From INSTITUTE OF ENVIRONMENTAL MEDICINE
Karolinska Institutet, Stockholm, Sweden

NEUTROPHIL EXTRACELLULAR TRAPS: FUNCTION IN INFECTIOUS AND NON-INFECTIOUS CONDITIONS

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Stockholm 2018

Published by Karolinska Institutet.
Printed by E-print AB

ISBN 978-91-7831-028-9

Neutrophil Extracellular Traps: Function in Infectious and Non-Infectious Conditions

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza.”

Dante Alighieri

Inferno, XXVI, vv. 119-120

ABSTRACT

Neutrophil extracellular traps (NETs) are composed of a backbone of chromatin, decorated with microbicidal peptides and proteolytic enzymes, and are used by neutrophils as a weapon against pathogens. Since their initial discovery in 2004, NETs have challenged scientists in terms of the nature of the triggers and the mechanisms of neutrophil release of NETs, as well as the implication of these extracellular structures in infectious and non-infectious human diseases. The work presented in this thesis, performed in an interdisciplinary perspective using an array of different methodologies, aims to deepen the knowledge on the functionality and dysfunctionality of NETs, including the mechanism of NET disposal and its immunological consequences. Once NETs have fulfilled their anti-microbial function, the timely clearance of these structures is needed in order to avoid a misguided autoinflammatory response. In Paper I, we showed that primary human macrophages employ TREX1, while dendritic cells use DNase1L3 to digest NETs purified from activated neutrophils. In addition, on the basis of cytokine profiling, we showed that NETs have immunomodulatory effects on phagocytes. In Paper II, we showed that JAGN1 is required for efficient fungal killing in NETs. Patients suffering from severe congenital neutropenia (SCN) present homozygous mutations in the gene encoding JAGN1 and are susceptible to bacterial and fungal infections. We found that JAGN1-deficient neutrophils isolated from an SCN patient, as well as neutrophil-like HL-60 cells with silenced JAGN1 expression, released NETs, but the expression of myeloperoxidase (MPO) was altered. In Paper III and IV we explored the interactions of NETs with graphene oxide (GO), a carbon-based 2-D material. Using ToF-SIMS, a mass spectrometry-based surface analytical method, we could show in Paper III that GO interacts with the plasma membrane of neutrophils, promoting cholesterol oxidation. In addition, we could define a size-dependent mechanism of GO induced release of NETs. Furthermore, we showed in Paper IV that GO undergoes efficient extracellular degradation through neutrophil degranulation or in NETs in a process dependent on MPO. Moreover, intermediate degradation products of GO did not cause DNA damage in lung cells. Overall, the work presented in this thesis has shed light on the clearance and degradation of NETs by phagocytic cells and the involvement of several endonucleases in a cell type-specific manner, and has confirmed the importance of NETs in fungal killing, with new evidence for a role of JAGN1 in this process. In addition, we have shown for the first time that GO triggers NETs and reported that GO may also undergo degradation in NETs in a similar manner as pathogens. Our studies have thus revealed that MPO expressed in NETs is a key element in eliminating infectious as well as non-infectious agents.

LIST OF SCIENTIFIC PAPERS

I. Lazzaretto, B. and Fadeel, B. Intra- and extracellular degradation of neutrophil extracellular traps by macrophages and dendritic cells. 2018. (manuscript, in revision)

II. Khandagale, A., Lazzaretto, B., Carlsson, G., Sundin, M., Shafeeq, S., Römling, U. and Fadeel, B. JAGN1 is required for myeloperoxidase-mediated fungal killing in neutrophil extracellular traps. 2018. (manuscript, in revision).

III. Mukherjee, S.P., Lazzaretto, B., Hultenby, K., Newman, L., Rodrigues, A.F., Lozano, N., Kostarelos, K., Malmberg, P. and Fadeel, B. Graphene oxide elicits membrane lipid changes and neutrophil extracellular trap formation. 2018. *Chem*. 4: 334-358.

IV. Mukherjee, S.P., Gliga, A.R., Lazzaretto, B., Brandner, B., Fielden, M., Vogt, C., Newman, L., Rodrigues, A.F., Shao, W., Fournier, P.M., Toprak, M.S., Star, A., Kostarelos, K., Bhattacharya, K. and Fadeel, B. Graphene oxide is degraded by neutrophils and the degradation products are non-genotoxic. 2018. *Nanoscale*. 10: 1180-1188.

CONTENTS

LIST OF ABBREVIATIONS

ACPAs	Anti-Citrullinated Protein Antibodies
AFM	Atomic Force Microscopy
AMP	Antimicrobial peptide
ANCAs	Anti-Neutrophil Cytoplasmic Antibodies
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
CD-	Cluster of Differentiation
CFU	Colony Forming Unit
CNT	Carbon Nanotube
CTXB	Cholera Toxin B
DAMPs	Damage (or Danger)-Associated Molecular Patterns
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic Cells
DMSO	Dimethyl sulfoxide
DNase	deoxyribonuclease
DNase1L3	DNase1-like 3
ECACC	European Collection of Authenticated Cell Cultures
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
FBS	Fetal Bovine Serum
G-CSF	Granulocyte- Colony Stimulating Factor
GM-CSF	Granulocyte-Monocyte-Colony Stimulating Factor
GO	Graphene Oxide
HBSS	Hank's Balanced Salt Solution
HMDMs	Human Monocyte-Derived Macrophages
IFN	Interferon
IL-	Interleukin
JAGN1	Jagunal homologue 1
M-CSF	Macrophage-Colony Stimulating Factor
MDDCs	Monocyte-Derived Dendritic Cells

MPO	Myeloperoxidase
mRNA	Messenger RNA
MβCD	Methyl-β-cyclodextrin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
NAMPs	Nanoparticle-Associated Molecular Patterns
NE	Neutrophil Elastase
NETs	Neutrophil Extracellular Traps
PAHs	Polycyclic Aromatic Hydrocarbons
PAMPs	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PMA	Phorbol-12-myristate-13-acetate
PMNs	Polymorphonuclear leukocytes
PRRs	Pattern Recognition Receptors
RA	Rheumatoid Arthritis
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SCN	Severe Congenital Neutropenia
SEM	Scanning Electron Microscopy
siRNA	Small Interfering RNA
SLE	Systemic Lupus Erythematosus
STING	Stimulator of Interferon Genes
TEM	Transmission Electron Microscopy
TLR	Toll-Like Receptor
ToF-SIMS	Time of Flight – Secondary Ion Mass Spectrometry
TREX1	3’-exonuclease 1
YPD	Yeast Peptone Dextrose

1 INTRODUCTION

1.1 INFLAMMATION

The classical definition of inflammation, compiled by Celsus and Galen, delineates the phenomoenon as the manifestation of pain (*dolor*), warmth (*calor*), swelling (*tumor*), redness (*rubor*) and ultimately loss of function (*functio laesa*). Nowadays, inflammation is generally described as a physiological response to pathogen infections or insults that perturb the organism´s homeostasis. The aim of an inflammatory response is to exclude or eliminate the source of injury while temporarily adapting to the atypical condition and to ultimately restore functionality and homeostasis in the tissue (Medzhitov, 2008). Inflammation can likewise occur when the integrity of the organism is lost, as in necrotic cell death, without the action of external pathogens, and is therefore named "sterile inflammation" (Kono et al. 2014). Due to the multifaceted panel of responses that occur during the inflammatory process, such as the release of an heterogeneicity of soluble mediators and complement activation, which can be tissue-specific and cause-related, an univocal definition of inflammation might result reductive and unappropriate (Netea et al. 2017). It is therefore important to consider both the basic mechanisms and key players of this phenomenon in light of its complexity, trying to understand "the bigger picture" rather than focusing on individual aspects.

1.1.1 Key players in inflammation

The inflammatory process requires the coordination of the interplay between several actors, which can be classified in three main categories: inflammation inducers and related sensors, mediators of inflammations and effectors (Medzhitov, 2008). Inflammation inducers are molecules either of exogenous origin or that can arise from the organism itself in situation of stress, malfunction or damage of the tissues. Pathogen-associated molecular patterns (PAMPs) are of non-self origin and are highly conserved domains of peculiar structures commonly occurring among pathogens, while self molecules, that do not generally present an hazard due to their compartmentalization in intact cells or that are modified in the stressed microenvironment, are defined as DAMPs (Medzhitov, 2008). With the advent of nanotechnology, interactions between engineered nano-scaled entities and the immune system started to be investigated. It should not surprise that engineered nanomaterials introduced in biological systems could be perceived as non-self entities or, after absorption and modification of biomolecules on their surface, inducing the formation of complexes containing "altered self" epitopes that can be sensed by the innate immune system. In this context, nanomaterial-based inflammation inducers have been defined as nanoparticle-associated molecular patterns, or NAMPs (Fadeel, 2012 and Farrera and Fadeel, 2015). A panel of receptors, so-called pattern recognition receptors (PPRs), senses the danger signals and modulates the production of soluble mediators, which in turn activate effector target cells in different tissues. The ultimate goal of soluble mediators is to modulate the status of targeted cells in order to transiently adapt it to the harmful condition and, eventually, tune it to restore tissue homeostasis (Medzhitov, 2008 and Netea et al. 2017).

1.1.2 Role in disease

Acute inflammation responses are displayed from the first moments after pathogen invasion or tissue damage and rely on the autocrine and paracrine effects of the soluble mediators, primarily cytokines as TNFα and IL-1β, and chemokines as IL-8. The pro-inflammatory milieu typical of the first phases of inflammation should be timely restricted in order to avoid tissue damage caused by the immune system itself. Resolution of inflammation, intended as the removal of the cause, as well as reestablishment of physiological homeostasis in the tissue, is a mechanism based on active reprogramming of immune-competent cells. This is achieved through a different panel of cytokines aimed to reprogram macrophages, through the depletion of chemokines and the clearance of apoptotic cells from the site of infection (Ortega-Gomez et al. 2013 and Sugimoto et al. 2016). When sustainment of the abnormal microenvironment is not transient, the affected tissue is pushed to adapt to a chronic inflammatory status, which is a hallmark of several diseases.

One of the prevailing chronic inflammatory diseases, with an incidence up to 1% of the population, is rheumatoid arthritis (RA). Chronic inflammation in RA mainly affects the synovial membrane in the joints, where massive infiltration of innate and adaptive immune cells occurs in response to a microenvironment rich in cytokines and chemokines, among which TNFα, IL-6 and GM-CSF are mayhap the most relevant (Smolen et al. 2016). The chronic inflammation is the result of an autoimmune response to citrullinated epitopes leading to the rise of anti-citrullinated protein antibodies (ACPAs) that can form immunocomplexes and activate the complement system. The sustained inflammatory milieu in the joints ultimately results in reprogramming chondrocytes and osteoclasts towards catabolism of the tissue (Smolen et al. 2016).

Another example is systemic lupus erythematosus (SLE), a systemic autoimmune disease that targets different organs and increases the mortality risk of the patients, in prevalence women, compared to the general population (Kaul et al. 2016). SLE patients suffer from chronic inflammation at skin, blood vessels and kidney level due to the overproduction of anti-neutrophil cytoplasmic antibodies (ANCAs) that form immunocomplexes deposited either in situ or in the kidneys, determining kidney failure in the worst cases. Current treatments for SLE include NSAIDs and corticosteroids; biological treatments aim to deplete or modulate B cells and T cells populations as well as target pro-inflammatory cytokines (TNFα, type I interferons) (Lisnevskaia et al. 2014). The nature of SLE is multifactorial, displaying disorders in both innate and adaptive immunity. T follicular helper cells from SLE patients are more prone to activate IgG production by B cells in response to auto antigens, and it has been reported that regulatory T cells display reduced number or function in SLE patients, thus impairing the ability to suppress T helper cells proliferation (Rahman and Isenberg, 2008).

1.2 INNATE IMMUNE SYSTEM

The innate immune system is the first line of defense that organisms use to counteract invading pathogens and its basic mechanisms are evolutionary conserved from plants to vertebrates.

Unlike the other branch of the immune system, innate immunity relies on a rather unspecific type of response, which is functional to a swift reaction and a broad-spectrum efficacy, orchestrated by a cellular component and a humoral component (Turvey and Broide, 2010).

Within the humoral section of the innate immune system, which includes proteins and peptides constitutively present in the serum or secreted *ad hoc*, a further subdivision can be done discriminating between the complement system and antimicrobial peptides (Beutler, 2004). Antimicrobial peptides (AMPs) are evolutionary conserved structures which, in mammals, are primarily present in neutrophilic granules and epithelial cells, consisting in general of up to 50 aminoacids. In several cases, they originate from an inactive precursor *via* proteolitic cleavage, and are released upon necessity, allowing for a multi-level controlled regulation of their activity (Mahlapuu et al. 2016). In humans, several classes of AMPs are present, however, the cathelicidin-derived LL-37 peptide has recently gained novel interest, as not only microbicidal properties are associated with it, but also immunomodulatory effects. It is not possible to define *a priori* whether LL-37 directs the immune response towards a pro- or an anti-inflammatory outcome, as concurrent microenvironmental factors have to be considered, nevertheless its effects on type I IFNs production are quite well defined (Khalenberg and Kaplan, 2013). Due to its capacity of binding DNA and enhancing its internalization in cells of myeloid origin, LL-37 promotes both the intracellular STING-mediated and the TLR 7/9-mediated sensing of the nucleic acid, triggering the release of type I IFNs (Chamilos et al. 2012 and Ganguly et al. 2009). Under the name "complement" falls a network of serum protiens that are sequentially activated by proteolitic cleavage, whose critical functions are proinflammatory signaling and promotion of phagocytosis (Ricklin et al. 2010). Three distinct patways (classical, alternative, lectin) converge in the activation of the C3 component, downstream of which the effector molecules are initiated to ultimately enhance phagocytosis and immune responses (Hajishengallis et al. 2017). The classical pathway is initiated by IgM or IgG-antigen immunocomplexes recognized by C1q, similarly, the lectin pathway relies on the recognition of carbohydrate mioeties by mannose-binding lectins and ficolins. The alternative pathway exploits a fraction of hydrolized C3 which actively probes cell surfaces and is activated by carbohydrate moieties on foreign cells, as well as properdin, as initiator ligands (Ricklin et al. 2010).

The cellular arm of the innate immune system genereally identifies with cells of hematopoietic origin, namely myeloid cells, however, endothelial cells and epithelial cells, for instance, are also immuno-competent and should not be disregarded. These different cell types share the function of using a panel of sensors developed to recognize similar epitopes on different pathogens (PAMPs), or endogenous molecular patterns (DAMPs) which determine a harmful signature. These receptors are globally defined as pattern recognition receptors (PRRs) and ultimately regulate transcription and processing of cytokines but also cell death, autophagy and phagocytosis (Brubaker et al. 2015). PRRs can localize on the plasma membrane or intracellularly, either in the cytosol or within membranes of endocytic inclusions. Proper

localization and compartimentalization of PRRs is an imprescindible feature in order to avoid undesired responses to self molecules (Brubaker et al. 2015).

The Toll-like receptor (TLR) family of PRRs comprises both cytosolic and membrane bound members, covering a wide assortment of ligands both from microbial or viral origin and self origin. TLR4 recognizes lipopolysaccharide (LPS) in a multi-receptor complex with the aid of CD14 and myeloid differentiation factor 2 (MD2), using myeloid differentiation primary response 88 (MyD88) to transduce the signal intracellularly promoting transcription of genes regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Takeuchi and Akira, 2010). A subset of TLRs, namely TLR3, TLR8 (TLR7 in mice) and TLR9, is deputed to the recognition of nucleic acids of different fashion and different origin. Signaling through these receptors ultimately leads to the production of type I IFNs, a hallmark of autoimmune disease as SLE. Compartimentalization of these TLRs, together with degradation of self-DNA by intra and extracellular nucleases is aimed to prevent the mounting of an autoimmune response to nucleic acids, directing it primarily towards DNA and RNA of bacterial or viral origin. Still, complexes of DNA or RNA with proteins or peptides, as LL-37, ease internalization of the nucleic acids into the cells, promoting TLR-mediated IFN responses (Takeuchi and Akira, 2010). Cytosolic DNA, both viral and self, can also be recognized by STING, which localizes at the endoplasmic reticulum membrane, and is able to interact with dsDNA directly, or via pre-processing of the nucleic acid by cyclic GMP-AMP synthase (cGAS). Although appearing to be independent of other TLR-DNA sensing machineries, STING also activates NF-κB dependent production of type I IFNs (Barber, 2015).

While the basic knowledge on the PRRs-PAMPs and PRRs-DMAPs network is already established and continues to expand, the pieces of the PRRs-NAMPs puzzle are still waiting to be resolved. To date, a large body of studies focuses on the outcomes of the encounter or exposure of innate immune cells and engeneered nanomaterials, yet experimental evidence of immune cells-mediated sensing of nanoparticles awaits to be expanded.

1.2.1 Neutrophils

Among the components of the innate immune system, neutrophils are the first-line defense against a wide variety of pathogens, including bacteria and fungi (Mócsai, 2013).
Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are produced in the bone marrow through a process named myelopoiesis. Neutrophils and monocytes share a common progenitor stem cell in the bone marrow, then, neutrophils undergo granulocytopoiesis that leads to the sequential maturation of granules, namely primary (azurophilic) granules, secondary (specific) granules, tertiary (gelatinase) granules and secretory vesicles (Borregaard et al. 1995). Granulopoiesis is fine-tuned by granulocyte colony stimulating factor (G-CSF) that regulates production of PMNs under physiological conditions or infection (Soehnlein et al. 2017). Mature neutrophils are eventually released in the blood stream in a process mediated by the CXCR4 receptor, which is essential for retaining the PMNs in the bone marrow (Borregaard, 2010). Accounting for the majority of all circulating leukocytes (50-70%) in

humans, neutrophils patrol the blood vessels where they can survive up to 12 hours, while in tissues site of infection their lifespan is around 1-2 days (Mayades et al. 2014).

A simplistic view of the neutrophil function in the innate immune system has portrayed PMNs as "dumb" cells, solely devoted to the disposal of pathogens. In the last decades, the interest in neutrophil biology has increased, revealing a more complex and multifaceted role for the polymorphonuclear phagocytes (Nathan, 2006). To date, neutrophils are known to mediate the immune response via regulation of other innate immune cells, monocyte/macrophages and dendritic cells for example (Soehnlein et al. 2017), as well as tuning the adaptive immune response via interaction with B cells and T cells (Mócsai, 2013). The cross talk with the other cell types of the immune system is bi-directional: PMNs receive signals modulating cell survival and effector functions by other cell types, while in turn they initiate, suppress or regulate the reaction of innate and adaptive immune cells, either via direct cell-to-cell interaction, or soluble mediators as cytokines and chemokines (Scapini and Cassatella, 2014). The diversity of neutrophilic functions is correlating with the concept of neutrophil heterogeneity, which is becoming of interest in the recent years. As for the other cells of the immune system, phenotyping of neutrophils has given information on the presence of different subsets of PMNs, putatively programmed to exert or be more proficient for discrete functions. Low density granulocytes (LDGs) for instance, named after the fact that they sediment within the PBMCs layer after density gradient centrifugation, are found with higher recurrence in patients with autoimmune diseases and are prone to employ proinflammatory functions (Scapini et al. 2016).

As for the disposal of pathogens, neutrophils can make use of a repertoire of killing strategies: phagocytosis, degranulation and release of extracellular traps (Papayannopoulos and Zychlinsky, 2009), as represented in Figure 1.

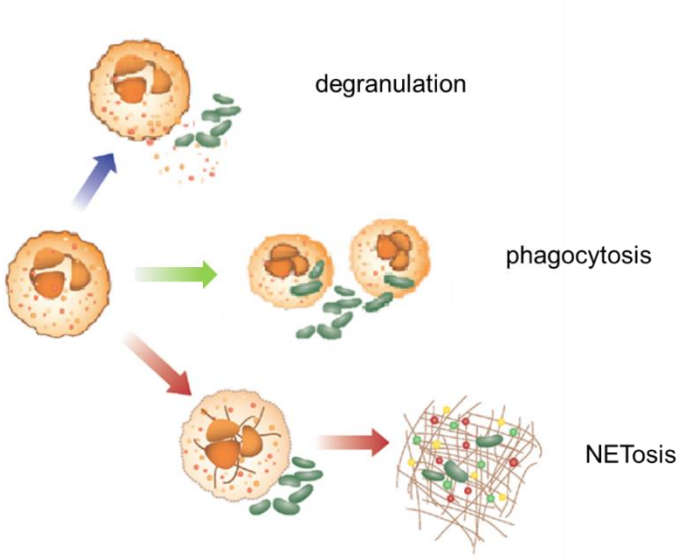

Figure 1. Strategies of pathogen killing operated by neutrophils. Adapted from: Gray et al. 2015. Antibiotics (Basel). 4: 62-75.

The phagocytic process in neutrophils is performed in a timeframe of seconds. The pathogens are internalized in phagosomes on whose membrane the NADPH oxidase complex is assembled to produce reactive oxygen species (ROS) that are released in the phagosome. The phagosome also fuses with the pre-formed cytosolic granules, which in turn discharge their pool of antimicrobial peptides and lytic enzymes (Nordenfelt and Tapper, 2011). The content of granules can also be released into the extracellular environment upon stimulation in order to create an unfavorable milieu for the pathogens. Generally, the content of secretory vesicles and tertiary granules is released extracellularly and the fusion of the granule membrane with the plasma membrane allows for the exposure of receptors involved in adhesion and chemotaxis (Mayades et al. 2014). Antimicrobial cationic peptides are capable of interaction with the negatively charged membrane of pathogens perturbing its stability via formation of pores that lead to the lysis of the microbes (Mahlapuu et al. 2016).

1.2.2 Macrophages and Dendritic Cells

Macrophages are professional phagocytes and they contribute to homeostasis by engulfing not only pathogens and toxic molecules, but also apoptotic cells, preventing them to enter secondary necrosis, thus playing a pivotal role in management of inflammation (Galli et al. 2011). The traditional paradigm about macrophages suggests that these cells, deriving from circulating monocytes, are recruited at site of infection where they can challenge pathogens and participate in the inflammatory responses. Else, tissue resident macrophages are primarily involved in resolution of inflammation and maintenance of tissue homeostasis, being replaced by monocytic precursors at need. A new body of evidence has started to show that tissue macrophages can develop within tissues biogenesis since embryonic development and are endowed with self-renewal properties (Italiani and Boraschi, 2014). Moreover, the traditionally used nomenclature employed to define different kinds of macrophages, attempting to define functions or phenotype, such as M1 *versus* M2 or alternatively *versus* classically activated macrophages, is based on discrete criteria thus delineating that the use of these terms might be misleading and prone to be confounding instead of clarifying. A consensus on nomenclature is not yet established, highlighting that an accurate description of the isolation, stimulation and analysis methods of macrophages used in each study are demanded in order to allow reproducibility, comparability and reliability of the findings (Murray et al. 2014).

Dendritic cells (DCs) are involved in the recognition and internalization of PAMPs and DAMPs (Kono et al. 2014), they can process and present them as antigens to the cells of the adaptive immune system that would in turn be activated and, in case of B cells, produce antibodies and autoantibodies involved in the onset and exacerbation of autoimmune diseases. As for the macrophage constellation, the nomenclature and classification of DCs needs to be implemented, especially regarding the human system (Haniffa et al. 2013).

Besides their prominent role in orchestrating the inflammatory process *via* the release of soluble mediators, macrophages and DCs are key players in keeping and restoring tissue homeostasis: phagocytosis of apoptotic cells triggers anti-inflammatory responses, tissue repair mechanisms and promotes immunological tolerance (Poon et al. 2014).

1.2.3 Soluble mediators

Cytokines, chemokines and growth factors can be described as soluble mediators, molecules that are produced by a cell, released into the extracellular environment and sensed through specific receptors on other cells, modifying the status of the latter in order to adapt it to the contingent situation. Among the main functions of soluble mediators are hematopoiesis, modulation of the inflammatory response and tissue remodelling. Although nomenclature and classification of soluble mediators is continuously evolving, some terms are worthy to be defined. Interleukins are secreted proteins that act in intercellular communication between leukocytes, and they are usually categorized in different families based on similar functionality, receptors to which they bind, or structure homology (Akdis et al. 2016). The term chemokine, a fusion between the words chemotactic and cytokine, identifies soluble mediators with chemotactic activity. Chemokines influence migration of immune cells in both physiological conditions (i.e. mobilization of neutrophils from the bone marrow into the blood stream) and infection, regulating localization and transmigration between the systemic circulation and the affected tissues (Griffith et al. 2014).

1.2.4 Defects in the innate immune system: Severe Congenital Neutropenia

Severe congenital neutropenia (SCN, also known as Kostmann disease) has been first described in 1956 by Rolf Kostmann, who identified a recurrent disease phenotype studying the population of a geographical isolate in the north of Sweden (Kostmann, 1956). SCN is a life threatening autosomal recessive disease characterized by an abnormally low neutrophil count in the blood stream, usually less than 200 PMNs / µl of blood (Glaubach et al. 2014). The onset of the disease is typically in the first months of life: patients suffer of recurrent infections characterized by a decreased formation of pus at sites of infection. The reduced number of circulating PMNs in these patients is due to a maturation arrest of the myeloid progenitor cells in the bone marrow, between the promyelocyte and myelocyte stage of differentiation. SCN is caused by genetic mutations affecting a panel of genes, resulting in the general feature of increased apoptosis of the granulocytes. Mutations of the ELANE gene, codifying for neutrophil elastase, have been described in more than half of SCN patients. Alterations in the ELANE gene, affecting both exons and introns, have been first shown in patients affected by cyclic neutropenia (Horwitz et al. 1999), then described in SCN patients who displayed greater diversity in the mutation pattern (Dale et al. 2000). Defects in the translation products have been shown to lead to misfolded NE rather than loss of function mutants. Misfolded NE induced ER stress, reduced clonogenic capacity and triggered apoptosis of the granulocyte precursors (Grenda et al. 2007). SCN patients with mutation in the gene codifying for HCLS1-associated protein X-1 (HAX1) have also been reported. Affected patients are deficient for HAX1 in hematopoietic cells, while in healthy individuals the protein is ubiquitously expressed and localizes mainly to mitochondria (Klein et al. 2007). The lack of HAX1 causes mitochondrial membrane potential dissipation, resulting in increased cell death, both spontaneous and TNF-α induced; the apoptosis-prone phenotype of HAX1 deficient cells could be rescued by retroviral gene transfer of a plasmid construct bearing the HAX1 gene in both myeloid precursor cells and fibroblasts (Klein et al. 2007). Observations in mice models (Chao

et al. 2008), as well as in the original pedigree described by Kostmann, highlighted a link between increased apoptosis, due to HAX1 deficiency, and cognitive impairment of various level, defining a complex phenotype in HAX1-deficient SCN patients (Carlsson et al. 2008).

In 2014 Botzug et al. described a novel gene implicated in SCN. The group identified nine distinct mutations in the Jagunal Homolog 1 (JAGN1) gene, defining an apoptosis-prone phenotype in the patients´ PMNs. Described for the first time in *Drosophila melanogaster*, *jagunal* was shown to be functional to the reorganization of ER during oogenesis, which was compromised by its loss (Lee and Cooley, 2007). Neutrophils with mutant JAGN1 displayed aberrant ER structure and scarcity of granules as well as impaired glycosylation of multiple proteins. The exact function of JAGN1 in humans is still to be investigated, but it appears to have a role in ER trafficking (Botzug et al. 2014). Moreover, selective deletion of Jagn1 in mice hematopoietic cells led to an impaired killing capacity of neutrophils in *Candida albicans* infections, without affecting their phagocytic capacity or ROS production (Wirnsberger et al. 2014). MPO levels and ability to release it upon challenge with *C. albicans* were also impaired in Jagn1 deficient neutrophils as well as in PMNs isolated from patients. Notably, administration of GM-CSF, but not G-CSF, to JAGN1 mutant PMNs restored their expression of MPO as well as killing capacity of *C. albicans* (Wirnsberger et al. 2014).

The current therapy for SCN patients is administration of recombinant human G-CSF (rhG-CSF), which reverses neutropenia, leading to a normal neutrophil count in the blood stream. SCN patients bearing ELANE mutations and treated with G-CSF presented neutrophils with normal phagocytic capacity but reduced NE and MPO levels, as well as impaired ability to control *C. albicans* infections (Donini et al. 2007). The reduced ability to resolve infections, despite the conservation of phagocytic capacity of neutrophils from SCN patients, suggests that other mechanisms of pathogen killing could be impaired: for example, NET production could be affected as well as NET composition, resulting in higher susceptibility to infection and less prompt resolution.

1.3 NEUTROPHIL EXTRACELLULAR TRAPS (NETs)

Brinkmann and colleagues reported for the first time the release of neutrophil extracellular traps (NETs) as a new killing strategy employed by neutrophils upon death (Brinkmann et al. 2004). NETs are three dimensional web-like structures consisting of a backbone of nuclear chromatin (DNA and histones) decorated with granule proteins, mainly neutrophil elastase (NE) and myeloperoxidase (MPO). NETs can be triggered by different stimuli, such as phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS), calcium ionophores and different kinds of bacterial and fungal pathogens (Kenny et al. 2017). The extracellular traps are capable of capturing bacteria and degrading them at the site of infection (Brinkmann et al. 2004).

1.3.1 Mechanisms of NET formation

The process of NET formation and release has been defined as “NETosis” (Steinberg and Grinstein, 2007), a kind of cell death displaying features that differ from both apoptosis and

necrosis. During NETosis, the nuclear envelope disintegrates first and nuclear content mixes with the cytosol, then loss of intracellular membranes leads to the disappearance of cytoplasmic organelles. Lastly, the integrity of plasma membrane is lost and the NETs are released (Fuchs et al. 2007). Starting from the observation that the nuclear membrane of neutrophils is broken down during this process, similarly to the one of cells undergoing mitosis, Amulic and colleagues showed that PMNs, although terminally differentiated, upregulate core proteins in the cell cycle machinery when triggered for NET production, in order to mediate disassembly of the nuclear envelope (Amulic et al. 2017). The concept of NETosis as a form of cell death has been partly challenged. Kubes and colleagues described "vital NETosis" showing that NET-forming neutrophils in vivo can conserve their chemotactic function and cytoplasmic structures, including granules, and speculated that PMNs retain the necessary features to kill bacteria through conventional mechanisms (Yipp et al. 2012 and Yipp and Kubes, 2013). Furthermore, Pilsczek et al. described an early form of NET release via nuclear blebs delivered through the cytoplasm into the extracellular environment, thus maintaining the integrity of the plasma membrane (Pilsczek et al. 2010). The latest recommendation by the Nomenclature Committee on Cell Death has now adapted in light of the larger body of knowledge, and suggests to avoid the term NETosis whenever supporting evidence of cell death is not provided, recommending the choice of NET release or NET extrusion instead (Galluzzi et al. 2018). The origin of NET DNA has also been object of debate. Yousefi and colleagues revealed the presence of mitochondrial DNA as NET backbone, without detecting nuclear DNA (Yousefi et al. 2009). Considering these findings, it has to be taken into account that NETs can be triggered by a variety of stimuli, and that NETosis, as well as NET production, can be studied at different time points, thus potentially explaining diverse outcomes.

Reactive oxygen species (ROS) are required for NET formation through the activation of NADPH oxidase, as patients suffering from chronic granulomatous disease (CGD) that have impaired NADPH oxidase function are defective in NET production (Fuchs et al. 2007). Conversely, an increasing number of reports indicate that NET release, for example in response to calcium influx or alteration in lipid composition in the plasma membrane, does not involve NADPH oxidase, but ROS of mitochondrial origin instead (Douda et al. 2015 and Neumann et al. 2014a). During NET formation, ROS trigger MPO-dependent dissociation of a granule complex containing NE, MPO and other granule proteins, so that NE is released into the cytosol and can digest F-actin allowing for the loss of cytoplasmic structure. NE can then translocate to the nucleus where it can partially cleave histones consenting chromatin decondensation (Papayannopoulos et al. 2010 and Metzler et al. 2014). Chromatin decondensation is also to be attributed to the calcium-dependent action of PAD4 that is capable of citrullinating histones (citrullinated histones are found in NETs) thus permitting a relaxed conformation of the nuclear chromatin (Rohrbach et al. 2012).

1.3.2 Role in killing of pathogens

Neutrophils release extracellular traps in response to a wide variety of bacteria, both against Gram-positive and Gram-negative bacteria (Brinkmann et al. 2004). The ability of NETs to

degrade pathogens *in situ* is to be attributed to the protease activity of NE, the peroxidase action of MPO (Parker et al. 2012a) as well as the antimicrobial properties of histones and LL-37 peptides. Nevertheless, some bacteria seem to be endowed with mechanisms to escape NETs. Both *Streptococcus pneumoniae* and *Staphylococcus aureus*, for example, are trapped in NETs but not killed, due to their capability of degrading NETs using surface nucleases (Beiter et al. 2006 and Thammavongsa et al. 2013). On the other hand, NETs-associated LL-37 has been described to protect NETs from degradation by nucleases from either *S. pneumoniae* or *S.aureus* (Neumann et al. 2014b).

Interestingly, neutrophils seem to have the ability to modulate their defense strategy according to the size of the pathogen. The internal pool of NE can be directed towards phagocytosis or NET formation, depending on the microbe being in small form or large aggregates, such as hyphae (Branzk et al. 2014). The dectin-1 receptor, a receptor that recognizes β-glucan, has been indicated to mediate the size recognition, but this role is debated (Bryd et al. 2013 and Bachiega et al. 2015). *Candida albicans* is a clinically relevant pathogenic fungus, which presents the dimorphic phenotype of both cellular and filamentous hyphae forms. The capacity of NETs to counteract both forms of *C. albicans* accounts for 20-30% of the overall killing capacity (Urban et al. 2006), however, when *C. albicans* forms highly dense aggregates and layers biofilms, extracellular polysaccharide matrixes that shield the fungal colonies, NETs seem to be ineffective (Kernien et al. 2017 and Johnson et al. 2017).

1.3.3 Role in diseases

The same properties of NETs that constitute a clear benefit in host defense against microbes, such as the mesh structure providing a platform for proteolytic activity, have a role in the onset or aggravation of several pathologies (Papayannopoulos, 2017).

A detrimental outcome linked to the NET structure is the promotion of thrombosis in the vasculature. Fuchs et al. showed how NETs provide a scaffold for platelet adhesion and aggregation promoting fibrin deposition. Treatment with DNaseI prevented thrombosis, thus confirming the role of NETs in this process (Fuchs et al. 2010). Furthermore, NE on NETs can use its proteolytical activity to cleave platelets receptors and coagulation inhibiting factors, thus promoting platelet accumulation and coagulation (Papayannopoulos, 2017). In addition, NETs have been described as a driving cause of pancreatitis. Similarly to the blood vessels, the pancreatic duct can be occluded by excessive formation of extracellular traps, which is triggered by bicarbonate or calcium carbonate crystals, highly present in the pancreatic juice (Leppkes et al. 2016).

Involvement of NETs in the formation of atherosclerotic plaques has been investigated in both human and mouse models. Oxidized low density lipoproteins, hallmark of atherosclerosis, can induce NET release through TLR2 and TLR6 (Awasthi et al. 2016). In turn, NETs were found to prime macrophages for pro-IL-1β production, and consequent exposure to cholesterol crystals activated inflammasome-mediated secretion of IL-1β (Warnatsch et al. 2015). Moreover, ApoE-deficient mice fed with high fat diet developed atherosclerotic plaques rich

in cholesterol crystals surrounded by NETs, and in a double knock-out model where NE was also deleted, a reduced presence of NETs was assessed (Jorch and Kubes, 2017).

Another area of study that is opening up in the last 5-6 years investigates the role of NETs in cancer. NET-rich microenvironments have been associated with a few kind of tumors in both mice and humans, but a clear evidence or link still needs to be delineated. Intestinal tumors with a disruptive phenotype for the intestinal barrier ease the leakage of LPS into the circulation, thereby promoting NET release and consequent coagulation in the surrounding vasculature, supporting the development of a tumorigenic microenvironment (Jorch and Kubes, 2017 and Papayannopoulos, 2017). An original study by Cools-Lartigue et al. suggested a role for NETs in the establishment of tumor metastasis. The NET platform appeared to trap circulating tumor cells, favoring settlement of these cells and consequent metastatic formation (Cools-Latigue et al. 2013).

High levels of glucose are capable of promoting NET release. PMNs isolated from patients with type I or type II diabetes were more prone to release the traps when stimulated with ionomycin, compared to healthy controls. Also, diabetic mice displayed high levels of citrullinated histones and a delay in wound healing, which were recovered by deletion of *Pad4* or administration of DNaseI, pointing at a role for NETs in the process (Wong et al. 2015). Moreover, in a cohort of type II diabetes patients related to a control population, significantly higher levels of circulating cell-free dsDNA and NE were assessed, compatible with a predisposition for NETs production (Menegazzo et al. 2015).

1.3.4 Role in autoimmune diseases

Autoimmune diseases are characterized by the development and production of antibodies directed against self-antigens. Anti-neutrophil cytoplasmic antibodies (ANCAs) are directed against intracellular neutrophil constituents: considering the known components of NETs (Urban et al. 2009), it is not difficult to imagine that a prompt removal from the extracellular environment is desirable in order to prevent antigen presentation and consequent auto-antibodies production. Kessenbroch et al. described the presence of MPO and PL3 containing NETs *ex vivo* in kidney biopsies of small-vessel vasculitis patients (Kessenbroch et al. 2009). In this study, ANCAs have been shown to cause NETs production, triggering a self-sustaining loop that can exacerbate the disease.

Similarly, neutrophils from patients suffering from rheumatoid arthritis (RA) presented enhanced NETosis compared to healthy controls or patients suffering from osteoarthritis. Furthermore, anti-citrullinated protein antibodies (ACPAs) present in high level in RA serum, were shown to induce NETosis and bind to NETs (Khandpur et al. 2013). Fibroblast-like synoviocytes, which play a major role in cartilage remodeling, were shown to have the capability of internalizing NETs through a RAGE-TLR9 mechanism, and consequently upregulate MHC-II molecules, compatible with the upregulation of pro-inflammatory cytokines (Carmona-Rivera et al. 2017).

The autoimmune disease that perhaps presents the tightest connection with NETs is SLE. Herrmann et al. reported reduced clearance of apoptotic cells by monocyte-derived macrophages from SLE patients, highlighting the deficiency of rapid and effective clearance of cell debris rather than abnormal apoptosis (Herrmann et al. 1998). Impairment in the clearance of apoptotic cells can lead to secondary necrosis thus exposing intracellular antigens triggering autoimmunity. Similarly, ineffective removal of NETs has been linked to the pathogenesis of SLE. According to the degradation rate of NETs by sera of SLE patients, two subpopulations have been described: one population whose sera can dismantle NETs efficiently and one not. The poor NET degradation has been linked to high levels of anti-NETs antibodies that shield NETs from DNaseI mediated digestion (Hakkim et al. 2010). Anti-NETs antibodies are also capable to recruit C1q on the NETs-immunocomplexes; C1q can in turn directly inhibit DNaseI thus further preventing NET degradation (Leffler et al. 2012). Conversely, Farrera and Fadeel showed that C1q enhances NET uptake by human monocyte-derived macrophages from healthy individuals and physiological concentration of DNaseI in serum are not sufficient to fully degrade NETs, thus highlighting the need for functional macrophage-mediated clearance of the extracellular traps (Farrera and Fadeel, 2013). The antimicrobial peptide LL-37 has been identified on NETs from SLE patients; the presence of LL-37 not only prevents DNaseI-mediated degradation of NETs but it is necessary to activate IFNα production by plasmacytoid DCs (Lande et al. 2011). Likewise, macrophages from SLE patients are more prone to release IL-1β via NLRP3 inflammasome activation upon LPS priming and exposure to LL-37, compared to healthy controls (Kahlenberg et al. 2013).

From these observations, it appears clear how a functional battery of DNAses is of paramount relevance in preventing onset or exacerbation of SLE. Present in both murine and human serum, DNase1L3, produced by macrophages and DCs, has been shown to have the capacity of digesting microparticle-associated DNA of apoptotic origin (Sisirak et al. 2016) and to prevent vascular occlusion by NET DNA in mice (Jiménez-Alcázar et al. 2017). Moreover, a loss-of-function mutant variant of *DNASE1L3* has been associated with SLE (Al-Mayouf et al. 2011). Tightly linked to the IFN signature in SLE, aberrant sensing of cytosolic DNA needs to be prevented in order to avoid the immune response. Cytosolic DNA can undergo degradation by TREX1, a mechanism exploited also by viruses, as HIV, to keep their genomic material below threshold of detection (Yan et al. 2010). TREX1 is an ER-bound nuclease with cytosolic activity aimed to dismantle self-DNA to avoid its recognition by the cGAS-STING machinery (Barber 2015). Mutations in the gene encoding TREX1 have been associated with mislocalization of the protein, leading to the rise of SLE phenotypes in both mice and humans (Lee-Kirsh et al. 2007, Fye et al. 2011 and Grieves et al. 2015).

1.4 ENGINEERED NANOMATERIALS

According to the definition proposed in 2011 by the European Commission, a nanomaterial is "a material with one or more external dimensions in the size range of 1-100 nm" (Commission Recommendation of 18 October 2011). This definition clearly focuses on just one aspect of the particles, leaving room for discussion on what features have to be considered relevant to define,

especially in the framework of risk assessment for regulatory purposes (Maynard, 2011 and Gallud and Fadeel, 2015). The nanotechnology era has dramatically increased the production and use of nanomaterials on a global scale in everyday life: nanomaterials are used in fuel and grease, cosmetics, catalysts, electronics and drug carriers (Nel et al. 2006). Humankind has been exposed to nano-sized matters throughout its existence: from campfire combustion products to viruses, the human body has learnt to deal with a wide variety of nanoscale particles. The nanotechnology era has exponentially increased the variety of particles available (metal-based, organic and carbon based), as well as the chances for exposure, both accidental and occupational, to such an extent that questions about the safety of nanomaterials and the need for scientific research have arisen, leading to the development of nanotoxicology as a new field of investigation.

1.4.1 Toxicity of nanomaterials

Nanotoxicology aims to investigate the interaction between engineered nanomaterials and biological systems, emphasizing the fact that life itself is made possible by biological systems interacting on a nanosized scale (Shvedova et al. 2010). This interaction happens as a consequence of the biological systems encountering nanomaterials, a situation that can occur in the scenarios of occupational or accidental exposure, as well as voluntary exposure in the case of nanoparticles employed for biomedical applications. Occupational exposure might pose a risk when considering the proximity of the materials as well as the frequency of potential exposure, mainly through skin and airways (Kagan et al. 2010a), however it presents the opportunity of prevention by proper risk assessments, based also on the possibility to characterize the nanomaterials at site of production. On the other hand, accidental exposure might occur due to the inadvertent generation of nanomaterials, for instance in vehicle exhaust, as in the case of carbon nanotubes found in bronchoalveolar lavage fluids of asthmatic children in Paris (Kolosnjaj-Tabi et al. 2015), where characterization of the materials, or mixtures of materials, cannot be easily performed.

When assessing the effects of nanomaterials on biological systems, the intrinsic properties of the material have to be considered, in particular when the ultimate goal is to exploit those properties for medical purposes, minimizing undesired effects (Fadeel et al. 2015). Size is of course relevant: the smaller the particle, the higher the surface area that can interact with the biological system. Also, different sizes in the nano-range can determine the ability of particles to cross barriers (blood-brain barrier, placenta, cellular membrane, nuclear membrane) and to be engulfed by professional phagocytes. Together with size, shape has also its relevance. Learning from the asbestos paradigm, high aspect-ratio particles, as fiber-shaped ones, that maintain a certain rigidity and are biopersistent in the biological environment bear characteristics that are at the base of their toxicity (Tran et al. 2011). As nanoparticles enter in contact with biological fluids, they gain a coating, or "corona" of biomolecules, such as protein and lipids, that determines the "biological identity" of the particle, or, in other words, what the cells "see" (Monopoli et al. 2012). Of note, the adsorbed proteins on the nanoparticle can change its surface properties, but the particle may also affect protein folding and induce

conformational changes that can lead to exposure of epitopes triggering unexpected cellular responses (Farrera and Fadeel, 2015). Surface charge and coating of the particle can determine the quality of the bio-corona, and different shapes of particles with the same chemical composition can lead to different responses.
When assessing biocompatibility of nanomaterials, it is of primary importance to exclude endotoxin contamination that can be due to the synthesis process as well as further manipulation of the material. The importance of endotoxin testing is even more relevant when studying the interaction between nanoparticles and the immune system (Bhattacharya et al. 2013). Excluding endotoxin contamination allows relating effects of the nanomaterial on the biological system to its intrinsic properties, disregarding confounding factors.

For the evaluation of toxicity of nanoparticles, some considerations on doses and endpoints need to be regarded. In the multitude of studies addressing toxic outcomes of engineered nanoparticles, different metrics to describe the administrated *in vitro* dose are used, and correlation between the various metrics results not always straightforward. Furthermore, doses used are often not calibrated in a benchmark dose perspective, frequently resulting unrealistically high. Another issue faced by nanotoxicologists is to consider whether the nominal dose (the amount administrated) corresponds to the delivered dose (the amount that effectively reaches the cells) and which share of the delivered dose is eventually interacting with the cells or is ultimately internalized (Fadeel et al. 2015).

Regarding the endpoints taken into account in nanotoxicology, an evaluation of cell death is often used as a first measure of toxicity. Returning a rough information, this evaluation is not very useful as unique endpoint, but it is functional to establish experimental conditions for investigating more fine parameters, as oxidative stress or damage to nucleic acids. For instance, genotoxicity driven by nanoparticles might arise from both direct interaction between the nanomaterial and the genetic material, as well as in an indirect form, *via* generation of nanoparticle-induced ROS, which in turn produce the oxidative damage on the nucleic acids. Nanomaterials in the 8-10 nm size range can cross the nuclear envelope and directly interact with DNA, while larger particles would gain access to DNA during the mitotic process (Magdalenova et al. 2014). Genotoxicity of nanoparticles (i.e. oxidation of DNA bases, single- or double-strand brakes, interference with DNA repair mechanisms) might not result in swift cell death, and have effects, for instance, on transcription of genes, cell proliferation and tumorigenesis, therefore, appropriate assays for endpoints other than cell death are to be employed.

1.4.2 Graphene-based nanomaterials

Carbon based nanomaterials are, to date, one of the most active fields of interest in nanotechnology due to their versatility and variety of potential applications (Bhattacharya et al. 2016). Graphene, isolated for the first time in 2004 by Novoselov and co-workers, is a planar monolayer of carbon atoms bound together in hexagonal pattern, bearing excellent thermal and electrical conductivity as well as outstanding mechanical features, like strength and lightness

(Tonelli et al. 2015). One of the most relevant derivatives of graphene is graphene oxide (GO) which presents, on the surface of the carbon monolayer, oxygen epoxide groups as well as carbonyl, hydroxyl and negatively charged reactive oxygen groups that provide good water solubility to the material (Tonelli et al. 2015).

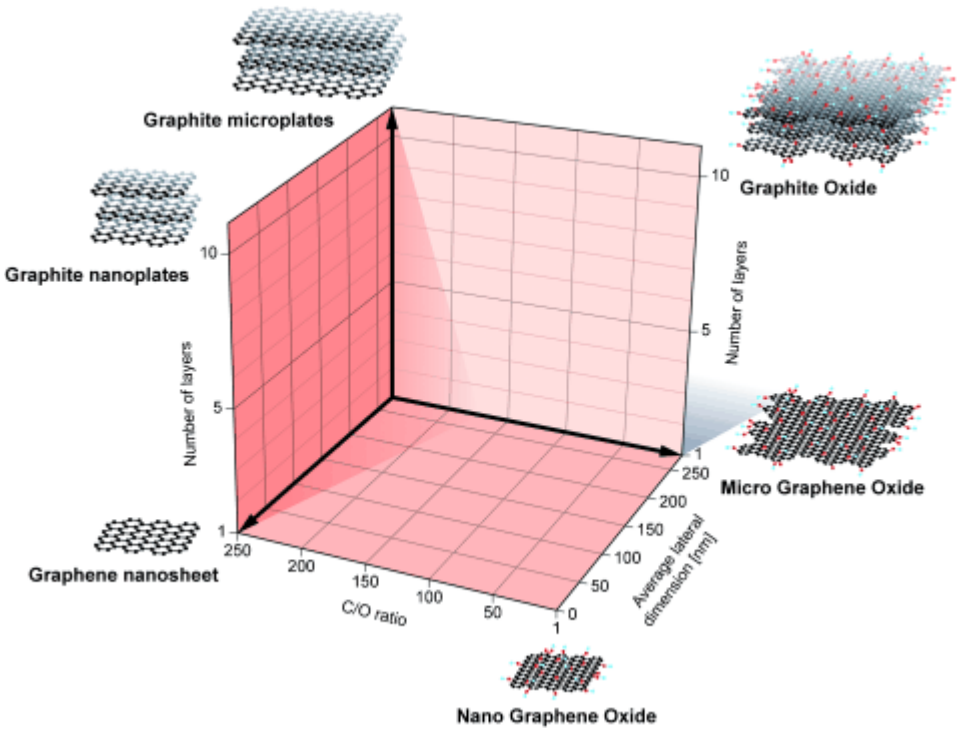

Figure 2: Schematic classification of different kinds of graphene based on their fundamental properties: number of layers, lateral dimension, and carbon/oxygen ratio. The schematic drawings at the corners represent ideal cases. Reproduced from Wick et al. 2014, with permission from John Wiley & Sons, Inc.

Besides the degree of oxidation, or carbon-to-oxygen ratio, which determines the hydrophobic/hydrophilic properties of the material and the consequent dispersibility in aqueous solutions, two other structural characteristics of graphene, with relevant implication for toxicity assessment, were taken into account by Wick et al. in proposing an approach to classify the different kinds of graphene, as depicted in Figure 2 (Wick et al. 2014). The lateral size determines the deformability of the material as well as the maximal dimension, which can vary from nanometers to micrometers and the number of layers defines both thickness and rigidity. These properties are key factors in the encounter with biological system as they influence cellular uptake and the ability to cross biological membranes (Wick et al. 2014).

Graphene-based materials of biomedical interest are not limited to the different kinds of graphene depicted above. For instance, carbon nanotubes (CNTs) are cylinders made by graphene sheets: they can be constituted either by a single graphene sheet (single-walled carbon nanotubes, SWCNTs) or by several concentric layers of graphene (multi-walled carbon nanotubes, MWCNTs). CNTs bear diameters within the nanometer range, while length can span up to the micrometer range (Bhattacharya et al. 2016).

Due to its properties, graphene can be easily exploited for further surface functionalization, being a versatile tool to develop biosensors or drug carriers (Tonelli et al. 2015). Amongst the different applications tested, graphene-based materials have been shown to present suitable scaffolds for tissue engineering, providing a platform for cell adhesion, proliferation and differentiation (Nayak et al. 2011 and Park et al. 2011). Furthermore, due its structure, graphene can interact with nucleic acids through its hydrophobicity and π-π stacking, protecting them from degradation by nucleases (Lei et al. 2011), thus presenting a promising tool for gene delivery.

1.4.3 Interactions of graphene-based nanomaterials with the innate immune system

The immune system has evolved to defend the organism from potentially harmful non-self or modified-self matter, recognizing a wide variety PAMPs and DAMPs through PRRs on innate immune cells. Similarly, the notion of NAMPs has been proposed, based on the rationale that the innate immune system may treat nanomaterials in a similar fashion (Farrera and Fadeel, 2015 and Mukherjee et al. 2018b). Once encountered an organism, nanomaterials for biomedical applications would interact with the immune system, either in the systemic circulation or in localized tissues.

GO has been shown to not significantly affect cellular viability, ROS production or pro inflammatory cytokine release by HMDMs, whereas promoting macrophage activation and CD86 expression (Russier et al. 2013). Conversely, GO could activate both mouse and human macrophage cell lines in a size dependent manner, with larger graphene flakes triggering more robust activation and pro inflammatory cytokine production. On the other hand, as no endotoxin testing was performed in the study, the possibility that the effects reported were dependent on contamination cannot be ruled out (Ma et al. 2015). In another study, where control for endotoxin contamination was performed and the material classified as "endotoxin-free", GO did not elicit loss of cell viability in primary HMDMs, but was promoting inflammasome activation and IL-1β secretion (Mukherjee et al. 2018a). Interestingly, evidence of large GO flakes lining along the plasma membrane of macrophages in a sort of "coating" has been reported and defined as "masking effect", postulating that this phenomenon would be instrumental for either internalization of the material or loss of function or viability. The encounter of GO with the lipid bilayer of macrophage plasma membrane resulted also in piercing of the membrane or lipid extraction, depending on the different kinds of GO used (Mukherjee et al. 2017). Remarkably, macrophages were shown to be capable of digesting graphene based nanomaterials, namely CNTs, in a superoxide-peroxinitrite dependent manner (Kagan et al. 2014). The effect of different types of GO on DCs has been examined in a few studies, both *in vitro* and *in vivo*, focusing on the outcome for antigen presentation. In one study, GO was found to decrease antigen presentation to T cells, while other studies using GO to adsorb the antigen of interest revealed enhanced internalization of the GO-antigen complexes by DCs and subsequent antigen presentation (Mukherjee et al. 2017). At the time of compiling this thesis, a lack of studies focusing on the interactions

between neutrophils and GO has been noticed, thus highlighting the relevance of the ones herein presented. However, effects of graphene based nanodiamonds have been investigated in a recent study, showing that the material would elicit NET formation in a mouse model (Muñoz et al. 2016). Moreover, in the same fashion as they capture conventional pathogens, NETs were shown to entrap and degrade oxidized SWCNTs in an MPO-mediated mode in an acellular system (Farrera et al. 2014). Furthermore, the administration of nanotubes to mice via pharyngeal aspiration elicited granuloma formation in the lung as well as a marked inflammatory response, whereas MPO-degraded nanotubes did not (Kagan et al. 2010b). Taken together, these studies suggest that the effects of graphene based nanomaterials on immune cells could be exploited when formulating vaccines, as they could act as adjuvants, and, as they present the possibility of not being biopersistent, could pose a limited risk for deleterious side effects.

2 AIM OF THE STUDY

Since their discovery in 2004, NETs have gained considerable attention in both basic cell biology and immunology as well as in clinical medicine, bridging together different areas of investigation. The overall aim of this study is to deepen the knowledge of NETs in a multidisciplinary context, with a focus on the mechanisms of formation and disposal of NETs, and their roles in both infectious and non-infectious conditions.

The specific aims are:

- To study the inflammatory responses to NETs in macrophages and DCs
- To identify the nucleases involved in degradation of NETs in phagocytic cells
- To assess whether JAGN1 is involved in the production or function of NETs
- To determine if and how GO sheets of different lateral dimensions trigger NETs
- To study extracellular biodegradation of GO and toxicity of the degradation products

3 METHODOLOGIES

In this section, critical considerations on methods and models used in the constituent papers of this thesis, as well as a description of the experimental protocols are provided. A detailed description of the experimental conditions is available in the method section of each constituent paper.

3.1 CELL MODELS

3.1.1 Primary human cells

Primary cells offer a number of advantages, as well as some downsides compared to cell lines. As *ex vivo* model, primary cells bear the characteristics of the tissue of origin and higher resemblance to the *in vivo* scenario, furthermore, in the case of non-cancerous primary cells, they present lower mutation rate compared to tumor cell lines. On the other hand, primary cells might require time-consuming isolation procedures and more careful culture methods, while presenting a generally shorter lifespan in culture. Additionally, the variability between batches of isolated primary cells reflects individual variability in the population of donors, which is lost with the use of clonal cell lines.

Primary human neutrophils from healthy blood donors were isolated from buffy coat using density gradient centrifugation followed by gradient sedimentation in a 5% dextran solution and hypotonic lysis of residual erythrocytes. The same procedure was applied for the isolation of primary neutrophils from the SCN patient. For NET production, freshly isolated neutrophils were maintained in serum-free RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and treated with 25 nM PMA for 2 or 3 hours. Production of NETs was performed in absence of serum, since heat-stable nucleases, capable of degrading NETs, are present in sera ordinarily used as cell culture supplement (von Köckritz-Blickwede et al. 2009). Purified NETs were obtained removing PMA-containing supernatants and adding fresh HBSS or RPMI. Thorough pipetting was carried out and the mixture was centrifuged at 1500 rpm for 5 min. Eventually, supernatant containing NETs were retrieved and used for further assays.

Buffy coat from healthy blood donors was the source for *primary monocytes*, isolated *via* density gradient centrifugation followed by positive selection with CD14 MACS magnetic beads. Monocytes were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in the presence of 10% FBS. Differentiation into *macrophages (HMDMs)* was performed using 50 ng/mL recombinant human M-CSF for 3 or 4 days, while culture medium was supplemented with 16 ng/mL recombinant human GM-CSF and 50 ng/mL recombinant human IL-4 in order to obtain *dendritic cells (MDDCs)*.

3.1.2 Cell lines

The human acute promyelocytic leukemia cell line *HL-60* (Collins et al. 1977) was purchased from ATCC and maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FBS in absence of antibiotics. HL-60 cells can be differentiated into neutrophil-like cells using various stimuli, like all-trans retinoic acid, dimethylformammide and dimethyl sulfoxide (DMSO). In our study (Paper II) we achieved optimal differentiation using 1.25% DMSO for 5 days, substituting the medium with fresh one after 3 days of culture (Arroyo et al. 2002). The HL-60 model was chosen for its particular suitability to our scope, being a promyelocytic cell line, which resembles the stage of maturation arrest in the development of myeloid progenitors in the bone marrow of SCN patients. Furthermore, we could establish an HL-60 model in which transient downregulation of *JAGN1* was significant and not interfering with the differentiation process.

The *BEAS-2B* human bronchial epithelial cell line was purchased from ECACC and cultured, according to the manufacturer's guidelines, in bronchial epithelial cell growth medium (BEGM) supplemented with GA-1000 (gentamicin sulfate and amphotericin-B), retinoic acid, epinephrine, transferrin, triiodothyronine, bovine pituitary extract, insulin, hydrocortisone and recombinant epidermal growth factor (EGF). Cells were seeded on surfaces pre-coated for 2 h with 0.01 mg/mL fibronectin and 0.03 mg/mL bovine collagen type I in BEGM basic medium with addition of 0.01 mg/mL bovine serum albumin and 0.2% penicillin-streptomycin. BEAS-2B cells are SV40-immortalized normal bronchial epithelial cells explanted from autopsies of cancer-free individuals (Reddel et al. 1989) that are not tumorigenic when injected in nude mice. In Paper IV, BEAS-2B cells were selected to study DNA damage originating from degradation products of GO, as this cell line is noncancerous and a relevant model for inhalation exposure.

3.2 NET FORMATION AND DEGRADATION

In order to evaluate formation of NETs, complementary methods have been used: qualitative imaging using scanning electron microscopy or confocal microscopy with staining for the major NET components, and quantitative measurements *via* SYTOX green assay or NE assay.

3.2.1 Scanning electron microscopy (SEM)

For SEM imaging (Paper III), freshly isolated neutrophils were seeded on poly-L-lysine coated coverslips and exposed to either PMA, control media or graphene flakes for 3 h. Fixation was performed with 2.5% glutaraldehyde in phosphate buffer, samples rinsed and sequentially placed in 70% ethanol for 10 min, 95% ethanol for 10 min, absolute ethanol for 15 min followed by acetone treatment. Samples were dried in a controlled CO_2 system, mounted on aluminium stub and coated with carbon prior to be analyzed in an Ultra 55 field emission microscope (Zeiss, Oberkochen, Germany).

3.2.2 Confocal microscopy

Freshly isolated primary human neutrophils were seeded in poly-L-lysine coated coverslips and stimulated with PMA for 2 h, then cells were fixed with 2% PFA in PBS and stained for MPO (Paper I and Paper II), LL-37 (Paper I), NE (Paper II and Paper III) and counterstained with DAPI. Visualization of the samples was performed on a ZEISS LSM510META confocal microscope. For the HL-60 cells, incubation with PMA was carried out for 3 h and, after fixation as described above, the sample were probed for MPO and counterstained with SYTOX green for visualization of the DNA fibers. The mere staining and imaging of extracellular DNA fibers can lead to controversial interpretation of the results, as extracellular DNA can be the product of other cell death modalities, as necrosis. It is therefore important, when assessing the presence of NETs, to combine DNA staining with immunostaining for at least one of the proteinaceous component of NETs, and verifying their co-localization (Jorch and Kubes, 2017).

3.2.3 SYTOX green assay

With the aim to have a measure of NET formation, HL-60 cells, transfected with either control siRNA or siRNA targeting JAGN1, were seeded in HBSS at a density of 0.1 x 10^6 cells / well in a black 96-well plate and incubated with or without PMA for 3 h. Incubation with SYTOX green (5μM) was performed for 15 min prior to analyze fluorescence emission at 523 nm in an Infinite F200 Tecan plate reader. SYTOX green is a DNA-intercalating dye, which can not cross the plasma membrane of living cells, thus being able to detect only extracellular DNA, upon the binding of which it becomes highly fluorescent (Vong et al. 2013).

3.2.4 NE assay

For measuring NET production in response to GO, the SYTOX green assay was found to be not suitable, as the material was interfering with the measurements, so the NE assay was chosen instead. Freshly isolated neutrophils were seeded at a density of 0.2 x 10^6 cells / well in 96-well plates and exposed to 12.5 μg/mL of small or large GO flakes, 25 nM PMA, 10 mM MβCD or control RPMI media for 3 h. In some experiments, the cells were pre-incubated for 30 min with one of the following inhibitors: 10 μM DPI, 500 μM Trolox or 10 μM MitoTEMPO, or the experiment was conducted in the presence or absence of 10 mM EGTA. After exposure, the samples were subjected to extensive pipetting and centrifuged at 1600 rpm for 5 min, then 150 μl of supernatants were retrieved. To digest NET DNA and let NET-bound NE be available for the assay, 50 μl of the so-obtained supernatants were incubated with 0.3 U/μL of DNAseI for 15 min. Then, 50 μl of assay buffer containing N-(methoxysuccinyl)-Ala-Ala-Pro-Val-4-nitroanilide were added and absorbance at 405 nm was detected with an Infinite F200 Tecan plate reader after 3 h of incubation. N-(methoxysuccinyl)-Ala-Ala-Pro-Val-p-nitroanilide is a specific substrate for NE, which enzymatically cleaves the substrate releasing 4-nitroaniline, whose absorbance can be detected at 405 nm (Castillo et al. 1979).

3.2.5 Extracellular degradation of NETs

Agarose gel electrophoresis was performed with the aim to check the extracellular degradation of NETs. After addition of purified NETs to MDDCs, supernatants were collected at different time points and enzymatic activity of DNase1L3 was promoted with addition of 2 mM $CaCl_2$ and 2 mM $MgCl_2$ for 1 h at 37°C. Samples were then subjected to 1% TAE agarose gel electrophoresis, detection was performed with Sybr green I and the gel was visualized in a Molecular Imager scanner (Bio-Rad). Undigested purified NETs, being of high molecular weight, would not run through the gel, packing instead close to the loading well, while fragmented digested NETs would run through the gel, resulting in the disappearance of the signal from the area close to the loading well.

3.3 UPTAKE OF NETs BY MACROPHAGES AND DENDRITIC CELLS

Uptake of NETs was evaluated using *confocal microscopy*. For some experiments, pre-incubation with CellTracker Orange for 30 min was performed, otherwise, HMDMs or MDDCs were seeded on glass coverslips and stained for 15 min with Hoechst 33342. Then, media containing Hoechst staining were removed, samples washed, and purified NETs, pre-incubated with SYTOX green for 15 min, were added to the cells. For some experiments, Lysotracker Red was added 30 min before fixation of the samples, in order to visualize lysosomes. Incubation of purified NETs with cells was carried out for 1 h, then fixation with 2% PFA in PBS was performed, coverslips were mounted on glass slides and visualized in a ZEISS LSM 880 confocal microscope. The CellTracker dye, which binds unspecifically to all the proteins in the cells, was used in order to confirm the intracellular localization of the engulfed NETs. The cell-permeable DNA dye Hoechst 33342 was chosen to mark nuclear DNA of the phagocytes, and removed before addition of the SYTOX green-labeled NETs in order to allow for the discrimination between DNA of phagocyte origin and NET DNA. Extranuclear green DNA dots were considered as engulfed NETs and cells presenting such phenotype were considered as positive when scoring the samples.

3.4 GENE EXPRESSION AND DOWNREGULATION

Constitutive expression of relevant genes in Paper I and Paper II was determined quantifying specific mRNA transcripts using quantitative Real-Time PCR (RT-qPCR). The same technique was adopted to assess the degree of downregulation of selected genes after transfection with specific siRNAs.

3.4.1 Real-Time quantitative PCR

In order to purify total RNA from samples intended to be analyzed with RT-qPCR, a silica membrane column based method was chosen over organic phase extraction methods. Besides avoiding the use of chloroform-containing reagents, the column purification yields high quality RNA, with minimal proteinaceous impurities. Furthermore, the QIAGEN RNeasy Mini Kit used for extraction of total RNA, allows for on-column DNA digestion, thus minimizing DNA contamination of the samples. Total purified RNA was quantified using the Nanodrop

platform; thereafter 1 µg of total RNA was subjected to reverse transcription with the Revert Aid-H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Samples were analyzed using specific primers and SYBR green probing on an Applied Biosystem 7500 Real-Time PCR System.

3.4.2 Silencing of DNaseII, TREX1, DNase1L3 and JAGN1

Silencing of *DNaseII* and *TREX1* in HMDMs was performed delivering 3 µM of specific siRNAs into the cells, after 2 days from isolation, using the Amaxa Nucleofector 2B electroporation platform, following the guidelines from the manufacturer. Silencing efficiency was evaluated after further 2 days of culture of the cells in the relevant media. For downregulation of *DNASE1L3* and *JAGN1* the method of choice for transfection of specific siRNAs was the Lipofectamine 3000 reagent used in complex with 75 nM siRNA targeting *DNASE1L3* or 50 nM of specific *JAGN1* siRNA. Electroporation is a quite harsh transfection method, which involves the use of high-voltage electric pulses, applied for short time, that perturb the plasma membrane structure creating pores, which allow the transfer of nucleic acids into the cells (Potter and Heller, 2018). This is quite a straightforward technique and allows for the transfection of a large number of cells in short time, however, cell viability might be markedly impaired. The use of cationic lipids, as Lipofectamine 3000, on the other hand, displays generally lower cytotoxicity in relation to electroporation. Cationic lipids are exploited to form complexes with the negatively charged nucleic acids, such as siRNAs, with an overall positive charge of the complex, allowing them to be attracted to the cell membrane and ease internalization (Kim and Eberwine, 2010).

3.5 PROTEIN EXPRESSION

3.5.1 Western blot

Western blot experiments were used in order to have semi-quantitative information on protein expression and / or release in purified NETs or cell culture supernatants and were carried out following standard procedures. For MDDCs (Paper I) and primary neutrophils (Paper II) cell pellets were lysed with Radioimmunoprecipitation assay (RIPA) buffer, while HL-60 cell pellets were lysed in CellLytic M buffer. RIPA buffer is the buffer of choice for the lysis of neutrophils, as it allows for thorough membrane extraction, however, its preparation is time-consuming and the shelf life short, compared with the more stable CellLytic M buffer. 4-20% Tris-Glycine gradient gels were used in Paper II, as they warranty higher resolution for low molecular weight proteins, as MPO (15 kDa subunit) and FLAG-tagged JAGN1 (~20 kDa), while 4-12% SDS-PAGE gels were chosen for Paper I. When using cell culture supernatants as samples for western blot, it is difficult to choose a housekeeping protein in order to normalize the results, thus a total protein staining intermediate step was carried out. Membranes were probed with specific primary antibodies and IRDye secondary antibodies, suitable for detection with an Odissey CLx LI-COR Biosciences scanner.

3.5.2 Enzyme-linked immunoassorbent assay (ELISA)

This assay was used to quantify MPO in cell lysates and purified NETs obtained from HL-60 cells (Paper II). A commercially available ELISA kit for MPO was purchased from eBioscience /Affymetric and used according to the manufacturer's guidelines, which are based on conventional Sandwich ELISA protocols. Sandwich ELISA offers high specificity, as it employs two antibodies directed towards different epitopes of the target, thus not requiring pre-processing purification steps of complex samples.

3.6 PHENOTYPICAL ASSESSMENT OF CELLS

3.6.1 Microscopy

With the aim to monitor morphological variation of HMDMs and MDDCs in response to NETs and LPS (Paper I), bright field microscopy was used. Cells were seeded at a density of 0.5 cells / well in 24-well plates, exposed to different stimuli for 1 h, then exposure media were washed out and replaced with fresh ones for further 24 h. After 24 h, the samples were visualized with an inverted fluorescence microscope (Nikon ECLIPSE TE2000-s) using the bright field option. In order to assess differentiation of HL-60 cells into neutrophil-like cells, nuclear morphology was evaluated (Paper II). Staining with the DNA-binding fluorescent dye DAPI was exploited for visualization of cell nuclei and samples were scored for round nuclear morphology (undifferentiated) *versus* kidney-shaped or multi-lobular morphology (differentiated).

3.6.2 Flow cytometry

Flow cytometry analysis was carried out on a BD Accuri C6 flow cytometer and data analyzed with BD Accuri software. For differentiation of HL-60 cells (Paper II), upregulation of CD11b was evaluated. CD11b is one of the subunit constituent of integrin $\alpha_M\beta_2$, which regulates adhesion and migration of innate immune leukocytes and whose expression is higher in mature neutrophils compared to the promyelocyte progenitors (Martinelli et al. 2004). In Paper I, activation and maturation of MDDCs in response to NETs and LPS was monitored using CD80 and CD86 as markers of activation and CD83 as maturation marker. Both CD80 and CD86 are capable of binding CD28 on the surface of T-cells and are required as co-stimulatory molecules in the activation of T-cells (Lanier et al. 1995), while CD83 is needed for T-cell maturation in the thymus (Breloer and Fleischer, 2008).

3.7 MULTIPLEX ASSAY FOR SOLUBLE MEDIATORS

The Luminex Bio-Plex Pro Human Cytokine Panel 27-plex (Bio-Rad) was utilized for analysis of cytokines, chemokines and growth factors released by HMDMs and MDDCs in response to NETs and LPS. The Luminex assay is a based on a principle that resembles a sandwich ELISA protocol, making use of magnetic beads, which present specific and well-defined fluorescence properties, coated with capture antibodies. In the kit used in Paper I, 27 different kinds of beads, coated each with specific antibodies against one of the soluble mediators in the panel, were mixed with the samples. Being the beads magnetic, washing steps performed on a magnetic

wash station allowed for elimination of all the sample components that not bind to the capture antibodies, or the excess of reagents. Biotinylated detection antibodies are added to the system and phycoerythrin (PE)-conjugated streptavidin is used for labeling the complexes. Samples are eventually read in a flow cytometry system. The fluorescence signal originating from the beads is associated to the specific soluble mediators while the fluorescence intensity of PE is proportional to the amount of the specific analyte (Bhattacharya et al. 2017). The Luminex platform offers the possibility to assess a large panel of soluble mediators using very limited volume of sample, even with complex composition, offering a quite broad dynamic range that allows minimal optimization steps. Furthermore, compared to conventional ELISA assays, detection limits for most soluble mediators are lower and the protocol is not more time-consuming.

3.8 FUNGI AND FUNGAL KILLING ASSAYS

Candida albicans (strain SC5314, ATCC MYA-286) was obtained from ATCC. For the experiments, *C. albicans* was subcultured at an OD595 of 0.1 in RPMI 1640 medium with 2% glucose at 37°C for 4 h in order to induce the formation of hyphae, while the culture was maintained in YPD (1% yeast extract, 2% bacto peptone and 2% glucose) at 30°C for the yeast form. *C. albicans* was chosen as a model pathogen for its susceptibility to neutrophil and NETs-mediated killing in both hyphae and yeast form in a size-dependent fashion (Urban et al. 2006 and Branzk et al. 2014). For the evaluation of fungal killing capacity of NETs (Paper II), purified NETs obtained from HL-60 cells transfected with either control siRNA or *JAGN1* siRNA were incubated with *C. albicans* at MOI of 0.01 for 2 h at 37°C. In some experiments, purified human MPO (0.066 µM) was added to the JAGN1 deficient purified NETs prior to incubation with the fungi. After 2 h, OD595 was used as a readout of the survival of *C. albicans* and from the same samples, 50 to 500 CFU were seeded on YPD agar plates and cultured for 24 h at 37°C, then colonies were counted.

3.9 CELL VIABILITY ASSAYS

For cell viability assessment of neutrophil exposed to GO (Paper III), the luminescence-based Cell Viability Kit SL (BioThema, Sweden), measuring *total cellular ATP* which is rapidly degraded upon cell death, was utilized. The kit exploits the D-luciferin-luciferase system: in presence of ATP and O_2, D-luciferin is converted by luciferase into oxyluciferin, with the reaction liberating pyrophosphate from ATP and light. The intensity of the light can be measured with a luminometer and is proportional to the amount of ATP, thus giving a measure of cell viability (Lundin, 2000). This assay was selected because it had no interference with the carbonaceous nanomaterial and, unlike other assays as Alamar Blue, it was well suited for the short lifespan of neutrophils in culture and time points selected in this study.

In order to assess cytotoxicity of the biodegradation products of GO to the BEAS-2B cell line (Paper IV), the *Alamar Blue* assay was utilized. In this assay, resazurin is added to the cell culture in order to monitor the redox state of the culture environment (Lancaster and Fields, 1996). Resazurin, a non-fluorescent blue-colored and oxidized redox indicator, is

reduced to resorufin, which is fluorescing at ~580 nm and red-colored, as a result of the metabolic activity of the cells that causes a chemical reduction of the culture environment. Data collection can be performed measuring either fluorescence (Ex. 530-560 nm, Em. 590 nm) or absorbance (570 nm and 600 nm). The Alamar Blue assay is well suited for assessment of cytotoxicity over medium to long time exposure, as it does not affect cell viability, thus being useful to monitor both cell death and cell proliferation.

3.10 CALCIUM DYNAMICS

With the aim to measure the variation of intracellular concentration of calcium ions (Ca^{2+}) in response to GO, freshly isolated neutrophils were treated with the Fluo-4 Direct Calcium Assay Kit (Invitrogen), according to the manufacturer's protocol. Then, neutrophils were exposed to 12.5 μg/mL of GO, small or large flakes, up to 180 min. The Ca^{2+} ionophore ionomycin (1 μM) was used as positive control. Measurements of fluorescence, which is directly proportional to the content of intracellular calcium ions, were taken at different time points using an Infinite F200 Tecan plate reader (Ex 485 nm; Em 535 nm). Intracellular calcium measurements, using molecules that fluoresce upon binding Ca^{2+}, present several challenges, as high background fluorescence and rapid extrusion of the probe from the cells. For these reasons, the above-mentioned kit was chosen, as it contains an additional dye meant to eliminate background fluorescence from cell culture media as well as probenecid, which inhibits organic anion transporters thus hindering active excretion of the probe from the cells and reducing background fluorescence due to extracellular Ca^{2+} binding.

3.11 DETECTION OF MITOCHONDRIAL SUPEROXIDE

For detection of mitochondrial superoxide production by neutrophils in response to GO, the MitoSOX Red probe (Invitrogen) was used according to the manufacturer's guidelines prior to exposure of the cells to small or large flakes of GO. The MitoSOX Red probe is rapidly permeating living cells and selectively localizing in the mitochondria, where it can be readily oxidized by mitochondrial superoxide, but not by other ROS. Oxidized MitoSOX Red displays high fluorescence upon binding to nucleic acids, which is proportional to the amount of superoxide generated by mitochondria. Exposure of MitoSOX-labeled neutrophils to GO was carried out in the presence or absence of the selective calcium-chelating agent EGTA (10 mM), to highlight the Ca^{2+}-induced superoxide production. Fluorescence emission was detected at different time points using an Infinite F200 Tecan plate reader (Ex. 540 nm; Em 590 nm).

3.12 CHOLESTEROL MEASUREMENTS AND DETECTION OF LIPID RAFTS

The Cholesterol Assay Kit (Abcam) was utilized, according to the manufacturer's instructions, for analyzing cholesterol dynamics in the membranes of neutrophils exposed to small or large flakes of GO or MβCD in the presence or absence of the antioxidant Trolox. The kit, optimized for detecting cholesterol in biological membranes, uses filipin III, a selective fluorescent cholesterol-binding macrolide antibiotic, as a probe. Filipin III is highly light sensitive and photobleaches rapidly, thus staining procedures and detection were performed minimizing the exposure of the samples to light. Detection of cholesterol-bound filipin III was performed using

either a Tecan Infinte F200 plate reader (Ex. 360 nm; Em 465 nm), for quantitative information, or a ZEISS LSM510 META confocal microscope for direct visualization of membrane cholesterol in neutrophils. Lipid rafts, functional protein-rich plasma membrane domains (Lingwood and Simons, 2010), were visualized staining PFA-fixed samples with Alexa Fluor 594-Conjugate recombinant cholera toxin B (CTXB) for 45 min and visualized using a ZEISS LSM510 META confocal microscope. The use of CTXB to visualize lipid rafts domains in the plasma membrane is due to its capacity of binding sphingolipids, one of the major lipid components of these domains, without posing toxicity, as the cytotoxic A subunit of the protein is not present in the recombinant form.

3.13 CHARACTERIZATION OF GRAPHENE OXIDE

Thorough characterization of the material is of outmost importance in order to understand its interactions with biological systems and the possible mechanisms of toxicity that the material could elicit. It is important to bear in mind that intrinsic properties of the pristine material can vary according to the dispersion media used; therefore, characterization in the relevant exposure media should not be neglected. To this aim, a panel of techniques has been exploited to evaluate different physicochemical properties of the graphene oxide flakes used in Paper III and Paper IV. The GO materials were synthesized at the University of Manchester, UK.

Lateral dimension and thickness. For the evaluation of structural properties of GO, optical microscopy, TEM and AFM were used. In order to determine lateral dimension, bright field *optical microscopy* using a Zeiss PrimoVert microscope was used. Furthermore, *TEM* analysis of GO samples was performed on a Tecnai 12 BioTwin (Philips/FEI) 100kV electron microscope, drop casting the samples on a formav-carbon-coated copper grid. *AFM* was employed for the assessment of both lateral dimension and thickness. Briefly, 20µl of sample were casted on a freshly cleaved mica surface coated with poly-L-lysine and allowed to adsorb for 5 min. Excess unbound GO was removed with Milli-Q water and the sample was air-dried prior to analysis using a multimode microscope in tapping mode equipped with a J-type scanner, OTESPA silicon probe (Bruker, Coventry, UK) and a Nanoscope V controller operating with NanoScope Analysis software v.1.40 (Veeco, Cambridge, UK).

Optical properties. *Absorbance* of both small and large GO flakes was evaluated using a Varian Cary winUV 50 Bio spectrophotometer scanning the peak wavelength and maximum absorbance ranging from 200 to 800 nm, while *fluorescence* emission spectra were collected with a LS-50B PerkinElmer spectrofluorometer at an excitation wavelength of 525 nm.

Surface charge and aggregation. Surface charge is a very relevant property of materials introduced in biological environment, which can be affected, together with aggregation, by the composition of the media utilized for dispersion of the material. With this in mind, *zeta potential* measurements were performed for both the pristine materials and material dispersion in the relevant exposure media. To this aim, electrophoretic mobility, which relates to zeta potential as per Henry's equation, was measured with a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). It is important to consider that this technique is optimized for measurements

of spherical particles (Retamal Marin et al. 2017). Nevertheless, it can give an approximate indication on the surface charge of non-spherical materials as well as information on the stability of the material in suspension in different dispersion environments.

Raman spectroscopy measurements were recorded using a Thermo Scientific DXR micro-Raman spectrometer equipped with a 50x objective at 633 nm laser excitation and an exposure time of 25 s, after air-drying of the samples on glass slides. This technique is highly suitable to study graphene based materials (Beams et al. 2015), which present a characteristic Raman signature peak at ~1580 cm^{-1} (G-band), representative of sp^2 carbon-carbon stretching, and, in case of any defect breaking the symmetry of the carbon monolayer structure, a disorder-induced D-band at ~1350 cm^{-1}.

Chemical composition and degree of functionalization. For the analysis of the chemical composition of GO, thus confirming its purity, *X-ray photoelectron spectroscopy* (*XPS*) was used in combination with *thermogravimetric analysis* (*TGA*) to define the degree of functionalization. These two methods allow to describe the precise atomic structure of the material, delivering information on the degree of oxidation, which is relevant for the dispersibility and the stability in aqueous suspension of the material. TGA was performed in a Pyris 6 analyzer instrument (PerkinElmer) using 1-2 mg of starting material, while XPS analysis was carried out at the NEXUS facility at the National EPSRC XPS Users' Service, nanoLAB, Newcastle-upon-Tyne, UK.

3.13.1 Endotoxin contamination

When evaluating effects of nanomaterials on immunocompetent cells, it is good practice to exclude contamination of the material with endotoxin, which could arise from non-sterile synthesis methods or improper conservation and handling practices. The endotoxin limit for products that enter in contact with the lymphatic or the cardiovascular system is 0.5 EU/mL (FDA guidelines, June 2012) corresponding to 0.05-0.1 ng of endotoxin per mL. Usually, endotoxin contamination of nanomaterials is assessed with the LAL (Limulus Amebocyte Lysate) assay, however, graphene based nanomaterials interfere with the absorbance wavelengths used as readout of this method (Mukherjee et al. 2016). In order to circumvent this issue, GO used in Paper III and Paper IV was tested using the TNF-α expression test. In brief, primary HMDMs (obtained as described above in this method section) were exposed to either LPS or GO in the presence or absence of the specific endotoxin inhibitor polymyxin B, and TNF-α release was measured after 24 h exposure using a commercially available human TNF-α ELISA kit (Abcam).

3.13.2 Carbon radicals and acellular oxidation

Electron paramagnetic resonance (EPR) was exploited for verifying the presence of carbon radicals on GO. Dry lyophilized GO sheets (1.5 mg) were placed in an EPR tube and measurements were performed at room temperature on a EMX XBand Bruker spectrometer. When applying a magnetic field, the gap between the energy states of paired electrons is widened, so that, while paired electrons reside within their energy levels, unpaired ones are

free to move between the two distinct levels. As more electrons are generally harbored at lower energy state, unpaired electrons can move to higher energy levels by absorption of energy, which can be recorded in an EPR spectrum. In order to achieve a higher signal-to-noise ratio, EPR spectra are presented as first derivative of the absorption spectrum. 2,7-dichlorofluorescein-diacetate (*DCF-DA*) is a fluorescein derivative often used for measuring intracellular ROS. Deacetylation of the probe, mediated by intracellular esterases, is functional to the retention of the compound inside the cells. The deacetylated compound (HDCF) is a non-fluorescent molecule, which becomes fluorescent upon oxidation (Bilski et al. 2002). In Paper IV, as aimed for an acellular assay, HDCF was used as a probe to detect oxidation mediated by carbon radicals on GO, incubating 25 µM of the probe with different concentrations of small or large GO flakes for 10 min. Fluorescence intensity (Ex. 485 nm; Em. 535 nm) was measured using a Tecan Infinite F200 plate reader and values normalized for auto-fluorescence of GO.

3.14 TIME-of-FLIGHT SECONDARY ION MASS SPECTROMETRY

Secondary-ion mass spectrometry (SIMS) is based on the principle that acceleration of high-energy ions (primary ions) onto a surface, results in the target surface scattering secondary ions, characteristic of its chemical composition, which can be analyzed in a mass spectrometer, using various analyzers, as Time-of-Flight (ToF) (Fletcher et al. 2011).

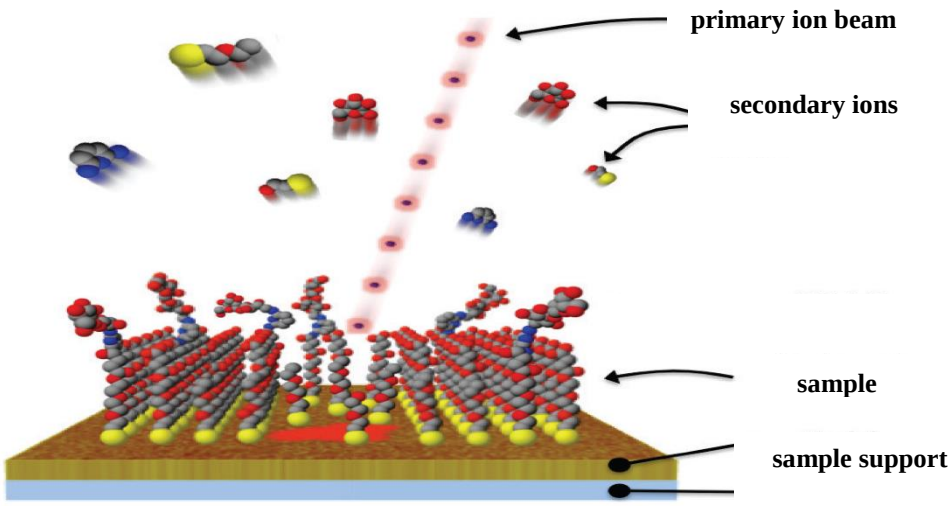

Figure 3: schematic representation of the ToF-SIMS principle. Adapted from Bolles et al. 2010. Materials (Basel). 3: 3948-3964.

As outlined in Fig. 3, imaging ToF-SIMS is a technique to analyze mass spectra in relation to each pixel of the ion image of origin, thus allowing to obtain information about localization of biochemical identities, which is otherwise lost with conventional mass spectrometry techniques, without the need of a labeling strategy (Nygren and Malmberg, 2007). Being

particularly useful in profiling sugars and lipids, ToF-SIMS can be employed to outline biological samples, as tissue sections or monolayers of cells, both at the surface (i.e. cell membrane) and for depth profiling analysis, a sort of "coring" into the cells. A current limitation of using imaging ToF-SIMS for the analysis of biological samples is related to the sources of primary ions. The optimal sources for bio-imaging purpose, as Bi^{3+}, produce secondary ions whose mass signature has yet to be completely mapped, and does not fully overlap with mass spectra obtained from conventional mass spectrometry techniques. However, use of standards for comparison purposes can overcome this difficulty (Nygren and Malmberg, 2007). In Paper III, Bi^{3+} were used as primary ion sources for surface mapping of PMNs exposed to small or large GO, MβCD, PMA or control media, or for analyzing cholesterol oxidation in acellular samples. Samples were examined with a TOF-SIMS V (ION-TOF GmbH, Münster, Germany) in positive and negative ion mode using The SurfaceLab 6 software (v.6.6, ION-TOF) for acquisition, processing and analysis. For depth profiling, sputtering with C_{60}^{3+} ions was employed, as it efficiently "exfoliates" the sample with minimal damage of the proximal layers of the material, and Bi^{3+} primary ions were used for analysis, reaching an approximate depth of >800 nm. Acquisition of the spectra from each layer of sample was recorded as a 2D image slice and the 3D image was reconstructed stacking the 2D images and normalizing the depth to the contour line to Z=0 (Robinson et al. 2012).

3.15 BIODEGRADATION OF GRAPHENE OXIDE

In order to confirm biodegradation of GO by degranulating neutrophils and purified NETs, a panel of techniques has been used (Kagan et al. 2010b and Farrera et al. 2014).

3.15.1 Raman confocal microspectroscopy

As described in section 3.13, the characteristic signature of GO with D and G band, at 1354 cm^{-1} and 1582 cm^{-1} respectively, was analyzed over time using *raman confocal microspectroscopy* to map the different samples with a laser of 532 nm wavelength and magnification set at 600x (WITec alpha300 system, Germany). 10 000 spectra per sample from 3 different areas were averaged to display the results. The D:G ratio was also calculated as it is a measure of the defects introduced in the graphene structure. A reduction in the intensity of the G band indicates a breakdown of the carbon-carbon bonds in the graphene structure, while an increased intensity in the D band correlates with the introduction of asymmetry, as resulting from oxidation, for instance (Farrera et al. 2014). The advantage of confocal Raman microspectroscopy, over conventional Raman spectroscopy, is to combine the features of a confocal microscope, which allows to scan specific areas of the sample, with a Raman spectrophotometer that can register the spectra from the designated areas. This is the technique of choice in order to monitor the presence of graphene-based nanomaterials in biological samples, however it is quite time consuming and requires expertise for operating the instrument and data analysis.

3.15.2 Microscopy-based methods and mass spectrometry

Complementary to Raman confocal microspectroscopy, transmission electron microscopy (TEM), atomic force microscopy (AFM) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) were used. *TEM* was performed dispensing dialyzed small and large GO flakes before and after 12 h of biodegradation onto TEM grids, and air-dried before analysis with a JEM-21000F JEOL TEM microscope. Although not returning quantitative measurements, TEM provides a qualitative assessment of material degradation. For *AFM* measurements, the samples were prepared as for TEM and analyzed with a Bruker Dimension Icon AFM equipped with a TESPA probe. As TEM, AFM can return a qualitative idea of material biodegradation, however it can also provide information on the number of layers and thickness of the material at atomic level, being able to map the surface to identify proteins associated with the graphene flakes or defects in its structure.

For the specific analysis of small flakes of GO and its biodegradation products, *MALDI-TOF MS* was performed without the assistance of a matrix using a Voyager-DE PRO MALDI TOF mass spectrometer (AB Sciex, Framingham, MA). Mass spectrometry brings the advantage of monitoring the decrease of the specific peaks of the material upon degradation and the consequent appearance of peaks attributable to degradation products in the spectra. Nevertheless, analysis and interpretation of the latter part is time consuming and correlating the spectra of degradation products to defined chemical structures is not a trivial process.

3.16 GENOTOXICITY (COMET ASSAY)

To measure DNA damage induced by biodegradation products of GO on BEAS-2B cells, the *alkaline comet assay* was performed. Cells were exposed to GO degradation products for 24 h, then washed, harvested using trypsin and mixed with 1% low-melting point agarose at 37 °C. The mixture was placed on microscope slides, and the slides subjected to lysis for 1 h, then transferred into alkaline (pH > 13) electrophoresis buffer, and subjected to electrophoresis. After neutralization in Tris buffer, samples were fixed with methanol and stained with SYBR green. At least 100 nucleoids per sample were scored using a Leica DMLB fluorescence microscope. The comet assay is a technique for detecting DNA damage at a single cell level, presenting several advantages: besides low costs, the method is sensitive to detect low levels of DNA damage and requires small amount of cells per sample. The alkaline version of the comet assay (pH > 13) allows for the cumulative detection of DNA damage of different kinds, single- and double-strand breaks as well as modification of sites that result in DNA brakes in the alkaline milieu, as bases that underwent alkylation or oxidation. Upon electrophoresis, migration of the negatively charged nucleoids results in a “comet” of shorter DNA fragments (Tice et al. 2000). Scoring of the comets is performed with a specific software and evaluation can be based on the length of migration or the proportion of DNA in the tail, expressed for example as tail length or % DNA in tail, as in Paper IV.

4 RESULTS

4.1 PAPER I: NETs HAVE IMMUNOMODULATORY EFFECTS AND ARE DEGRADED INTRA- AND EXTRACELLULARLY BY MACROPHAGES AND DENDRITIC CELLS

The current research on NETs is mostly focused on the molecular signaling involved in NET formation and on the subsequent role of NETs in disease (Jorch and Kubes, 2017 and Papayannopoulos, 2018). However, much less is known about the clearance of NETs and their interplay with other immunocompetent cells. With this study, using a human *ex vivo* system (Farrera and Fadeel, 2013), we aimed to highlight, in a systematic and thorough way, the relevant response that HMDMs and MDDCs mount in response to NETs. Moreover, using the same model, we aimed to study which nucleases are responsible for the degradation of NET DNA.

We first confirmed that NETs produced in our system bore not only a DNA scaffold, but also the characteristic proteinaceous components that define extracellular traps of neutrophil origin, like MPO and LL-37.

The use of the cell-impermeable and DNA-intercalating dye SYTOX green was instrumental for the tracking of internalized NETs in both HMDMs and MDDCs without affecting lysosomal or cytoskeletal dynamics. With this tool, we were able to visualize internalized NETs in our model system and define their non-lysosomal localization, hinting that extra-lysosomal nucleases could be involved in the degradation of NETs.

The expression of the lysosomal nuclease DNaseII in our model system was evaluated together with the expression of the ER-bound and cytosolic active TREX1 (also known as DNaseIII) and the extracellular nuclease DNase1L3, which is mainly produced by macrophages and DCs. In our model of MDDCs, DNase1L3 resulted to be prominently expressed over the other nucleases and in respect to the other cell types, while TREX1 seemed to be more relevant for the macrophage model. After successfully achieving transient downregulation of both TREX1 and DNaseII in HMDMs, we could observe that TREX1-deficient HMDMs were not proficient in degrading internalized NETs, while downregulation of DNaseII was not affecting the process. Furthermore, concomitant silencing of both DNaseII and TREX1 lead to similar results as the TREX1 deficient scenario, thus confirming that TREX1, but not DNaseII is responsible for NET degradation in HMDMs. Next, we showed that MDDCs were indeed capable of releasing DNase1L3 both constitutively and, to a greater extent, in response to the NET stimuli. Secreted DNase1L3 was capable of dismantling NETs in a time-dependent way and downregulation of this nuclease resulted in minor effectiveness in degradation of the extracellular traps.

Taking into consideration that a variety of experimental models and conditions have been to date used with the aim to describe interactions between NETs and other immune cells, we

sought to use our human *ex* vivo model for the purpose to compile a systematic overview of the response that HMDMs and MDDCs mount when exposed to NETs. Our study revealed that NETs induce morphological changes in both cell types, with a dramatic modulation of the phenotype of MDDCs, which however does not result in activation or maturation, as per expression of the surface markers CD80, CD83 and CD86. Moreover, when considering co-stimulation with LPS, NETs were markedly able to reduce the effect of endotoxin stimuli to levels comparable to exposure to NETs alone.

The immunomodulatory potential of NETs was confirmed by the analysis of cytokines, chemokines and growth factors secreted by MDDCs and HMDMs in response to NETs and LPS. Exposure of both cell types to the traps did not elicit a remarkable production of cytokines, while co-exposure with endotoxin modulated the effects of the latter on the cells. Co-exposure to NETs and LPS triggered significant production of IL-1β by both cell types, hinting at inflammasome activation, whereas reducing LPS-activated secretion of the immunomodulatory cytokine IL-10 and of the T-cell stimulatory molecule IL-12. NETs were capable of inducing secretion of the neutrophil chemoattractant IL-8 (CXCL8) as well as MIP-1α (CCL3) and MIP-1β (CCL4) by both MDDCs and HMDMs. Furthermore NETs reduced the endotoxin-mediated release of IP-10 (CXCL10) by MDDCs and abolished it in HMDMs. Concomitant exposure to NETs and LPS triggered the release of G-CSF and GM-CSF by HMDMs and G-CSF by MDDC, conversely, NETs abolished the LPS-mediated release of VEGF by both cell types. Overall, NETs appear to have specific modulatory effects on macrophages and DCs.

As summarized in Figure 4, these findings shed light on the mechanisms of intra- and extracellular NET disposal, and provide a systematic overview of the immunomodulatory effects of NETs on LPS-activated macrophages and DCs.

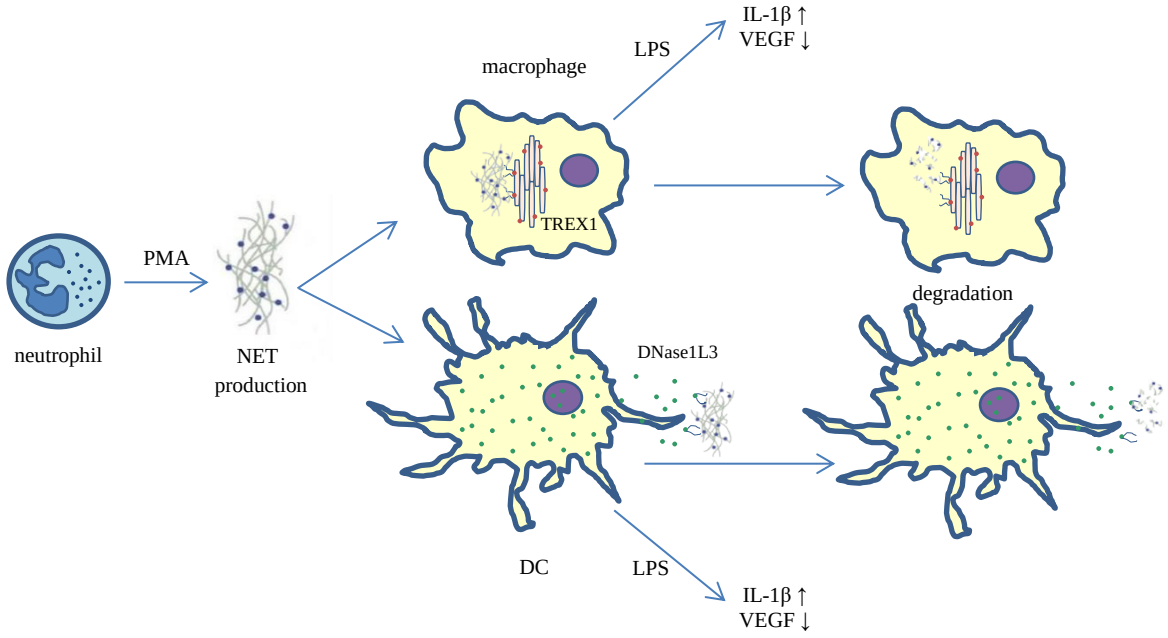

Figure 4: Schematic summary of the findings in Paper I.

4.2 PAPER II: MYELOPEROXIDASE-MEDIATED FUNGAL KILLING IN NETs REQUIRES JAGN1: IMPLICATIONS FOR SEVERE CONGENITAL NEUTROPENIA

NETs are believed to be required for efficient fungal killing, including the killing of the clinically relevant *Candida albicans* (Urban et al. 2006).

Homozygous mutations in *JAGN1* have been recently reported to be at the base of SCN in humans (Botzug et al. 2014), and the deletion of *Jagn1* in the hematopoietic lineage of mice highlighted that counteraction of *C.albicans* infection by neutrophils in those individuals was ineffective (Wirnsberger et al. 2014).

In this work, we were able to sample neutrophils from a SCN patient with *JAGN1* mutations and were able to detect NET formation, irrespectively of the genetic defect. However, both NETs and neutrophils from the patient displayed lower levels of MPO in relation to the healthy control.

Next, we established an *in vitro* model, using the hematopoietically relevant cell line HL-60, in which *JAGN1* was transiently downregulated, and could confirm in this model that the production of NETs was not affected by JAGN1 deficiency, although the presence of MPO on NETs was visibly lower compared to the control. Furthermore, overexpression of JAGN1 in the same model resulted in a recovery of the NET phenotype. Downregulation of JAGN1 in HL-60 did not interfere with the process of differentiation into neutrophil-like cells, as per CD11b expression and nuclear morphology variation, while affecting MPO expression at both transcript and protein level.

The current therapy for SCN is administration G-CSF, which restores a normal neutrophil count, yet the functionality of the circulating neutrophils seems not to be optimal in respect to the defense against microorganisms (Donini et al. 2007). Additionally, administration of GM-CSF, but not G-CSF, has been shown to recover the impairment in killing of *C.albicans* by Jagn1 deficient mice (Wirnsberger et al. 2014). We therefore sought to investigate the role of GM-CSF in our *in vitro* model, and we showed that pre-treatment of HL-60 cells transfected with specific JAGN1 siRNA could recover both the MPO presence on NETs and restore MPO expression in the cells.

Ultimately, we studied the performance of NETs, purified from HL-60 transfected with *JAGN1* siRNA or control siRNA, in killing of *C.albicans* in both yeast and hyphae form. Using two different assays to determine fungal growth, we could observe that both yeast and hyphae exposed to NETs from JAGN1 deficient HL-60 cells were proliferating to a greater extent compared to the fungi exposed to NETs purified from control HL-60. Administration of GM-CSF to the cells prior to NETs purification recovered killing capacity of NETs from HL-60 cells with reduced expression of JAGN1 to an extent comparable to control NETs. Moreover, as we pinpointed that the defect in NETs from JAGN1 deficient cells is in the MPO decoration

of the traps, we added purified human MPO to the system, which restored the killing capacity of those NETs.

In conclusion, the present study illustrates how JAGN1 deficiency is reflecting into an impaired functionality of NETs resulting in a compromised MPO-mediated killing of fungi within the traps. In addition, we could elucidate how GM-CSF restores both phenotype and function on NETs in our JAGN1 deficient HL-60 model, suggestive of future directions to be considered in the therapy of JAGN1-dependent SCN.

4.3 PAPER III: GRAPHENE OXIDE PERTURBATES LIPID HOMEOSTASIS IN THE PLASMA MEMBRANE OF NEUTROPHILS AND TRIGGERS SIZE-DEPENDENT FORMATION OF EXTRACELLULAR TRAPS

Graphene Oxide (GO) is a two-dimensional nanomaterial currently gaining tremendous attention for its physicochemical properties that render it potentially suitable for a variety of applications, including biomedical use for clinical imaging and drug delivery (Yang et al. 2013). While several reports have focused on macrophage interacting with GO, there are no studies on the interactions of GO with neutrophils. In the present study we investigated the impact of GO of different sizes on neutrophils and the interactions that GO entertains with the lipids in the plasma membrane of these cells.

First, thorough characterization of the material was carried out, highlighting that small and large flakes of GO used in the study differed only for the lateral dimension, maintaining other properties constant. In addition, assessment of endotoxin contamination showed that the material was endotoxin-free, thus the effects displayed on cells were to be related to the intrinsic properties of the material.

In order to determine cell viability of neutrophils exposed to GO, we performed dose-response studies, which showed that cell survival at 3 h was maintained up to 80% at the lower doses. Next, we assessed NET production and could detect formation of extracellular traps by neutrophils exposed both to small and large flakes, with the large flakes inducing NET formation to a greater extent.

We then explored mechanisms of NET formation induced by GO and used MβCD, known cholesterol-depleting agent able to trigger NADPH oxidase-independent NET formation (Neumann et al. 2014a), for comparison. As expected, NET production triggered by PMA was blocked by inhibition of NADPH-oxidase, while MβCD mediated NET formation was not. Large GO flakes did also induce NET production independently of NADPH-oxidase, while small flakes required it. Furthermore, we studied the role of mitochondrial ROS and calcium in GO-triggered release of NETs and could observe calcium influx mediated by large flakes together with mitochondrial reactive oxygen species production. As the release of NETs was suppressed by both calcium chelation and mitochondrial ROS inhibition, we could show that the mechanism of NET production triggered by large GO flakes is dependent on calcium and reactive oxygen species of mitochondrial origin.

As we noticed similarities between the behavior of MβCD and large GO, and saw that GO was interfacing with the plasma membrane of neutrophils by TEM imaging, we proceeded to look at lipid perturbations in the plasma membrane elicited by GO. To this aim, we analyzed lipid rafts stability using CTXB staining and filipin III staining for cholesterol, and showed that large GO flakes induced lipid raft disruption and cholesterol depletion in the plasma membrane of neutrophils, in analogy with MβCD treatment. With the use of ToF-SIMS we were able to characterize modifications of lipids in the plasma membrane of neutrophils exposed to large

GO flakes and we could show that GO elicited a reduction in cholesterol, but augmented the presence of oxidized cholesterol species, therefore, we asked whether GO was capable of directly oxidizing cholesterol in the plasma membrane. To this purpose, after confirming the presence of single carbon radicals on GO *via* EPR analysis, we showed that GO was indeed capable of directly oxidizing a model probe, while no direct oxidation of cholesterol was detected in an acellular model system, suggesting that cholesterol oxidation by GO requires multiple steps and players.

Last, we asked whether inhibition of cholesterol oxidation in the plasma membrane would prevent membrane perturbation and the subsequent release of NETs in neutrophils exposed to GO. In order to address this question, the vitamin E analog bearing antioxidant properties, Trolox, was pre-incubated with neutrophils, which were then exposed to GO. Indeed, the use of Trolox reduced both cholesterol oxidation and production of extracellular traps by neutrophils exposed to large GO flakes.

With this study, we were able to describe the outcome of the interaction between neutrophils and GO, and define the mechanism of NET formation related to the exposure. Furthermore, the use of ToF-SIMS to analyze cell surfaces allowed detailed lipid analysis of the biological specimen, highlighting the usefulness of this technique for investigating biological material. Taken together, the findings in this study describe not only toxicity mechanisms of GO on neutrophils, but also suggest a way to scavenge the adverse outcome that can potentially arise.

4.4 PAPER IV: GRAPHENE OXIDE BIODEGRADED BY NEUTROPHILS IS NOT GENOTOXIC FOR LUNG CELLS: IMPLICATIONS FOR RISK ASSESSMENT

One *caveat* that has to be considered regarding the use of engineered nanomaterials in medically relevant employment is their persistency in the body, after fulfillment of their function. It is to date well understood that biopersistency in the lungs of asbestos-like materials is cause of granuloma, and ultimately cancer and loss of functionality. It has been shown previously that single walled carbon nanotubes, a graphene-based material, can be degraded *in vivo* and *in vitro* by neutrophil myeloperoxidase (MPO) (Farrera et al. 2014 and Kagan et al. 2010b). In this paper, we investigated whether GO could be degraded by neutrophils and whether the degradation products would bear genotoxic side effects.

For the present study, the same GO, with two different lateral dimensions used in Paper III, was analyzed. Size distribution of the materials was defined and endotoxin contamination was excluded.

Degradation of GO flakes was first carried out in an acellular system, where GO was incubated with purified human MPO together with NaCl and H_2O_2, relevant substrates for the enzymatic catalysis, in the presence or absence of MPO inhibitor I. Over a 12 h period of time, the GO signature, as per the characteristic D-band and G-band on the Raman spectrum, was completely removed by MPO treatment, while in the presence of the specific MPO inhibitor the intensity of D-band and G-band remained unvaried, confirming that the biodegradation process is myeloperoxidase-dependent. Moreover, compiling the D:G ratio, a measure of the defects introduced in the pristine structure of graphene, and confirming our observation using atomic force microscopy, we showed that MPO-mediated degradation was proceeding over time for both small and large flakes.

Neutrophils are known to engage both intracellular and extracellular strategies in order to counteract infections, hence, in the present study, we focused on degranulation and formation of extracellular traps as the means that neutrophils could exploit to degrade GO. To this end, degranulation was triggered in freshly isolated primary human neutrophils and exposure to small or large GO flakes was carried out up to 6 h. For GO flakes of both sizes, we could observe a time-dependent reduction in the GO specific signature in the Raman spectra, indicating that degradation was progressing over time. Additionally, NET production was triggered in freshly isolated neutrophils, purified NETs were collected and co-incubated with GO, together with the relevant substrates for MPO activity, in an acellular system up to 12 h. Confocal Raman analysis confirmed biodegradation of the material, which was completely constrained by the addition of MPO inhibitor I.

Toxicity of intermediate biodegradation products obtained from degradation of small and large GO flakes with purified MPO was tested on the epithelial lung cell line BEAS-2B, as this is a relevant model to study carcinogenesis related to inhalation exposure. Cell viability and genotoxicity, which was studied using the comet assay for evaluation of DNA damage, were

assessed after 24 h exposure to biodegradation products obtained at different time points, in order to assess toxicity of various digestion intermediates. Cell viability was maintained upon exposure to the different GO products and DNA damage elicited by the pristine materials as well as by degradation products was comparable to control levels for both small and large flakes.

In summary, this study shows that neutrophils can mediate degradation of GO in the extracellular environment in a MPO-dependent manner and that the resulting intermediate products do not reduce cell viability nor induce DNA damage in lung epithelial cells. These findings may be relevant for the risk assessment of these materials for human health, as they suggest that inhalation exposure to GO would not pose a carcinogenic risk, provided that the degradation of the material occurs.

5 DISCUSSION

5.1 ORCHESTRATED CLEARANCE OF NETs

To date, much of the literature on NETs has been focused on describing mechanisms of NET formation and pathogen killing. Only in recent years, the interest in mechanisms of NET clearance has grown. Investigating how NETs are disposed of is not a trivial process. First, protocols used to separate NETs from neutrophils or neutrophil debris vary amongst different studies, implying that what is defined as "NETs", "purified NETs" or "isolated NETs" presents inter-study variability that can lead to contrasting results. On the other hand, no "perfect protocol" that yields pure NETs, as defined per a DNA backbone decorated with a specific panel of proteins, without any other contaminant, has been established. A way-out to this issue could be the use of purified DNA artificially complexed with proteins or peptides of interest, but the lipid or sugar component of NETs, which is to date yet to be studied, and the complexity of the NET structure would be lost. Second, conditions for extracellular degradation with serum nucleases, as DNaseI, might be easier to control, as, in principle, separation from the neutrophils would not be necessary, or easier to replicate in test-tube experiments compared to studying intracellular degradation within the phagocytes. To this end, a NET-specific labeling tool, which should not interfere with either degradation or enhance cellular uptake, would be of great help in monitoring the intracellular journey of the traps. For the work in this thesis, isolation of NETs was aimed mainly to obtain a preparation that would exclude whole cells and cell debris, while labeling of the NETs, when incubated with mononuclear phagocytes, was carried out with a DNA dye as the point of interest was the tracking of the DNA component of the traps inside the phagocytes.

The fact that various intra- and extracellular nucleases are arrayed to minimize the risk of inadvertent immune responses triggered by self-DNA, highlights the importance of these mechanisms, underlined also by the evidence that mutations in such enzymes are linked with autoimmune SLE (Yasutomo et al. 2001, Lee-Kirsh et al. 2007 and Al-Mayouf et al. 2011). While the capacity of DNaseI in degrading NETs and factors inhibiting this process, as autoantibodies and C1q (Hakkim et al. 2010 and Leffler et al. 2012), have been studied, not much is known about other nucleases nor intracellular degradation (Farrera and Fadeel, 2013).

DNAse1L3, also known as DNaseγ, was first implicated in internucleosomal DNA fragmentation in necrotic and apoptotic cells (Mizuta et al. 2013 and Errami et al. 2013) and lymphoma cell lines deficient in DNAse1L3 expression were described as apoptotic-resistant (Boulares et al. 2006). In their recent work, Sisirak and colleagues showed that DNase1L3 was produced by human and murine mononuclear phagocytes and was able to prevent the development of SLE phenotype by digesting DNA associated with apoptotic macrovesicles (Sisirak et al. 2016), but no correlation with NET DNA was reported. In a mouse model, sera from *Dnase1l3*$^{-/-}$ mice and *DnaseI*$^{-/-}$ mice showed no impairment in degrading NETs, while the double knock-out was ineffective, suggesting that the two nucleases might be exchangeable and compensate each other (Jiménez-Alcázar et al. 2017). Conversely, a comparison between the activity of DnaseI and Dnase1l3 suggested a higher propensity of the latter to proficiently

process chromatin at an internucleosomal level, while the degradation by DnaseI had a random pattern (Napirei et al. 2005). In paper I, we confirmed that human DNase1L3 is capable of degrading NET DNA and it is secreted by MDDCs both constitutively and, to a greater extent, in response to NETs. Moreover, considering our observation on DNase1L3 localizing both in the nucleus and in the cytoplasm of DCs, as well as the known sequence of the protein, which contains nuclear localization domains (Shiokawa et al. 2003), one can speculate that two isoforms of the protein coexist, being involved in chromatin fragmentation during apoptosis (nuclear pool) or secreted and consequently involved in extracellular DNA fragmentation.

Although the link between mutations or deficiency of TREX1 and SLE or IFN signature has been made, not much is known regarding endogenous substrates of TREX1. A couple of studies in mouse models hypothesized that endogenous retroelements could be processed by Trex1 and impairment in this process would lead to a sustained production of interferons, however opposing findings were disclosed (Stetson et al. 2008 and Achleitner et al. 2017). Gehrke et al. observed that oxidized DNA is resistant to degradation by TREX1 and that DNA purified from NETs presents a relevant degree of oxidative damage, thus conferring hindrance to degradation (Gehrke et al. 2013). The preparation described as "purified NETs" in Paper I refers to "pristine" NETs, which were not subjected to DNA extraction or purification, as oxidative damage can occur when DNA purification methods are used (Costello et al. 2013), and allowed us to unravel a role for TREX1 in degradation of NET DNA in HMDMs. In our model system, we observed that NETs internalized by macrophages were not co-localizing with the lysosomes, thus potentially justifying a role for the non-lysosomal activity of TREX1 in the intracellular degradation process. Interestingly, LL-37 in complex with genomic DNA was reported to be capable of shuttling the nucleic acid through the plasma membrane into the cytosol of mammalian cells (Chamilos et al. 2012) and synovial fibroblasts were found to internalize NETs in a RAGE-TLR9 dependent mechanism without the involvement of actin and cytoskeleton reorganization (Carmona-Rivera et al. 2017). In this scenario, it is intriguing to think that NETs could possibly be "transfected" through the plasma membrane in a LL-37 dependent manner.

The study in Paper I provides new insights into the strategies that macrophages and DCs utilize to dismantle NETs and describes two nucleases involved in the process. These findings broaden the currently limited knowledge on the mechanisms of NET degradation and suggest new lines of scientific investigation with potential clinical relevance regarding impaired mechanisms of NET disposal in autoimmune diseases.

5.2 IMMUNOMODULATORY EFFECT OF NETs

Cell death can occur in different flavors, each one of them tuning the surrounding microenvironment to respond accordingly. While the immunological consequences of apoptotic cell death have been intensively studied (Hochreiter-Hufford and Ravichandran, 2013 and Gordon and Plüddemann, 2018), very little is known about the effects of NETs released by dying neutrophils on other immune cells. On one hand, macrophages exposed to NETting neutrophils, activated with pathogens or PMA, were capable to release

pro-inflammatory mediators and were ultimately undergoing cell death (Nakazawa et al. 2015 and Braian et al. 2013). On the other hand, Farrera and Fadeel described the response of macrophages to engulfed NETs as "silent" based on the observation that NETs alone were not able to trigger relevant production of soluble mediators (Farrera and Fadeel, 2013). Here we confirmed that, for the majority of the soluble mediators taken into account, this still holds true. However, we noticed, amongst other chemokines, a remarkable release of IL-8 by both HMDMs and MDDCs, pointing at the fact that the traps represent a signal to other immune cells. Original findings showed that NETs offer a platform for degradation of pro-inflammatory cytokines, as IL-1β or TNF, and chemoattractive factors for macrophages, eventually limiting inflammation (Schauer et al. 2014). Conversely, NETs were found to promote IL-1β release by macrophages in combination with cholesterol crystals or LPS, and that this effect was more pronounced in lupus macrophages (Warnatsch et al. 2015, Hu et al. 2017, and Khalenberg et al. 2013). In Paper I, we confirmed that IL-1β was secreted by HMDMs in response to NETs in co-exposure with LPS and that MDDCs responded to this co-stimulation in the same way. Similarly, we found that NETs were relevant in modulating the effects of LPS in both HMDMs and MDDCs, reducing LPS-triggered release of IL-10 and IL-12 by both cell types. Furthermore, we confirmed the findings by Barrientos et al. that described NETs reducing LPS-triggered activation of DCs in terms of both maturation markers on the surface and cytokine production (Barrientos et al. 2014). Of note, NETs from mice with collagen-induced arthritis or from patients with RA were capable of activating DCs from a healthy counterpart (Papadaki et al. 2016) while NETs from healthy donors were not effective (Barrientos et al. 2014). Besides their role in mounting an inflammatory response, macrophages play a great part in the resolution of inflammation and tissue regeneration. In this context, it was interesting to observe how NETs could abolish LPS-mediated release of VEGF by both HMDMs and MDDCs. In contrast with clearance of apoptotic cells, which induced VEGF release by macrophages that in turn promoted proliferation of neighbouring endothelial cells (Golpon et al. 2004), NETs were reportedly responsible to induce profound damage at endothelial level (Villanueva et al. 2011 and Kolaczkowska et al. 2015). Our observation that NETs affect LPS signalling in tissue regeneration gives an interesting insight and room for new studies to address how NETs might dysregulate tissue repair processes in infectious conditions.

Observations that NETs *per se* seemed not to affect other immune mechanisms might have led to a limited interest investigating their immunostimulatory potential, however, in light of the findings discussed above, a new line of research considering the immunomodulatory effects of NETs in combination with other stimuli might be more relevant to understand both pathological and physiological processes.

5.3 JAGN1 IN NET-MEDIATED KILLING OF FUNGI

Homozygous mutations in *JAGN1* are reportedly causative of SCN and *JAGN1* mutations seem to affect the functionality of the protein. Although a role for jagunal has been described in the oogenesis of *Drosophila melanogaster* (Lee and Cooley, 2007), a precise function for the human homolog remains to be defined. SCN patients bearing mutations in this gene display an

aberrant glycosylation pattern of proteins processed by the ER (Botzug et al. 2014) and mice deficient for the protein in the hematopoietic lineage display high susceptibility to fungal infection by *C. albicans*, as well as lower levels of MPO (Wirnsberger et al. 2014). Of note, the MPO precursor protein undergoes N-glycosylation (Hansson and al. 2006), however in the study presented in Paper II, no evidence of altered glycosylation patterns of MPO were discovered when *JAGN1* was silenced in HL-60 cells or in neutrophils from SCN patients with mutated *JAGN1*.

NETs account for about one third of the ability of neutrophils to counteract fungal infections (Urban et al. 2006), in particular regarding the hyphae form (Branzk et al. 2014). Besides capturing the pathogens within their webs, the extracellular traps are endowed with an array of microbicide weapons, of which MPO is of paramount relevance in counteracting *C. albicans* infections (Lehrer et al. 1969). Furthermore, MPO seems to be necessary for NET production (Metzler et al. 2014) and individuals lacking MPO were found to be unable to release NETs. However, individuals that presented only a partial deficiency in MPO were still capable of producing NETs (Metzler et al. 2011). In Paper II we confirmed a reduction of MPO levels in a SCN patient bearing *JAGN1* mutations and, similarly, JAGN1 deficient HL-60 cells displayed lower levels of MPO transcripts and protein. Both the SCN patient and JAGN1 deficient neutrophil-like cells were capable of producing NETs, yet, while the DNA content was similar in the presence or absence of JAGN1, MPO on NETs was less abundant compared to the respective controls. Assessing the fungicidal potential of NETs in our model, we observed that NETs from JAGN1 deficient HL-60 cells were less efficient in counteracting *Candida* growth and that this defect was rescued by addition of purified MPO or by pre-incubation of the cells with GM-CSF. The current therapy for SCN is administration of G-CSF, which restores a physiological number of neutrophils in the circulation. Albeit reestablishing the neutrophil count, the current therapy does not recover full functionality of the cells. Donini et al. showed that SCN patients conserve low MPO levels despite the therapy and, even though the molecular machineries involved in phagocytosis are intact, they display an impaired ability to kill *C. albicans* (Donini et al. 2007). These results were confirmed in a transgenic mouse model, in which G-CSF treatment was compared to GM-CSF. Indeed, the use of GM-CSF was able to not only increase MPO levels, but also to prolong life expectancy reducing susceptibility to *C. albicans* infection in the mouse model (Wirnsberger et al. 2014). Taken together, these findings show that JAGN1 is not necessary for NET release as such, but is required for their functionality as it regulates MPO levels on the traps. Furthermore, recovering the defects induced by JAGN1 deficiency, GM-CSF administration seems to be a promising alternative for SCN patients that poorly respond to G-CSF, but further research is due in this respect. Also, we could show that JAGN1 influences MPO abundance in SCN neutrophils and HL-60 neutrophil-like cells, however whether this is an MPO-specific effect or a broader effect on other granule proteins and which are the exact mechanisms regulating the process, remains to be revealed.

5.4 GO-INDUCED PRODUCTION OF NETs

To date, many studies have addressed the outcomes of interactions between graphene-based nanomaterials and immunocompetent cells (Mukherjee et al. 2017), yet reports on how neutrophils interact with these kind of materials are lacking. A couple of studies have claimed that neutrophils can release extracellular traps in response to either gold nanorods or cationic solid lipids (Bartneck et al. 2009 and Hwang et al. 2015), yet the supporting evidence was rather weak, whereas Liz et al. convincingly showed that neutrophil responded to silver nanoparticles with ROS production and NET release (Liz et al. 2015). Regarding graphene-based materials, nanodiamonds were able to induce NET production (Muñoz et al. 2016), while SWCNTs were not proficient in triggering NET release (Farrera et al. 2014), however no studies have previously investigated the effects of GO on neutrophils.

In Paper III, we describe that GO of different lateral dimensions elicits NET production in a size-dependent manner, with the small GO flakes being less effective in triggering the process. In addition, we showed that small GO flakes require the action of NADPH oxidase to trigger ROS-dependent NET release, while inhibition of NADPH oxidase did not affect NET production by the large flakes. As mitochondrial ROS can be a downstream event of calcium influx and ultimately lead to NET release, we compared GO with a calcium ionophore, as these kind of trigger elicit NET formation in mitochondrial ROS-dependent way (Douda et al. 2015), and observed that GO promoted calcium influx and mitochondrial ROS production, ultimately leading to the release of NETs.

It has been repeatedly reported that different stimuli can result in NET release through distinct mechanisms, which can involve NADPH oxidase or not (Parker et al. 2012b and Kenny et al. 2017). As we observed close interaction between GO, especially the large flakes, and the plasma membrane of neutrophils, we compared, at the plasma membrane level, the effects of GO with MβCD, a cholesterol-depleting agent able to induce NET release without the involvement of NADPH oxidase (Neumann et al 2014a). In both cases, we observed disruption of lipid rafts and decided to use ToF-SIMS in order to analyze changes in lipids at the cell membrane on a surface level as well as in depth into the membrane (Brison et al. 2011). A general decrease in cholesterol and a rise in oxidized cholesterol species was detected upon exposure to GO. 7-ketocholesterol, a derivate of cholesterol resulting mainly from non-enzymatic oxidation (Girotti, 1998), was predominately found in GO-exposed cells and could be responsible for the lipid raft disruption observed, as it reduces organization of phospholipid membranes compared to cholesterol (Massey and Pownall, 2005). We also observed that GO was not directly oxidizing cholesterol, implying that a more complex process involving also cellular components might be required, as shown in the schematic in Figure 5, and that administration of a vitamin E analog as antioxidant could prevent both cholesterol oxidation and NET production. These observations suggest that the harmful effects of the

material can be limited and properly controlled, making it a promising candidate for biomedical use.

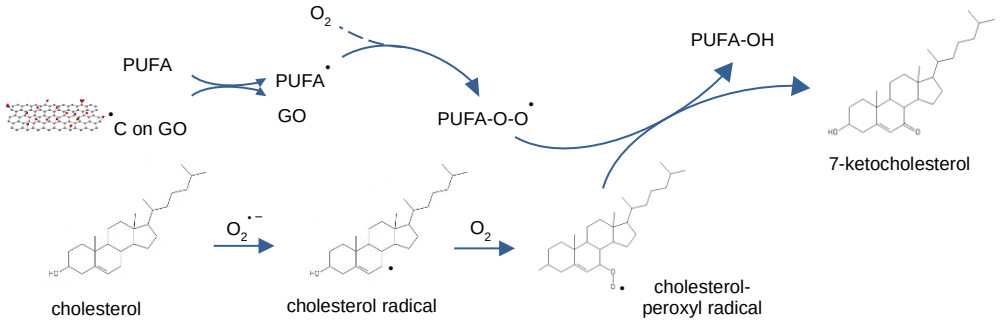

Figure 5: Proposed mechanism of multi-step non-enzymatic cholesterol oxidation by carbon radicals on GO. PUFA: polyunsaturated fatty acids. Reproduced from Mukherjee et al. 2018, Chem. 4: 334-358.

Descriptions of GO nanosheets forming pores and causing ruffling and shedding of plasma membrane in mammalian cells, ultimately affecting cell adhesion or viability (Duan et al. 2017 and Sun et al. 2016) have been reported. In that cases, the suggested mechanism of action was mechanical interaction or lipid extraction from the membranes, supported by molecular modeling. Conversely in Paper III, we showed that the material is chemically active in perturbing the membrane, although other kinds of interactions cannot be ruled out.

Mukherjee et al. report experimental evidence, supported by *in silico* simulations, that sensing of endotoxin-free SWCNTs, but not GO, by macrophages is mediated by TLR2 and TLR4 (Mukherjee et al. 2018b). Indeed, in the present study, GO was shown to chemically modify lipids in the cell membrane of neutrophils, ultimately triggering NETs, possibly excluding the need for a receptor-mediated mode of action. Similarly, it has been illustrated that both the adjuvant alum and uric acid crystals could elicit effects on DCs by altering lipid homeostasis of the plasma membrane (Flach et al. 2011 and Ng et al. 2008). Taken together, these findings suggest that GO could modulate responses of innate immune cells through different effects on lipids of plasma membrane.

5.5 IMPORTANCE OF BIODEGRADATION OF GO

In Paper IV, the same small and large flakes of GO used in Paper III were taken into account for testing their susceptibility to biodegradation by neutrophils. Previous studies demonstrated biodegradation of oxidized SWCNTs by neutrophils both *in vitro* and *in vivo* (Kagan et al. 2010b) as well as that biodegradation could occur within NETs in a MPO-dependent fashion (Farrera et al. 2014). Indeed, MPO was responsible also for biodegradation of GO and, using AFM, we could show that the protein was attaching directly on the surface of the material.

While several biological effects of graphene-based nanomaterials have been reported, a lack of information is still present regarding the effects of degradation products originating from these materials. Taking into account that peroxidase-mediated degradation of SWCNTs can proceed to the extent of yielding CO_2 and water, intermediate degradation products might present structures comparable to oxidized polycyclic aromatic hydrocarbons (PAHs) (Allen et al. 2009 and Kagan et al. 2010b). Although intermediate products obtained by MPO-mediated degradation of SWCNTs seemed to induce a greater impairment in cell viability of neutrophils, compared to cells exposed to the same amount of pristine material (Farrera et al. 2014), intermediate degradation products of GO did not impair viability of BEAS-2B cells. In the case of GO, which could directly trigger NET formation, assessing toxicity of degradation products on neutrophils might not be ultimately relevant for an *in vivo* exposure scenario, as GO-induced NET release can ultimately result in the death of PMNs producing the NETs. Therefore, assessing toxicity on BEAS-2B cells might be of higher interest considering a scenario in which inhalation exposure occurs, followed by neutrophil infiltration and *in loco* GO degradation. Of note, after 24 hours from exposure, GO seemed to increase metabolic activity of the cells, which could hint to mechanisms involved in longer-term toxicity. At the same time point, degradation products did not produce DNA damage either, contrasting with former studies indicating DNA fragmentation and chromosomal aberration in spermatozoa of mice, however these effects were consequent to administration of high doses (2000 µg/mL) or repeated exposure to GO (Akhavan et al. 2015).

Prompt and fast, within hours from exposure, degradation of GO are desirable qualities for the use of this material as drug carrier or for diagnostic purposes, limiting the risk of biopersistence. Moreover, absence of DNA damage by degradation products is also a promising feature; however, a thorough characterization of the compounds in the degradation mixture is due and needed, being helpful to design further experiments to investigate different endpoints of toxicity.

6 GENERAL CONCLUSIONS AND OUTLOOK

The work herein presented highlights the complex and multifaceted aspects of neutrophil extracellular traps (NETs), from their "birth", throughout their "active age" to their "death". In the present section, general conclusions are drawn and some reflections for future work are presented.

Versatility is the key. Release of extracellular traps occurs in response to a variety of stimuli: self or altered-self objects, and non-self entities. From the findings in this thesis, it is possible to conclude that neutrophils are induced to release NETs in response to conventional pathogens, as fungi, and non-conventional foreign objects, as two-dimensional nanomaterials. Even though the specific pathways involved in the release of the traps may vary, we were able to show that, in both circumstances, the ultimate function of NETs is dismantling the foreign object to prevent harmful outcomes. Notably, but not surprisingly, we could demonstrate the enzymatic oxidative activity of MPO, which is maintained on NETs, is a common key element in both counteracting fungal infections and degrading 2D-nanomaterials, corroborating the idea that the innate immune system can efficiently handle a variety of items using identical strategies.

Non-conventional problems require non-conventional solutions. Investigating the interactions of a two-dimensional nanomaterial with a biological system might seem, at first, an exercise in futility, however the increasing industrial use of these materials rises the risk of exposure and their desirable properties useful in medical applications deserve to be exploited in a safe manner. Analyzing the interactions between the material and the biological system requires the use of techniques that are not routinely employed in molecular biology, thus challenging to adapt them to the biological substrate. In this thesis, we could show that a combination of conventional assays and ToF-SIMS imaging complemented each other in describing a novel mechanism of NET formation in response to an "unconventional pathogen" acting on disruption of plasma membrane homeostasis instead of on a ligand-receptor base. This kind of approach, bringing together very different expertise, would be definitely useful also in "conventional" research, as observing a phenomenon from a different point of view would enrich the knowledge of it. Moreover, a thoroughly characterized nanomaterial, which interacts with the biological system in the same size range, can represent a useful tool to understand the behavior of nanosized entities in living organisms.

In the right place and at the right time. Impairment in the clearance of NETs from the extracellular milieu is tightly connected with their role in the onset of an autoimmune response, therefore it is not unexpected that more than one mechanism, possibly complementing each other, need to be arrayed to degrade the traps. Here we could show that mononuclear phagocytes arrange both intracellular and extracellular nucleases to the scope, with macrophages, being professional phagocytes, degrading the traps intracellularly through the action of TREX1, and DCs contributing to the extracellular degradation through DNase1L3.

Brinkmann and Zychlinsky ask whether immunity would be the second function of chromatin (Brinkmann and Zychlinsky, 2012). Indeed, we could show that NETs present immunomodulatory capacity, tuning the activation and the release of soluble mediators by macrophages and DCs in response to LPS. Considering that NETs can modulate the action of other immune cells in an infectious condition, but they need to be removed in order to circumvent an aberrant autoimmune response, it would be of interest to assess which is the time frame that harbors only beneficial properties of NETs, in other words, their useful lifespan, in order to exploit and modulate their function in counteracting infections. Another point of interest, to date not much studied, is the ability of peptides and opsonizing factors, as C1q or immunoglobulins, to hinder nucleases-dependent degradation of the extracellular traps. It has been reported that graphene based nanomaterials of interest for gene delivery, when in complex with nucleic acids, prevent the degradation of the latter. As we reported that graphene oxide is able to trigger relevant NET production, it would be of interest to include engineered nanomaterials in the panel of factors to investigate when studying inhibition of NET degradation.

Of mice and men. Soon after NETs were discovered, the scientific community started questioning whether NETs visualized *in vitro* were representing an event reflecting an *in vivo* behavior, or were just a beautiful artifact. Reports that extracellular DNA structures could be found in biopsies from humans or live tissue imaging of rodents, suggested that production of extracellular traps could happen in living organisms, however the relevance of this phenomenon in mice and men might largely differ. The susceptibility to infecting microorganism is quite different in the two species and, accounting for up to 70% of total circulating leukocytes in human, neutrophils represent only up to 25% of the white blood cells in mice (Mestas and Huges, 2004), suggesting that mechanisms other than NET release might be more relevant to counteract infections. An interesting model to study defects in NET production or clearance is the human *ex vivo* model, largely employed in this thesis. Particularly interesting to study NET dynamics, co-cultures of primary human immune cells exposed to NETs, together with other relevant stimuli or pathogens, could yield a simplified, yet representative and relevant model. Furthermore, studies using primary neutrophils from patients with defined genetic defects, as patients with chronic granulomatosus disease or bearing MPO deficiencies, have to date provided solid evidences in describing mechanisms of NET formation. Likewise, primary mononuclear phagocytes from patients with mutations in nucleases, as TREX1 or DNase1L3, or defects in vesicular trafficking linked to phagocytosis processes, could be used to investigate in deeper detail how intra- and extracellular disposal of NETs occurs in the human organism.

To eat or not to eat. To date, a few studies were able to show that NETs are internalized by other immune competent cells, however the mechanisms through which this is achieved remain mostly on a speculative level. Thorough characterization of NET composition, including lipids and carbohydrates, can hint to the components that might act as “eat me” signals for the phagocytes, thus indicating which receptors could be involved. On the other hand, the internalization process might not require a receptor-ligand mechanism at all, hence a

physicochemical characterization of the “NET-particles” could nevertheless pinpoint which components would allow the “transfection” process.

The work presented in this thesis wants to encourage an interdisciplinary approach in the investigation of neutrophil extracellular traps, suggesting that a combination of nanomaterial-based probes and advanced microscopy and spectroscopy techniques can be successfully applied to investigate biological processes. Furthermore, building up knowledge on mechanisms of NET release, networking with other immune cells, and degradation is the starting point to aspire to modulate and control those dynamics in order to exploit them for therapeutic purposes in autoimmune diseases or as microbicide weapons to be used in the antibiotic-resistant conditions. Overall, the present work has shed light on the role of NETs in both infectious and non-infectious scenarios.

7 ACKNOWLEDGEMENTS

The work presented in this thesis was performed at the Unit of Molecular Toxicology, Institute of Environmental Medicine, Karolinska Institutet, under the supervision of Prof. Bengt Fadeel, to whom I am deeply grateful for the continuous support, guidance and inspiring devotion to scientific research.

I also would like to acknowledge my co-supervisors, Prof. Johan Frostegård and Dr. Malahat Mousavi, for their support and for stimulating discussions, and all the external collaborators that have been involved in the research projects herein described for their availability in sharing their precious expertise.

Last, but not least, I am grateful to all the current and former colleagues in the unit and at the department for creating an enjoyable and stimulating work environment.

This work was funded by the Swedish Research Council (Vetenskapsrådet) and the European Commission through the GRAPHENE Flagship Project.

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