their respective marker with TNF did not change significantly. This observation indicated that they do not appear to contribute to extracellular trafficking of TNF upon LPS stimulation.

4.6 Neutrophil granular membrane proteins possess tyrosine-based adaptor motifs

Transmembrane proteins often contain a short amino acid stretch at the C-terminal end, which helps to sort them into different cellular compartments. Three short linear motifs, YXXΦ, [D/E]XXXL[L/I] and FXNPXY, are known to be required for clathrin-mediated endocytosis. Short linear motifs bind to protein complexes called adaptors. The adaptors also interact with clathrin and thus provide a link between the cargo and the clathrin coat

(Nakatsu *et al.,* 2003).

CD63 (granulophysin) is an abundant membrane-bound granule protein of neutrophils, and possesses the YXXΦ motif. CD63 is a member of the tetraspanin superfamily that has two extracellular domains and three relatively short cytoplasmic regions. They interact with one another to form structural platforms referred to as tetraspanin-enriched microdomains (TERM) or tetraspanin webs. These are involved in physiological processes such as immunological responses (Levy *et al.,* 2005) and tissue differentiation (Hemler *et al.,* 2005). Early studies identified CD63 as a marker for late endocytic/lysosomal organelles, and the C-terminal cytoplasmic domain of the CD63 contains a tyrosine-based lysosomal targeting motif (Marks et al., 1997). Like lysosomes, azurophilic granules contain CD63 in their membrane (Cham *et al.,* 1994).

However, cytokines secreted by neutrophils such as CXCL8, CXCL2, IFNγ, IL-1α/β, IL-6, IL-12, MIP-1α/β, TNF, TGFβ and VEGF do not possess adaptor motifs. This indicates that they do not bind directly to known adaptors for their trafficking, and instead may depend on other membrane-bound granule proteins, such as CD63, for directional sorting and trafficking to the cell membrane.

Summary

This work is focused on identifying secretory components in neutrophils that may contribute to cytokine trafficking, since trafficking and secretion of cytokines in neutrophils has not yet been characterized.

1. In this thesis, 64 cytokines were analyzed in culture supernatants (from LPS-treated neutrophils). This allowed rapid and abundant acquisition of data on cytokine release. A multiplex cytokine analysis showed that the release of 27

cytokines (including ENA-78, eotaxin-2, GRO, IL-8, IL-15, IL-20, MCP-1, PDGF-AA, PDGF-BB, RANTES, sCD40L, TNF, TRAIL and VEGF) was significant at 2-24 h post LPS induction. Interestingly, the multiplex cytokine analysis showed that TNF secretion in stimulated neutrophils was significantly higher in LPS-treated cells than for CHX+LPS.

2. As an inflammatory cytokine, TNF has many potent effects, and its excess or deficiency has many adverse clinical consequences. Hence we focused on the release of TNF from neutrophils upon LPS induction and its trafficking.
3. In order to evaluate whether secreted TNF had been synthesized and stored by neutrophils prior to release, cells were pretreated with cycloheximide followed by LPS. The result show that the secretion of TNF was significantly reduced upon the cycloheximide treatment, suggesting that 30% of TNF was secreted from stored sources. We concluded that LPS induced the release of both newly synthesized as well as stored TNF from neutrophils.
4. Although it is anticipated that neutrophils have early endosomes (EEs) and REs like many other cells, the presence of these compartments and their role in TNF trafficking in neutrophils have never been studied. Thus, it is very important to identify and characterize these compartments in neutrophils. To investigate this, we identified endosomal compartments (REs and EEs) in neutrophils by performing transferrin and dextran uptake as markers for REs and EEs, respectively.
5. To determine whether TNF is stored in granules or/and in other compartments of resting neutrophils, colocalization of TNF was performed with transferrin-Alexa

488 and anti-VAMP-3 (markers for RE), anti-CD63 and anti-CD66b (membrane markers for the primary and secondary granules, respectively) and Rab5 and Rab7 (markers for early and late endosomes, respectively). Qualitative, yellow punctate staining (combination of red and green fluorescence) as well as quantitative analysis (Pearson's correlation coefficient) shows that there was partial colocalization of TNF with transferrin, VAMP-3, CD63, CD66b, Rab5 and Rab7 in unstimulated neutrophils. These observations suggest that in the unstimulated state, neutrophils express TNF and store it in granules as well as in other intracellular organelles.

6. Upon LPS stimulation, colocalization of VAMP-3 with TNF was increased in comparison to that of unstimulated cells, which suggests that TNF traffics through REs upon stimulation by LPS. Conversely, colocalization between TNF and CD63 decreased with LPS induction shows that it may be released from primary granules with LPS treatment. The marker for secondary granules (CD66b) showed increased colocalization with TNF upon LPS stimulation, which was an interesting and novel pathway for TNF release. We also determined that there was no significant difference in the colocalization of Rab5 and Rab7 with TNF after LPS stimulation, suggesting that early and late endosomes are not involved in the trafficking of TNF to the cell periphery. From these results it can be concluded that TNF transits through REs in LPS-activated neutrophils to the cell surface, although secondary granules may also be a major conduit for TNF trafficking.
7. The amino acid sequences of granule proteins in neutrophils were examined to find out those that have adaptor motifs, which may allow their sorting to specific

granular compartments. Membrane proteins in primary granules, CD63, possess the YXXΦ motif, which interacts with the AP-3/AP-4 complexes. These adaptor proteins sort cargo either from endosomes or directly from the TGN. We found that other proteins present in the matrix of neutrophil granules, such as cathepsin L1, sialidase-1, cysteine-rich secretory proteins 3, and amiloride-sensitive amine oxidase, also have the YXXΦ motif, indicating that this motif is not exclusive to membrane-bound molecules.

Conclusions

Cytokines are important mediators of neutrophils, and their mechanisms of release have not yet been well characterized. We have found strong evidence that neutrophils contain REs, as determined by transferrin uptake and VAMP-3 labeling, and further, our observations have suggested a role for REs in TNF trafficking in neutrophils. These exciting observations compel us to determine the precise role of REs in cytokine trafficking and secretion in neutrophils. Further, we can hypothesize that REs are required for the transport of numerous cytokines from neutrophils for secretion. Characterizing and understanding these pathways is important because they have many therapeutic implications. In many autoimmune diseases in which inflammatory cytokines are produced in excess, models of cytokine release may help to provide novel approaches to reducing the release. For example, in rheumatoid arthritis and Crohn's disease, TNF has widespread deleterious effects, and anti-TNF antibody, an effective therapy, has many adverse side effects (Van Dullemen *et al.*, 1995). Understanding neutrophil cytokine secretion may allow for the development of more effective therapies that block TNF secretion. This is relevant to many areas of biology and will contribute to a new understanding of how cells package, transport, and release secretory products that have a fundamental role in immune homeostasis and host defense.

Future directions

Our study provides evidence suggesting that constitutive cytokine release occurs in neutrophils, but further studies are required to appropriately describe the trafficking of cytokines in these cells. A cohort of released cytokines from neutrophils could be

compared with LPS by inducing neutrophils with other inducers, such as platelet-activating factor, leukotriene B_4, and f-Met-Leu-Phe. After cytokines are characterized using multiplex cytokine analysis, immunofluorescence and colocalization with RE and other organelle markers could examine prominent cytokines. An interesting outcome for such a study would be to determine whether different cytokines might localize to different RE sub-compartments, providing insight into mechanisms for how cytokines are released from neutrophils.

Our results show that VAMP-3^+ REs colocalizes with TNF in neutrophils. Therefore, we hypothesize that SNAREs mediate RE trafficking and release of cytokines in neutrophils. Hence it is important to examine the role of VAMP-3 in signaling pathways regulating RE trafficking and exocytosis in neutrophils. In future studies, signaling could inhibit by using small interfering RNA (siRNA) or small hairpin RNA (shRNA) to VAMP-3. We predict that knocking down the expression of SNAREs will result in diminished cytokine secretion from neutrophils.

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