Priming and activation of neutrophils from blood and tissue

Lisa Davidsson

Department of Rheumatology and Inflammation Research
Institute of Medicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2023

Cover illustration by Susanna Németh

Priming and activation of neutrophils from blood and tissue

lisa.davidsson@vgregion.se

ISBN 978-91-8069-103-1 (PRINT)
ISBN 978-91-8069-104-8 (PDF)

Printed in Borås, Sweden 2023
Printed by Stema Specialtryck AB

When perusing joint fluid, take care,
'cause you're not on the right ch'min de fer
The proximal issue
is found in the tissue
(An exudate comes from elsewhere)

On the neutrophils' likely emotion,
If we actually watch them in motion:
their, to crystals, fixation
smacks of flirtation,
to monos, we'd venture, devotion

The neutrophils act like marauders,
overrunning synovium´s borders
´Tho seemingly bold
they do what they´re told
They´re soldiers, just following orders

When neutros beguin their beguin-ery,
possessing exquisite machinery,
we will probably find
it´s the monos behind
their engaging in Idi ami-ery

On Acute Gouty Arthritis, by Stephen E. Malawista, MD
Arthritis and Rheumatism 2012 (cited with permission)

ABSTRACT

Inflammation is a powerful process, involved in many disease states, as well as in physiological situations. Neutrophils, the main characters of this thesis, are potent immune cells with the capacity to regulate inflammatory responses.

In health, circulating blood neutrophils are regarded as quiescent, with limited responsiveness to stimuli. When needed to combat infection, or during, e.g., flares of inflammatory disease, neutrophils have to leave the circulation to reach the inflamed tissue. The process of passing through, i.e., transmigrate, the blood vessel wall is believed to functionally alter and pre-activate the neutrophils, an event referred to as priming. Primed neutrophils are able to respond forcefully to activating stimuli, including the performance of efficient phagocytosis, maximal release of microbicidal molecules, production of reactive oxygen species (ROS) and formation of neutrophil extracellular traps (NETs). In certain disease states also circulating blood neutrophils can be primed, due to the presence of, e.g., proinflammatory cytokines or bacterial products in the bloodstream. While properly regulated priming of tissue neutrophils is beneficial for fighting bacteria, priming of neutrophils in the circulation often results in adverse outcomes, since uncontrolled neutrophil activation can damage the surrounding vasculature. Priming is thus to be seen as a control mechanism, important in the regulation of neutrophil activation.

Despite tissue neutrophils being the main effector cells, blood neutrophils represent the main source of knowledge on neutrophil biology, due to the simplicity of obtaining these cells. The overall aim of this thesis was to increase the current knowledge on neutrophil priming and activation, complex processes that participate in regulating inflammation, in blood and tissue, in health as well as in inflammatory disease.

In **paper I,** a simple skin blister technique to obtain and study *in vivo* transmigrated neutrophils was described and characterized.

In **paper II,** in contrast to neutrophils obtained from skin blisters and skin chambers (two *in vivo* models of aseptic inflammation) and the leading dogma, synovial fluid neutrophils derived from patients with inflammatory arthritis were shown to display no, or only mild, signs of priming.

In **paper III**, the interactions between neutrophils and monosodium urate (MSU) crystals, triggers of the inflammatory disease gout, were studied. Neutrophil ROS production induced by the crystals was shown to be strictly intracellular and clearly primed in tissue neutrophils derived from skin chambers. NET formation induced by the crystals was not dependent on ROS and not primed in any of the studied tissue neutrophils.

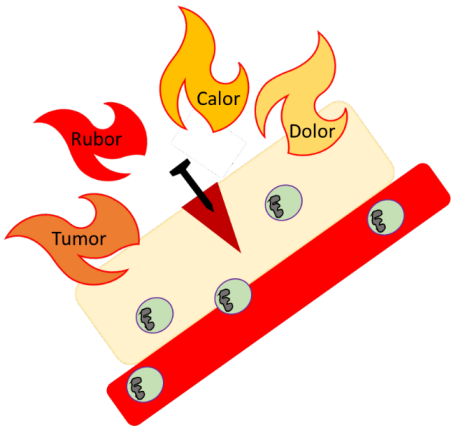
In **paper IV,** blood neutrophils from gout patients were shown to be primed for baseline and MSU crystal triggered ROS production, while NET formation and receptor expression were similar between neutrophils from gout patients and controls.

In conclusion, this thesis highlights the complexity of neutrophil priming and activation and the changes that neutrophils undergo during transmigration to different inflamed tissues.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Inflammation är den reaktion som uppstår när kroppen försvarar sig mot något som upplevs som främmande och/eller skadligt. Benämningen härrör från det latinska ordet *inflammare* som betyder tända eld på och symtomen kan helt klart liknas vid att bränna sig; det blir rött, varmt, svullet och gör ont. Reaktionen drivs av vårt immunsystem och utgör ett nödvändigt skydd mot infektioner och behövs för att städa upp vävnadsskador. Målet med den akuta inflammationsreaktionen är att döda invaderande mikroorganismer och att läka skadad vävnad. De beskrivna symtomen uppkommer framförallt till följd av att blodkärlen i området vidgas vilket leder till ökad blodgenomströmning (som ger värme och rodnad) och att blodkärlen blir mer genomsläppliga (vilket leder till svullnad). Vidare rekryteras vita blodkroppar och det frisätts en mängd olika inflammatoriska substanser som bland annat retar smärtreceptorer (vilket leder till att det gör ont). Ibland påverkar inflammationen hela kroppen och kan då ge symtom som feber och trötthet. Inflammation kan i vissa fall utvecklas så att kroppens celler och vävnader skadas och kronisk inflammation är involverad i många olika sjukdomstillstånd, till exempel ledgångsreumatism. Man liknar ofta inflammation vid ett tveeggat svärd – den är på både gott och ont.

Akut inflammation är kroppens skydd mot infektion och/eller vävnadsskada. Blodflödet i den inflammerade vävnaden ökar och vita blodkroppar tillkallas. Fyra vanliga symtom vid akut inflammation är tumor (svullnad), rubor (rodnad), calor (värmeökning) och dolor (smärta).



Immunsystemet - kroppens egen försvarsmakt

Vårt immunsystem/immunförsvar utgörs till stor del av olika vita blodkroppar och lösliga komponenter och brukar delas in i två (överlappande) delar; det medfödda och det förvärvade, eller adaptiva, immunsvaret. Det medfödda immunsvaret utgör den första försvarslinjen och har som sin främsta uppgift att snabbt vara på plats vid hotande invasion/infektion, utan att egentligen veta vem det är som anfaller. Det adaptiva immunsvaret som utgörs av T-lymfocyter och antikropps-producerande B-lymfocyter, specialförbanden om man vill fortsätta att vara metaforisk, befinner sig ett steg upp på gradskalan och kräver lite mer mobilisering. Väl på plats använder de sig av sina respektive spetskompetenser för att göra processen kort med fienden. Specialförbanden upparbetar även kunskap (immunologiskt minne – principen som vaccination grundar sig på) om fiender de mött genom åren. Denna kunskap gör att de både snabbt och effektivt kan nedkämpa inkräktaren om den återkommer.

Neutrofiler – avhandlingens huvudkaraktärer

Den här avhandlingen fokuserar på en särskild sorts vita blodkroppar - neutrofiler - vilka tillhör det medfödda immunsvaret och är livsviktiga för vår förmåga att försvara oss mot infektioner. Låga nivåer av neutrofiler i blodet, som till exempel vid cellgiftsbehandling, gör oss mycket infektionskänsliga. Neutrofiler patrullerar normalt sett blodbanan i ett vilande (fredligt) tillstånd, men vid en invasion, som

till exempel infektion, vävnadsskada, eller akuta skov i inflammatoriska sjukdomar (ledgångsreumatism eller gikt för att ta några vanliga exempel), mobiliserar de snabbt till den inflammerade vävnaden. Processen att ta sig över blodkärlsväggen och ut i vävnaden försätter neutrofiler i beredskapsläge, vilket är en förutsättning för att effektivt kunna nedkämpa fienden. Beredskapsläget brukar benämnas ”priming” och innebär bland annat att vissa receptorer - molekyler som binder till sig signalsubstanser och överför ett budskap till cellen - uttrycks på neutrofilens yta, medan vissa andra försvinner. Neutrofiler besitter en bred vapenarsenal och kan såväl äta upp (fagocytera) inkräktare, som använda sig av kemisk krigsföring, vilket bland annat innebär att de frisätter toxiska fria syreradikaler (ROS) och enzymer (proteiner som är inblandade i de flesta av kroppens processer, bland annat kan de bryta ner andra proteiner). Vidare kan neutrofiler i vissa lägen utföra självmordsattacker, vid vilka de kastar ut sitt kärninnehåll vilket fungerar som toxiska fångstnät, så kallade neutrophil extracellular traps (NETs). Vid vissa sjukdomstillstånd, till exempel vid blodförgiftning, kan neutrofiler försättas i beredskapsläge/primas inne i blodbanan, vilket är förenat med negativa konsekvenser eftersom toxiska substanser då skadar blodkärl och omkringliggande vävnad. Man har också sett att neutrofiler kan försättas i beredskapsläge i blodbanan vid vissa inflammatoriska sjukdomar.

Syfte och resultat

Trots att vävnadsneutrofiler generellt är de aktiva soldaterna, så utförs forskning framförallt på blodneutrofiler, eftersom dessa kan fås fram genom ett enkelt blodprov. Denna avhandling syftar till att öka kunskapen om priming och aktivering, komplexa processer som bidrar till inflammationsreglering, av neutrofiler och vad som skiljer blod- från vävnadsneutrofiler.

Vi började med att ta fram och karaktärisera en enkel, skonsam hudblåse-modell för att utvinna och studera vävnadsneutrofiler (**studie I**). Hudblåsorna kan jämföras med de man får vid skoskav, fast utan att göra ont (de kliar bara lite). Genom att suga ut blåsvätskan får man tillgång till såväl vävnadsneutrofiler som andra immunceller och lösliga inflammatoriska substanser som man kan studera. I **studie I** visade vi hur sammansättningen av inflammatoriska celler och substanser i blåsorna förändrades över ett dygn samt att dessa blås-neutrofiler - i likhet med den rådande dogmen – är kraftigt primade. Däremot visade vi sedan (**studie II**) att neutrofiler kan ta sig från blod till inflammerad ledvätska (det vill säga ledvätska som aspirerats ur akut inflammerade leder hos patienter med kroniska ledsjukdomar som till exempel ledgångsreumatism) utan att primas. Vi har även kunnat visa att beredskapsläge/priming inte behöver innebära att hela neutrofilens vapenarsenal omfattas, till exempel så kan ROS frisättningen vara kraftigt primad, men inte NETs frisättningen (**studie III** och **IV**).

Den vanligaste inflammatoriska ledsjukdomen i världen är gikt - kanske mer känd som portvinstå? - vilken uppkommer till följd av förhöjda värden av urinsyra i blodet. Patienter med gikt har en klart ökad risk för kardiovaskulär sjukdom, som till exempel hjärtinfarkt och stroke. En relativt ny (liten) studie utförd på patienter med kronisk gikt påvisade en möjlig koppling mellan tecken till neutrofilaktivering i blod och en ökad risk för kardiovaskulär sjukdom. Vi ville därför studera neutrofilaktivering i blod från patienter med gikt och jämföra dessa med neutrofiler från friska frivilliga (**studie IV**, ännu ej publicerade data). Vi har visat att blodneutrofiler från giktpatienter har en jämförelsevis (med kontrollgruppen) ökad ROS-frisättning, men vi såg ingen skillnad mellan grupperna vad gäller NETs-frisättning och receptoruttryck. Orsaken till, och betydelsen av, detta fynd behöver studeras ytterligare.

Avhandlingen belyser priming och aktivering av neutrofiler och de förändringar som neutrofiler genomgår när de transporterar sig från blodbanan till olika inflammerade vävnader. Det är vår övertygelse att ökad kunskap om neutrofiler och deras reglering kommer att bidra till en bättre förståelse för inflammatoriska processer och förhoppningsvis leder detta till utveckling av nya behandlingsstrategier i framtiden.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **A simple skin blister technique for the study of in vivo transmigration of human leukocytes**.

Davidsson L, Björkman L, Christenson K, Alsterholm M, Movitz C, Thorén FB, Karlsson A, Welin A, Bylund J.

Journal of Immunological Methods 2013; 393 (1-2): 8-17

II. **Neutrophil recruitment to inflamed joints can occur without cellular priming.**

Björkman L, Christenson K, Davidsson L, Mårtensson J, Amirbeagi F, Welin A, Forsman H, Karlsson A, Dahlgren C, Bylund J.

Journal of Leukocyte Biology 2019; 105 (6): 1123-1130

III. **In vivo transmigrated human neutrophils are highly primed for intracellular radical production induced by monosodium urate crystals.**

Davidsson L, Dahlstrand Rudin A, Sanchez Klose FP, Buck A, Björkman L, Christenson K, Bylund J.

International Journal of Molecular Sciences 2020; 21 (11): 3750

IV. **Neutrophil ROS production is primed in blood neutrophils from gout patients.**

Davidsson L, Dahlstrand Rudin A, Christenson K, Björkman L, Bylund J.

In manuscript

CONTENT

ABBREVIATIONS

AAV	Anti-neutrophil cytoplasmic antibodies (ANCA) associated vasculitis
ANA	Anti-nuclear antibodies
ANCA	Anti-neutrophil cytoplasmic antibodies
APP	Acute phase protein
CGD	Chronic granulomatous disease
CL	Chemiluminescence
CLR	C-type lectin receptor
CR	Complement receptor
CRP	C-reactive protein
CXCR	Chemokine receptor
DAMP	Damage associated molecular pattern
EGPA	Eosinophilic granulomatosis with polyangiitis
ELISA	Enzyme-linked immunosorbent assays
FcR	Fc-receptor
FMF	Familial Mediterranean fever
fMLF	N-formylmethionyl-leucyl-phenylalanine
FPR	Formyl-peptide receptor
GPA	Granulomatosis with polyangiitis
GPCR	G-protein-coupled seven-transmembrane receptor
IL	Interleukin
LDN	Low-density neutrophil
LPS	Lipopolysaccharide
MPA	Microscopic polyangiitis
MPO	Myeloperoxidase

MSU	Monosodium urate
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NK	Natural killer
NLR	Nucleotide oligomerization domain (NOD)-like receptor
NSAID	Non-steroidal anti-inflammatory drug
OLFM4	Olfactomedin 4
PAMP	Pathogen associated molecular pattern
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphnuclear cell
PR3	Proteinase 3
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SOD	Superoxide dismutase
TLR	Toll-like receptor
TNF	Tumor necrosis factor

INTRODUCTION

Already a long time ago, it was recognized that people who survived severe infectious diseases, such as the plague or smallpox, then appeared to be immune (from Latin *immunis*, meaning not susceptible to infection) to the disease in question. In the 18th century, it was noticed that dairymaids often were protected from smallpox. In 1796 Edward Jenner was able to demonstrate that infection with cowpox trigger immunity to smallpox by subcutaneously introducing (inoculating) material from a fresh cowpox lesion to a young boy. Two months later, the same boy could be inoculated with material from a fresh smallpox lesion without developing disease. The Latin word for cowpox is *vaccinia,* and Jenner therefore decided to name the procedure *vaccination* (1). Hundred years later, Louis Pasteur and his colleagues discovered that injections of attenuated (weakened) rabies virus offered protection against this disease. Pasteur was by that the first to create a vaccine in a laboratory setting and is by many named as the father of immunology. Pasteur died in 1895, six years before the first Nobel Prize in physiology and medicine was awarded to Emil Adolf von Behring for his discovery that injection of diphtheria toxin in animals trigger the formation of protecting serum-antitoxins (later named antibodies). These antitoxins could then be transferred to humans, providing protection against diphtheria (2). Soon after, it was realized that there were different types of immunity and that antitoxin (antibody) formation only was one of the body´s defense mechanisms.

Our immune system consists of two overlapping parts, the innate and the adaptive immunity. The innate immunity is an evolutionarily conserved system that represents our first line of defense, initiating an acute protective inflammatory reaction when triggered by infection and/or tissue damage (3). Inflammation (from Latin *inflammare*, meaning to set on fire) is usually manifested by redness, swelling, pain, increased temperature and systemic symptoms such as fever and fatigue. The adaptive immunity, sometimes called the required (or specific) immune system, composes T-lymphocytes and antibody-producing B-lymphocytes with the ability to recognize and respond to a wide variety of antigens. The adaptive immunity takes a little more time to mobilize (and to mature), but after a first exposure it possesses the ability to create immunological memory, meaning that the next time a specific pathogen is encountered, the adaptive immune response is quicker, stronger, more specific and longer-lasting (4). The concept of immunological memory is the basis for vaccination; vaccines mediate their protection mainly by the induction of highly specific antibodies produced by B-lymphocytes, or via the induction of T-lymphocyte mediated immune memory (5). Sometimes the adaptive immunity wrongly reacts against endogenous (harmless) structures, giving rise to chronic autoimmune diseases, such as rheumatoid arthritis (RA) (6). There is however a vital interplay between the innate and the adaptive immunity and the innate immune system is an important co-player in the pathogenesis of autoimmune diseases. The innate immunity can also become incorrectly activated, i.e., when there is no threat, due to dysregulated secretion of proinflammatory cytokines (small proteins that are important mediators of homeostasis and inflammation) and this is referred to as autoinflammation (4, 6, 7).

While acute inflammation is required for fighting microbes, chronic inflammation, the outcome, e.g., if the acute inflammatory event fails to resolve, is implicated in a wide range of human diseases, including, e.g., autoimmune diseases (8). Inflammation is therefore to be seen as a double-edged sword, having both favorable and unfavorable consequences. Neutrophils, the main characters of this thesis and potent effector cells and regulators of inflammation, belong to the innate immunity and are the most abundant leukocytes in the human bloodstream (9). When needed to combat infection, or during, e.g., flares of inflammatory disease, neutrophils have to leave the bloodstream, i.e., pass over, or transmigrate, the blood vessel wall, to reach the inflamed tissue and are typically functionally altered and pre-activated, or primed, by this process (10). In certain disease states also circulating blood neutrophils can be primed, due to the presence of, e.g., proinflammatory cytokines in the bloodstream (11-13). Primed neutrophils are able to respond forcefully to activating stimuli and while properly regulated priming of tissue neutrophils is beneficial for fighting bacteria, priming of neutrophils in the circulation often results in adverse outcomes. Despite tissue neutrophils being the main effector cells, blood neutrophils represent the main source of knowledge on neutrophil biology. *The overall aim of this thesis was to increase the current knowledge on neutrophil priming and activation, complex processes that participate in regulating inflammation, in blood and tissue, in health as well as in inflammatory disease.*

INNATE IMMUNITY

The innate immunity is an evolutionarily conserved system built up by phagocytosing cells of myeloid origin, epithelial cells and soluble molecules (3). The innate immunity was traditionally considered a primitive part of the immune system until Charles Janeway Jr. in 1989 proposed the game-changing hypothesis of specific innate immune receptors that recognize microbial patterns (14).

Pattern recognition receptors

Innate immune cells are equipped with pattern recognition receptors (PRRs) that recognize specific microbial structures, conserved among microbial species, also referred to as pathogen associated molecular patterns (or PAMPs) and molecular signals indicative of tissue damage (damage associated molecular patterns; DAMPs). The recognition of PAMPs and/or DAMPs by innate immune cells triggers microbicidal and proinflammatory activity and provides co-stimulatory signals for the adaptive immune cells, particularly T-lymphocytes (15). PPRs include both intra- and extracellular receptors that are classified into five types, of which the toll-like receptors (TLRs) were discovered first and are the best described (15, 16). In 1998, TLR4 (17) was shown to be required for the recognition of lipopolysaccharide (LPS), a membrane-component of Gram-negative bacteria and a potent bacterial endotoxin that can lead to septic shock. Since septic shock is a potentially life-threatening condition, TLRs were given a central role in immunity and the finding was later awarded the Nobel prize in Physiology or Medicine in 2011 (18).

Cells of the innate immunity

Innate immune cells of myeloid origin compose monocytes, macrophages, dendritic cells, granulocytes and natural killer (NK) cells. Macrophages (“big eaters”), derived from blood monocytes, are found in essentially all tissues, acting as guarding posts and directors of the inflammatory response, possessing the ability to produce several cytokines (19). Macrophages and even more so, the closely related dendritic cells, are efficient antigen presenting cells, meaning that they introduce antigens from the phagocytosed prey to T-lymphocytes, enabling adaptive immune responses (20-22). Granulocytes, i.e., phagocytes containing cytoplasmic granules that can be released upon activation, include neutrophils, basophils, eosinophils and the tissue resident mast cells, the latter most known for their involvement in allergic responses (23). Neutrophils, basophils and eosinophils are also named PMNs (polymorphnuclear cells), after their multilobulated nucleus, but since neutrophils represent the vast majority of all PMNs, the designation most often aims solely at neutrophils. Neutrophils are the main characters of this thesis and will be thoroughly described in the next chapter. NK cells are leukocytes that resemble lymphocytes, but lack the antigen-specific cell surface receptors that define adaptive immunity. NK cells are equipped with cytolytic function and are mainly involved in the defense against virus infections and tumors (24, 25).

The complement system

An important part of the innate immune response is made up of the complex system of small proteins known as the complement system. Research on the complement system started over a century ago and the name ‘‘complement’’ originates from the idea that these proteins supported (complemented) the effects of antibodies. The complement system is closely connected with the coagulation system (26) and constitutes an important link between innate and adaptive immune responses with both direct and indirect influence on B- and T-cell activity (27, 28). Activation of the complement system involves enzymatic cleavage of the proteins and results in many different outcomes. Important tasks for the complement system include the opsonization (pre-coating) of microbes, which facilitates phagocytosis, the formation of potent chemo-attractants (signals that guide cells to the inflamed site) and direct attacks on microbial cell membranes, leading to cell lysis. The complement system is also important for the clearance of immune complexes and apoptotic cells (29, 30).

The complement cascade can be activated via three different pathways. The classical pathway was defined first and is activated when the recognition molecule C1q binds to immunoglobulin-coated antigens (31). The alternative pathway was discovered next and is triggered directly by microbial structures. The third, lectin, pathway is activated by mannose-binding lectin interacting with microbial glycoproteins and glycolipids. All pathways activate cleavage of the central and most abundant complement component C3 (32) into C3a and C3b, which in turn leads to the cleavage of factor C5 into C5a, which is a potent chemotactic factor for neutrophils, and C5b and formation of the lytic membrane attack complex (30).

The fact that deficiencies in the complement system may result in increased susceptibility to infections, especially encapsulated bacterial infections (29), is probably not surprising. However, defects in the complement system may also result in autoimmune diseases (29, 33), e.g., systemic lupus erythematosus (SLE), a chronic disease with a broad spectrum of clinical manifestations in which autoantibodies against different cell nuclear components (anti-nuclear antibodies; ANA) are produced. Deficiency of early complement proteins, C1q in particular, is strongly associated with SLE and reduced clearance of immune complexes and apoptotic cells is shown to contribute to the loss of self-tolerance in the disease. Deposition of immune complexes in several organs is important in the pathogenesis of SLE and triggers complement activation (reflected by low serum levels of C3 and/or C4), inflammation and tissue destruction (34, 35).

ACUTE INFLAMMATION

Acute inflammation is our immediate immune response to infection (induced by e.g., bacteria, fungi, viruses and other microorganisms) and/or injury, irritants or damaged cells, with the ultimate goal of fighting infection and restoring and healing the affected tissue. Acute inflammation, and subsequent resolution of inflammation, are complex processes, involving several cell types and soluble mediators. The acute immune response to infections is the best characterized and will be described below (36, 37).

If microorganisms manage to overcome the physical barriers provided by the skin and mucus membranes, several mechanisms cooperate to initiate an acute inflammatory response. Recognition of PAMPs by resident tissue cells (macrophages, dendritic cells, mast cells and epithelial cells) will activate the complement system and mediate release of proinflammatory mediators, including, e.g., cytokines, vasoactive amines and eicosanoids, e.g., leukotrienes and prostaglandins (produced from arachidonic acid). The enzyme responsible for the chemical synthesis of prostaglandins, cyklooxygenas (COX), constitutes the target for non-steroid anti-inflammatory drugs (NSAIDs), widely used to treat pain, fever and inflammatory processes (38). Altogether, the mechanisms that collaborate to trigger the acute inflammatory response result in increased blood flow and permeability of the blood vessels, with the goal of facilitating local delivery of soluble mediators and immune cells, predominantly neutrophils (9, 39).

The innate immune response is closely connected to coagulation; the formation of blood clots at the site of tissue injury provides antimicrobial barriers that reduce the risk of infection and the acute inflammatory response, in particular proinflammatory cytokines, promotes a pro-thrombotic state (26, 40-42). Proinflammatory cytokines, mainly interleukin 6 (IL-6), interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α), also trigger other systemic responses such as fever and release of acute phase proteins (APPs) produced by the liver. APPs partake in different ways in the acute inflammatory response, for example by opsonizing microorganisms, and include e.g., C-reactive protein (CRP), complement proteins and fibrinogen. APPs are also commonly used clinical markers of ongoing inflammation, e.g., fibrinogen, as reflected in the erythrocyte sedimentation rate, and CRP (43, 44). CRP both peaks and decreases quickly and is well suited for monitoring acute inflammatory conditions, such as infections. The name was given in 1930 when it was noted that proteins in the sera of patients with pneumonia decreased as they recovered. Since the protein was shown to react with the C-polysaccharide of pneumococcal cell walls, it was named "C-reactive protein" (45). The CRP value is primarily controlled by IL-6 and it is therefore important to be aware of the fact that treatment with IL-6 receptor antagonists reduce the use of CRP as biomarker for detection of infections (46, 47). Drugs targeting the IL-6 receptor are commonly used for the treatment of inflammatory diseases (e.g., RA) (48) and life-threatening excessive immune responses (“cytokine storm”) induced by, e.g., coronavirus disease 2019 (49, 50).

Resolution of inflammation is an active process that is important to protect us from uncontrolled inflammation that can become chronic and tissue damaging and drive the progression of many common diseases. The resolution-phase is initiated by the biosynthesis of several different pro-resolving mediators, including e.g., pro-resolving lipids that are produced upon lipid-mediator class switching; proinflammatory lipid mediators (e.g., prostaglandin) switch from being proinflammatory to instead initiate the production of pro-resolving lipids (51). Pro-resolving mediators act on inflammatory key events, including termination of the recruitment of neutrophils and promoting neutrophil apoptosis. Apoptosis is a controlled form of cell death and apoptotic neutrophils have an intact plasma membrane that protects the surroundings from cytotoxic components. Apoptotic neutrophils express “find-me” and “eat-me” signals and are cleared by resident macrophages, an event referred to as efferocytosis, which triggers a switch in macrophages from a pro- to an anti-inflammatory phenotype. Anti-inflammatory macrophages produce anti-inflammatory cytokines and pro-resolving lipids and promotes restoration of tissue homeostasis (52-54).

NEUTROPHILS

Neutrophils make up the largest proportion of circulating leukocytes and are the first to be recruited to an inflamed tissue. Neutrophils possess multiple effector mechanisms to fight down pathogens, including phagocytosis, production of reactive oxygen species (ROS), release of microbicidal molecules and formation of neutrophil extracellular traps (NETs).

Maturation and life cycle

Neutrophils originate from myeloid stem cells and are produced in the bone marrow. Several growth factors, including the master regulator granulocyte colony-stimulating factor (G-CSF), stimulate the production. When neutrophils are released into the circulation after approximately two weeks, they are fully matured, with a segmented nucleus, around 10 μm in diameter and packed with storage organelles, i.e., granules, containing multiple proteins, such as antimicrobial peptides, enzymes and receptors (39). Neutrophils possess a restricted capacity to perform *de novo* protein synthesis, but activated neutrophils are able to synthesize some cytokines/chemokines, e.g., IL-8 (55). In circulation, neutrophils compose 60-70% of the leukocytes, at concentrations around 1.8-7.5x10^9 cells/L. Not included in the measured concentration of "free-floating" neutrophils is the marginated pool, comprising blood neutrophils adherent to the endothelium, residing mainly in the spleen, liver, lungs and bone marrow. This pool can be quickly mobilized during, e.g., infection, inflammation, stress or treatment with corticosteroids (56-58). During conditions with high demand for neutrophils, such as severe systemic infections, the bone marrow needs to rapidly increase its *de novo* production of neutrophils. Clinically, this is reflected in high peripheral neutrophil counts and the appearance of immature neutrophils in the bloodstream, known as left-shift (59). Low peripheral neutrophil counts can be congenital (60), or, more commonly, acquired due to bone marrow suppression, e.g., blood malignancies and chemotherapy. Severely lowered neutrophil counts (<0.5x10^9 cells/L) result in immunodeficiency, characterized by high risk of severe bacterial or fungal infections, supporting the role of neutrophils as crucial players of the innate immune response (61).

Neutrophils are short-lived cells with a half-life of 6-8 hours in circulation (57), believed to undergo apoptosis and be cleared by macrophages in the bone marrow, liver or spleen, if not needed to combat infection. *In vitro* studies and studies in mice have described a natural drift in neutrophil phenotype and function (in blood samples taken at different times of the day), including the neutrophil capacity to phagocytose bacteria and produce ROS (62), a phenomenon referred to as neutrophil aging (63). Aged neutrophils have been shown to exhibit phenotypical changes quite similar to those of the primed/activated phenotype, such as decreased expression of L-selectin and lower granularity, as compared to recently released neutrophils (64, 65) and to increase their expression of the maturation marker chemokine receptor 4 (CXCR4), guiding them back to the bone marrow where they are cleared (64, 66). Neutrophil turnover *in vivo* remains however poorly defined (9, 58, 67).

During inflammatory conditions, the lifespan of tissue neutrophils is thought to be extended two- to threefold over blood neutrophils, due to factors that will delay apoptosis, e.g., a diversity of PAMPs as well as several cytokines and chemokines (57, 68, 69). This would ensure continued presence of (primed) neutrophils at the site of infection. After neutrophils have fulfilled their functions in the tissue, they are cleared by resident macrophages (8, 70), although it might be that some of the neutrophils in fact migrate back into the circulation, a process known as reversed transmigration (71, 72). The first observations on this process were made 2006 through microscopic observations in zebrafish (73) and *in vitro* models of human cells (74). More recently, these observations were also made in mice (75-77). Reverse migrated neutrophils have been reported to reside in the lungs where they upregulate CXCR4, followed by homing back to the bone marrow (77).

Granules

Neutrophils are filled with granules that are formed in a specific order during maturation in the bone marrow; azurophil (or primary) granules are formed first, thereafter specific (secondary) granules, gelatinase (tertiary) granules and last out, the secretory vesicles (78). When neutrophils are activated, the granules will mobilize in the opposite order of formation; minimal stimulation, such as getting in contact with the inflamed endothelium prior to extravasation, is sufficient to mobilize the contents of the secretory vesicles and gelatinase granules to the cell surface, e.g., surface receptors and adhesion molecules (79). Increasing activation is associated with the release of specific granules, containing highly toxic components and these granules fuse, to a large degree, with the phagosome. Specific- and gelatinase granules both contain the membrane-bound part of the NADPH oxidase, needed for ROS production (described in more detail below) (80). Azurophil granules are lysosome-like organelles, containing the most cytotoxic mediators and mainly fuse with the phagosome. Azurophil granules mobilize to the cell surface to a very limited extent, despite high activation (81). The defining protein of azurophilic granules is myeloperoxidase (MPO), that is involved in ROS processing and NET formation (82). Other important proteins found in most neutrophils granules, although most abundantly in azurophil granules, are the serine proteases, proteinase-3 (PR3), cathepsin G and neutrophil elastase (NE), with the potency to digest proteins, bacteria and bacterial virulence factors (83).

Granule-derived neutrophil proteins as targets for anti-neutrophil cytoplasmic antibodies (ANCA)

For clinicians, the neutrophil enzymes MPO and PR3 are (perhaps) mainly known as major targets for ANCA, which are antibodies associated with a group of systemic small vessel vasculitis called ANCA-associated vasculitis (AAV). The name ANCA is however a bit misleading since MPO and PR3 are in fact granule-localized, and not cytoplasmic, proteins. AAV include microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) and eosinophilic granulomatosis with polyangiitis (EGPA) (84). The vast majority of patients with GPA and MPA, and about half of patients with EGPA, present with ANCA, which is used as a diagnostic and prognostic marker of AAV.

The origin and role of ANCA is not clear, although one hypothesis is that ANCA autoantigens are generated/exposed through increased NET formation, leading to generation of autoimmune responses and formation of antibodies and growing evidence also support a pathogenic role of ANCA in AAV. It has been suggested that primed neutrophils expose MPO and PR3 on their surface, whereupon ANCAs bind to these antigens and neutrophil Fc-receptors (FcRs) simultaneously, leading to excessive neutrophil activation, including ROS production and NET formation with harmful effects on small vessels (85, 86). In support of the importance of neutrophil activation in AAV, avacopan, an inhibitor of the C5a receptor, blocking C5a-mediated neutrophil activation and infiltration into the vascular endothelium (87), became in 2021 the first new treatment for patients with severe and active MPA and GPA in over a decade.

NEUTROPHIL HETEROGENEITY

Traditionally, neutrophils have been regarded as homogenous cells with a single property of being efficient killers of the innate immunity. In recent years this view has changed and increasing evidence support the fact that neutrophils possess different phenotypes and functions, including the ability to regulate adaptive immune responses (9, 88-90).

One neutrophil subtype that has received much attention recent years constitutes the low-density neutrophils (LDNs), so-called because they are found within the low-density fraction during Ficoll-gradient separation. LDNs were first reported 1986 in SLE and RA patients (91) and hypothesized to reflect primed/activated (degranulated) neutrophils. At present, the term LDN refers to a wide variety of neutrophils, immature and mature, in health (89, 92) and in different pathological settings (89, 92, 93). In a recent study, pregnant women with SLE, known to face an increased risk of adverse pregnancy outcomes, were shown to possess higher proportions of circulating LDNs, as compared to healthy pregnant women, and LDNs in both peripheral and intervillous blood were more activated relative healthy pregnancies (94).

LDNs have been linked to T-cell suppression and these immunosuppressive neutrophils, also called PMN-myeloid-derived suppressor cells (PMN-MDSCs), are shown to be involved in many pathological conditions and to be associated with poor clinical outcomes in cancer (95). Potential regulating mechanisms involve, e.g., ROS production and direct cell-cell contact (96), while the precise identity and origin of PMN-MDSCs remains unclear (95). An additional neutrophil population linked to cancer and found within tumor tissues have been described, the so-called tumor-associated neutrophils (TANs), The function of these cells, as well as the connection between them and LDNs/PMN-MDSCs, remains unclear (97, 98).

Most neutrophil subsets, including those mentioned above, are typically found only during disease and they are quite often impossible to identify strictly based on the presence/absence of specific protein markers. There are, however, certain distinct neutrophil subsets that are defined by the presence/absence of protein markers and that in fact co-exist in circulation of healthy individuals. One such neutrophil subset marker is the glycoprotein CD177 that is expressed in 0-100% of circulating neutrophils in a given individual (99, 100). CD177 is located both in granule membranes and in the plasma membrane and our group recently reported that *in vitro* priming of neutrophils triggers further upregulation of CD177 in $CD177^+$ (but not in $CD177^-$) neutrophils (99). The function of CD177 is not fully understood, but higher proportions of $CD177^+$ neutrophils have been associated to different medical conditions, including autoimmune diseases such as AAV and SLE (101) and *in vitro,* the protein has been linked to neutrophil transmigration (102, 103). Our group recently showed that $CD177^+$ neutrophils have an advantage in terms of recruitment to the gingival crevice of periodontitis patients, which is a microbe-driven inflammatory process, while no such advantage could be seen in inflamed synovial fluids, or experimental skin chambers (99).

Another neutrophil subset marker is olfactomedin 4 (OLFM4). This glycoprotein is located within specific granules and present in 10%–30% of circulating neutrophils in healthy adults (104, 105). The role of this glycoprotein is unclear, but increased percentage of $OLFM4^+$ neutrophils have been independently linked to organ dysfunction and death in sepsis (106, 107). In a recent study, the proportion of $OLFM4^+$ neutrophils were elevated in septic shock, but there was no correlation to septic shock severity (108). *In vivo* transmigration to skin blisters (**paper I**), skin chambers and inflamed synovial fluids (105) did not influence the relative abundance of $OLFM4^+$ neutrophils in each individual, indicating that the presence/absence of OLFM4 does not affect tissue recruitment of neutrophils.

PHAGOCYTOSIS

The primary task for neutrophils is to recognize and eliminate invading microbes by phagocytosis, and it has now been more than a century since the Russian scientist Metchnikov proposed this concept for the first time. Metchnikov discovered that foreign pathogens, such as bacteria, are being engulfed and destroyed (phagocytosed) by our cells, thereby protecting our body from infectious diseases. This finding later awarded him the Nobel prize in Physiology or Medicine in 1908 (109).

Many cell types possess the ability to phagocytose particles, such as microbes and apoptotic cells, but only a specialized group of cells perform phagocytosis with high efficiency and these are called professional phagocytes (110), mainly including neutrophils (***Fig. 1***), macrophages and dendritic cells. Professional phagocytes are equipped with phagocytic receptors, opsonic or non-opsonic, that sense the microbe or particle as a target (111, 112). Opsonic receptors detect targets that are opsonized by immunoglobulins or complements, to facilitate phagocytosis. The best characterized opsonic receptors are FcRs (113), binding to immunoglobulins, and complement receptors (CR), binding to activated complement factors deposited on the particle surface. Among the complement receptors, CR3 is the most efficient phagocytic receptor, belonging to the integrin family of receptors (114). Non-opsonic receptors, directly recognizing molecular patterns, constitute some of the PRRs, e.g., the C-type lectin receptors (CLRs) (115), while for example TLRs are not able to induce phagocytosis. TLRs can however bind to their specific molecular pattern and by that, bring the neutrophil into a hyper-responsive (primed) state, for instance seen as an increase in surface expression of CR3, which will enable more efficient phagocytosis (112).

Once the target is recognized, receptor signaling triggers the neutrophil plasma membrane to extend around it, creating a membrane-bound vacuole called the phagosome. The process is quick, neutrophils can internalize an IgG-opsonized target in less than 20 seconds (116). Once created, the phagosome matures and fuses with cytosolic granules, mainly azurophilic and specific granules, creating an acidic phagolysosome filled with antimicrobial peptides and proteases. In addition, specific granules bring the membrane-bound part of the NADPH oxidase, needed to form ROS, to the membrane of the phagolysosome and a combination of these weapons kills and destroys the prey (117).

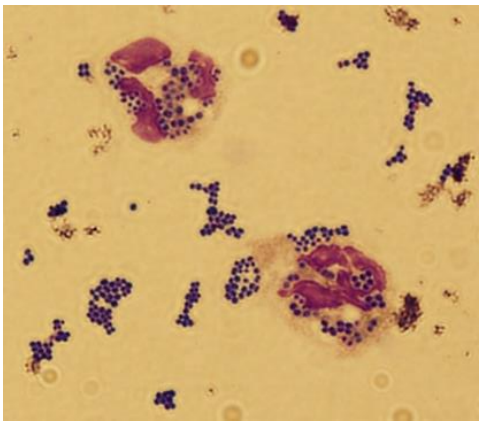


Figure 1. Phagocytosis. *Microscopy image of May-Grünwald-Gimsa stained cytospin slide, showing serum-opsonized* Staphylococcus aureus *(dark dots) being phagocytosed by neutrophils (with purple nuclei) after 20 minutes incubation at 37ºC.*

ROS PRODUCTION

ROS are highly reactive molecules that are produced in small amounts in all our cells, as a side product from mitochondrial respiration. At low levels, ROS are involved in many vital physiological processes, on the other hand, too much ROS are harmful to our cells. To regulate the balance and to protect us from the damaging effects of high amounts of ROS, the presence of antioxidants, such as superoxide dismutase (SOD) and catalase, help regulating the balance (118). In contrast to most other cell types, neutrophils are equipped with the ability to produce massive amounts of ROS upon activation, known as "the respiratory burst", primarily aimed for killing microbes in the phagosome, but also to function as signaling molecules, regulating immune reactions (119). Responsible for the neutrophil capacity to reduce molecular oxygen into ROS is the specialized, multicomponent, electron transporting enzyme system, the NADPH oxidase. The importance of ROS production is most obviously seen in patients with chronic granulomatous disease (CGD), a rare, inborn, immunodeficiency with defects in the proteins that form the oxidase. Neutrophils from CGD patients are unable to produce ROS and patients typically suffer from bacterial and fungal infections, the most prevalent being recurrent pneumonia (120, 121), as well as a variety of inflammatory symptoms (122, 123).

The NADPH oxidase

The NADPH oxidase (***Fig. 2***) consists of a membrane-bound part, cytochrome b558, and components in the cytoplasm, that all have to be put together to make the oxidase functional (124). Cytochrome b558 consists of two subunits (gp91phox and p22phox) and is primarily located in membranes of specific granules, but also, to some extent, in the plasma membrane and membranes of gelatinase granules (80). Upon activation, these granules translocate to, and fuse with, the plasma membrane, and/or intracellular membranes, providing cytochrome b558, whereupon the cytoplasmatic components (p47phox, p67phox, p40phox and GTPase Rac) translocate to the cytochrome b558-containing membrane to form the active oxidase. The active oxidase transports electrons across the membrane to molecular oxygen on the other side, reducing it into superoxide anion (O_2^-), which spontaneously dismutates into hydrogen peroxide (H_2O_2), a reaction that also can be catalyzed by SOD (125). These primary ROS can then be further processed by MPO into more potent secondary ROS, such as hypochloric acid (126). Hence, ROS can be produced both intra- and/or extracellulary depending on the location of the NADPH oxidase (127, 128). *In vitro,* one of the most frequently used agents to potently activate the NADPH oxidase, both intra- and extracellularly, is the chemical agent phorbol myristate acetate (PMA) (**paper III** and **IV**).

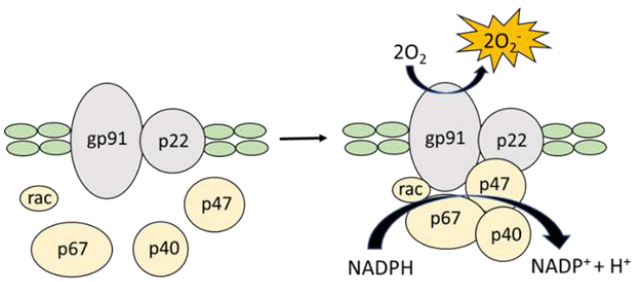


Figure 2. The NADPH oxidase mediates ROS production in neutrophils. *The membrane-bound part, cytochrome b558, constitutes the electron-transporting component of the oxidase and consists of gp91phox and p22phox. Upon activation, components in the cytoplasm (p47phox, p67phox, p40phox and the GTPase Rac) translocate to cytochrome b558-containing membranes, intra- and/or extracellularly, to form the active oxidase.*

During phagocytosis, specific granules provide cytochrome b558 to the membrane of the phagolysosome, and upon activation, the oxidase produce massive amounts of ROS to kill the ingested prey. In the phagolysosome there is also MPO, brought about by azurophilic granules, to process these primary ROS into more toxic secondary ROS, as described. Additionally, ROS can be produced intracellularly and be processed by MPO in situations where phagocytosis is not taking place, e.g., when triggered by soluble stimuli (127, 128). Why, and in which cellular compartment, such ROS is produced is not yet known, although an organelle formed as a result of the fusion between specific- and azurophilic granules has been suggested (129, 130).

When the oxidase is assembled in the plasma membrane, ROS are released extracellularly. The role of these ROS is not entirely clear, although it has been proposed, that when neutrophils fail to engulf the target, such as large bacteria or fungi, they instead produce and release ROS extracellularly (131, 132). Extracellular ROS are traditionally regarded as harmful and tissue damaging, but on the other hand, lack of ROS production (as in CGD) has been shown to *enhance* inflammatory symptoms, indicating that ROS are involved in cell signaling and regulation of the immune response (119, 133-135).

Measuring neutrophil ROS production

In vitro, assesment of ROS production is often used as read-out for neutrophil activation and there are several techniques to measure ROS (136). Extracellular levels of hydrogen peroxide (H_2O_2) can be measured by substrates that (catalyzed by horseradish peroxidase) oxidizes in the presence of H_2O_2, one of which being the frequently used fluorogenic Amplex Red reagent (**paper III**). The luminol-/isoluminol-amplified chemiluminescence (CL) technique used in **paper III** and **IV** is based on the emission of light (chemiluminescence) resulting from luminol/isoluminol reacting with O_2^- in the presence of a peroxidase (**Fig. 3**). Isoluminol cannot cross biological membranes and reacts only with extracellular O_2^-, while luminol reacts with both intra- and extracellular O_2^-. To detect intracellular O_2^- specifically, luminol is used together with membrane-impermeable ROS scavengers (SOD and catalase) that neutralize all extracellular ROS. Extracellular CL is measured in the presence of exogenously added horse radish peroxidase, whereas intracellular CL is utterly dependent on endogenous MPO activity (137).

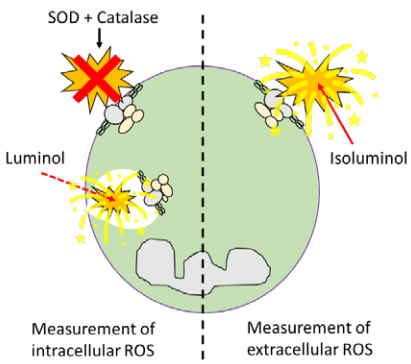


Figure 3. Schematic drawing of the chemiluminescence reaction. *When luminol/isoluminol reacts with ROS in the presence of a peroxidase, light (chemiluminescence) is emitted. Luminol crosses biological membranes and thus measures both intra- and extracellular ROS. To detect intracellular ROS specifically, luminol is used together with SOD and catalase (membrane-impermeable ROS scavengers) that neutralize all extracellular ROS. Extracellular ROS is measured by isoluminol which cannot cross biological membranes.*

NET FORMATION

Neutrophils can meet their end via active and non-lytic (apoptosis) or passive and lytic (necrosis) cell death, the latter characterized by loss of membrane integrity and proinflammatory responses. In 1996, a new form of cell death was described by Takei *et al* (138). PMA treated neutrophils were shown to demonstrate morphological changes different from those of typical apoptosis or necrosis. The cytotoxicity induced by PMA was possible to inhibit by the additive of antioxidants, suggesting an important role of ROS in this process. In 2004, this finding was confirmed and described as NET formation by Brinkmann *et al* (139) and has since received enormous interest.

NETs are web-like structures that enable neutrophils to capture microbes extracellularly, consisting of DNA strands clad with granule derived enzymes (e.g., MPO and NE) and antimicrobial peptides, expelled by neutrophils. Once captured, there is growing evidence that NETs also kill the microbes, at least *in vitro* (140). Although the majority of all studies on NET formation are made *in vitro* and quite few actually visualize NET release *in vivo* (141-146), NET formation is considered a real phenomenon. Stimuli shown to induce NET formation include microorganisms (139, 141, 147-149), immune complexes (150), certain cytokines (151), activated platelets (152) and sterile triggers such as MSU crystals (**paper II** and described in detail later). NET formation has also been linked to several non-infectious diseases. In autoimmune diseases, such as vasculitis (85, 86) and SLE (153), increased levels of NETs have been shown to promote inflammation and to be potential sources of autoantigens (154). NETs have also been shown to be involved in the formation of thrombi (both venous and arterial) (155, 156) and to contribute to the progression and metastasis of malignant tumors (142, 157).

NET formation occurs via different pathways, of which (NADPH oxidase derived) ROS dependent NET formation was discovered first and is the best described (139, 158, 159). *In vitro*, the standard procedure to trigger NET formation by this ROS dependent pathway, is to expose cells to the artificial stimulus PMA, often used as positive control for *in vitro* NET studies (**paper III** and **IV**). PMA triggers neutrophils to decondense their chromatin, disintegrate their nuclear envelope and mix nucleic acids with granule proteins, whereupon NETs are released via plasma membrane perforation. Our group recently showed that the ROS needed to promote PMA triggered NET formation has to be formed and processed by MPO intracellularly (137). Also ROS *independent* NET formation in response to certain stimuli is described (160-165) (**paper III**), although some studies argue that (NADPH-oxidase derived) ROS *independent* NET formation in fact relies on mitochondrial ROS (166, 167).

Certain types of NET formation have also been shown to consist of mitochondrial DNA (166, 168-171), to be dependent on mitochondrial ROS and not to be followed by cell death (168-171). In addition to this, Yipp and Kubes (141) proposed the concept of vital NET formation, in which the neutrophils are still viable and able to perform chemotaxis and phagocytosis, after rapidly throwing out NETs in response to gram positive bacteria, *in vivo*. In their study, nuclear (and not mitochondrial) DNA was described as the main source of DNA.

NET formation has indeed been subject for much investigation, providing an abundance of, sometimes contradictory, data. It is important to address the inconsistent view of the research community regarding the origin of the DNA (mitochondrial or nuclear) and delimitation from other forms of lytic cell deaths that have been described in the last decades (159, 172, 173). Two examples of these are necroptosis, which is a form of programmed necrosis (172), and pyroptosis, which is a form of programmed cell death triggered from intracellular pathogens, associated with osmotic swelling and pore formation (174). The different cell death pathways have been shown to overlap/crosstalk with each other to ensure that a cell that is committed to die will do so. Under certain conditions, NET formation has been shown to involve components/proteins of the pyroptosis and necroptosis pathways and it is likely that NET formation, to some extent, summarizes different lytic cell deaths leading to chromatin release from neutrophils (173).

There are several techniques to measure NETs, including, e.g., microscopy techniques, immunohistochemistry, enzyme-linked immunosorbent assays (ELISAs) and flow cytometry (175), but since neutrophil DNA and other NET component proteins can be present extracellularly due to other forms of lytic cell deaths as discussed, the detection is not specific for NETs. Improved techniques for the detection and quantification of NETs are needed to fully understand the role of NETs in health and disease.

NEUTROPHIL RECRUITMENT TO TISSUES

The recruitment of leukocytes to inflamed tissues follows a multistep pathway (**Fig. 4**) (9, 39). Once the acute inflammatory response is initiated, endothelial cells will upregulate adhesion molecules, including pre-stored P-selectin and *de novo* synthesized E-selectin, in response to proinflammatory mediators. Circulating neutrophils in postcapillary venules will then slow down and bind loosely to the inflamed endothelium through adhesion molecules on their surface, e.g., P-selectin glycoprotein ligand 1 (176). This weak binding, known as tethering, is further mediated by the neutrophil receptor L-selectin and promotes neutrophils to roll along the inflamed endothelium. After binding, L-selectin is proteolytically cleaved off and accompanied by the presence of soluble L-selectin in the circulation (177). Interactions between neutrophils and endothelial cells stimulate neutrophil granule mobilization, whereupon receptors necessary for attachment, e.g., the integrin CR3 (178), and chemotaxis, will be expressed on the cell surface. Firm attachment to the endothelium occurs through neutrophil-endothelium integrin-binding, and neutrophils then elongate and crawl through (transmigrate) the endothelium and migrate along the chemotactic gradient towards the inflamed site.

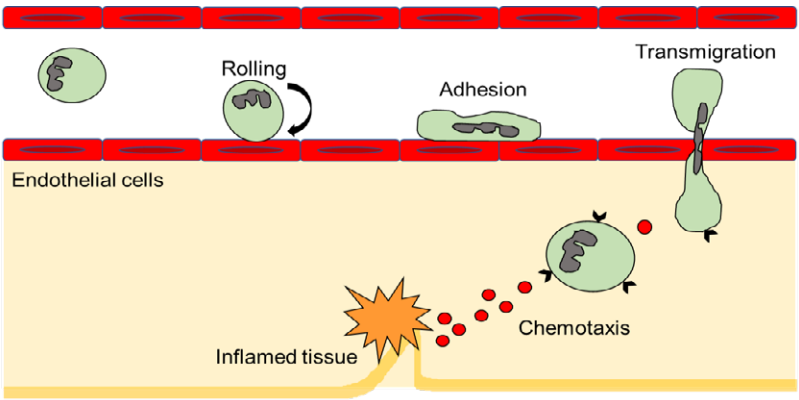


Figure 4. Transmigration from blood to tissue. *Circulating neutrophils bind loosely (tether) to the inflamed endothelium, start rolling and mobilize granules containing receptors necessary for attachment and chemotaxis to the cell surface. Firm attachment to the endothelium occurs through neutrophil-endothelium integrin-binding and neutrophils then transmigrate the endothelium and migrate along the chemotactic gradient towards the inflamed site.*

Chemotactic receptors

The crawling and chemotactic migration depends on cytoskeletal reorganization and interactions between neutrophil integrins and the extracellular matrix and is mediated by chemotactic G-protein-coupled seven-transmembrane receptors (GPCRs) expressed on the neutrophil surface (179, 180). Examples of such receptors are chemokine receptors, e.g., CXCR 1 and 2, mediating the effect of e.g., IL-8 which is highly chemotactic for neutrophils (181). In fact, neutrophils themselves amplify additional recruitment through release of IL-8 (182). Other GPCRs include e.g., formyl-peptide receptors (FPRs) that sense bacterial products such as the bacterial peptide formyl-methionyl-leucyl-phenylalanine (fMLF) (183) and receptors for a diverse set of chemo-attractants such as leukotriene B_4, platelet activating factor and complement fragment C5a (87).

The interaction with the inflamed endothelium and the exposure to proinflammatory mediators, put neutrophils in a state of high alert, called priming, necessary for subsequent activation and described next.

NEUTROPHIL PRIMING

Excessive ROS release into the surrounding tissues are believed to contribute to tissue injury and it is therefore important that neutrophil activation is properly regulated and that circulating neutrophils are restrained from unprovoked inflammatory response (184, 185). In health, to protect the surrounding tissues, circulating neutrophils are quiescent, with limited responsiveness to activating stimuli. To be able to forcefully respond to danger, including prolonged survival, morphology changes, maximal degranulation, enhanced phagocytosis, ROS production and NET formation, neutrophils must first get into a pre-activated condition known as a primed state (10, 186). Neutrophil priming is triggered by interactions with the inflamed endothelium during the transmigration process and by pro-inflammatory mediators or bacterial products at the inflamed site (10) and include a variety of events, e.g., proteolytic shedding of L-selectin, granule mobilization with upregulation of surface receptors (187), phosphorylation and translocation of the NADPH-oxidase cytosolic components (185) and an increased content and release of proinflammatory cytokines, such as IL-8 (182) (***Fig. 5***).

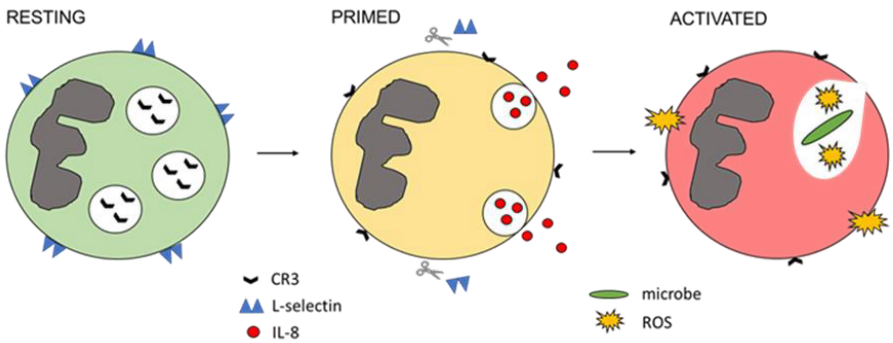


Figure 5. Priming and activation of neutrophils. *Circulating neutrophils are quiescent and characterized by limited responsiveness to activating stimuli. To get ready to respond to danger, neutrophils must first get into a pre-activated condition known as a primed state, normally induced by the neutrophil transmigration process and local factors at the inflammatory site. Activation of primed neutrophils through a second stimuli, results in reinforced neutrophil executive functions.*

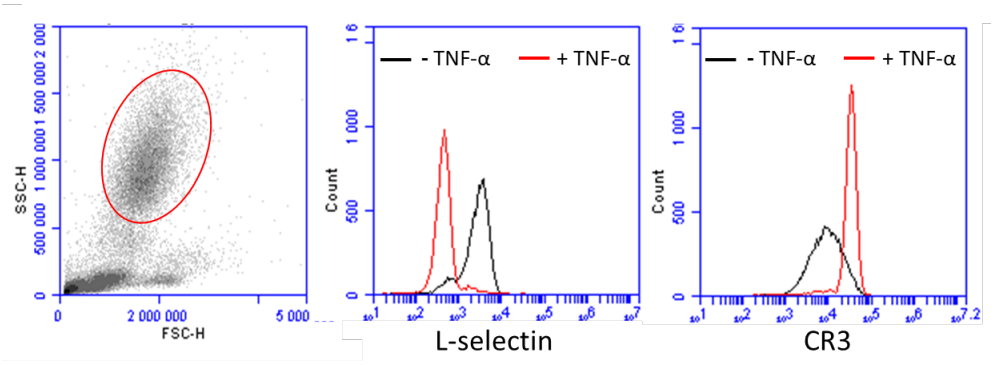
Priming of neutrophils in the bloodstream

In certain systemic conditions, such as e.g., sepsis (11-13), priming can occur within the circulation and while properly regulated priming of tissue neutrophils is beneficial for fighting bacteria, simultaneous systemic priming in severe sepsis is known to induce collateral damage (188). Signs of intravascular neutrophil priming have also been reported in different inflammatory conditions, such as elevated levels of ROS in RA and vasculitis (118, 185, 189, 190). In **paper IV**, blood neutrophils from gout patients were shown to be primed with regards to intracellular ROS production, but not receptor expression or NET formation (further discussed later).

Tools to assess neutrophil priming

Neutrophil priming *in vivo* is a dynamic and context-depending process that is not easy to study in humans and there is no exact definition of priming (10). In addition, *in vitro* experiments have demonstrated the neutrophil potential to recover from a primed phenotype ("de-priming"), although little is known about the importance of this phenomenon and the destiny of these cells (186). Much of our knowledge on neutrophil priming comes from *in vitro* experiments based on single primer/activation

combinations, often at high concentrations. Frequently used priming agents include for example the proinflammatory cytokine TNF-α and the bacterial toxin LPS and commonly used tools to assess neutrophil priming include the determination of surface receptor expression, e.g., CR3 (high) and L-selectin (low) (187) and measurement of ROS-responses (***Fig. 6***).



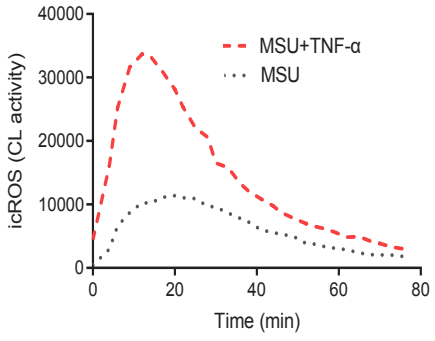


Figure 6. The differences in receptor expression and ROS production in primed versus resting neutrophils. *Receptor expression (above) was measured by flow cytometry. The gate (red circle) identifies the neutrophil population and is applied in the analysis of receptor expression. The black line shows resting neutrophils and the red line shows (primed) cells pre-treated (20 min) with TNF-α. L-selectin is cleaved off in primed cells, while CR3 is up-regulated. Intracellular ROS production (left) was measured using luminol-enhanced CL upon stimulation with MSU crystals. The black dotted line shows resting neutrophils and the red dashed line shows primed neutrophils. TNF-α does not itself trigger a ROS response.*

DAMAGED SKIN AS AN INFLAMMATORY MODEL

Studies on *in vivo* transmigrated human neutrophils include e.g., pus (141, 191), BAL fluid (192, 193), cerebrospinal fluid (194, 195), gingival crevicular fluid (99), saliva (196), sputum (197-199), peritoneal dialysis effluents (200), pleural effusion (201) and aspiration of synovial fluid from patients with arthritis (**paper II** and **III** and described later). Yet, much of our knowledge on *in vivo* transmigrated human neutrophils comes from experimental skin chambers, a controlled model of aseptic inflammation (202, 203); skin blisters (quite similar with those arising from, e.g., chafed feet) are created on the volar part of the forearm by application of negative pressure using a vacuum pump and blisters form after a little less than 2 hours (***Fig. 7***), whereupon the blister roofs are removed and collection chambers filled with autologous serum are adjusted over the (non-bleeding) lesions. Production of highly chemotactic mediators, e.g., IL-8 and the complement product C5a (203), in the skin chamber fluid creates a chemotactic gradient that guides neutrophils into the chambers, from where they can be collected and analyzed. Neutrophils account for about 90%–98% of the exudate cells after 10–24 h (204). Transmigration from blood to tissue is known to induce neutrophil priming and skin chamber derived neutrophils display a highly primed phenotype with increased extracellular ROS production in response to chemo-attractants (203, 205), upregulation of CR3, shedding of L-selectin and increased content of IL-8 (**paper II**), in line with the prevailing view and those seen at natural sites (191-193, 199, 200). In addition, skin chamber derived neutrophils were shown to be highly primed (respond much stronger) with respect to MSU crystal induced intracellular ROS production, as compared to blood neutrophils from the same donor (**paper III**).

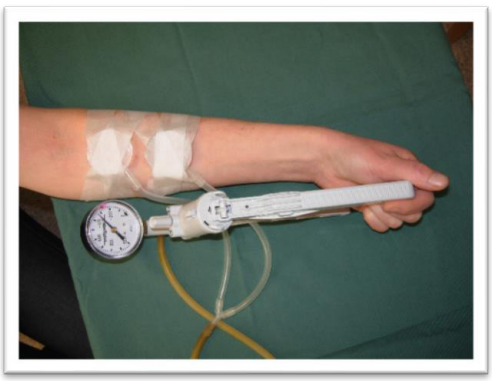


Figure 7. The creation of skin blisters. *Skin blisters are created on the volar part of the forearm using suction chambers connected to a vacuum pump. Blisters form after a little less than 2 hours.*

Paper I – a simple skin blister model

In **paper I**, a simple and useful skin blister technique to obtain and study *in vivo* transmigrated human leukocytes was described and characterized. The described technique is based on the formation of skin blisters identical to those used for the skin chamber technique, but without the more complicated (and slightly artificial) part including removal of the blister roofs and attaching plastic collection chambers filled with serum; immune cells are recruited directly into the blister fluid, from which they can be collected at different time-points using a micropipette. The collected blister fluid can also be used to analyze soluble inflammatory mediators at different time points (206-208). The total leukocyte count (neutrophils, monocytes and lymphocytes) were shown to increase in the blister fluid over the first 6-8 hours, after which the numbers were consistent up to the final analyzed time-point (24 h). Furthermore, and true to the text book, neutrophils were shown to be recruited first, followed by monocytes and lymphocytes. Neutrophils obtained from our skin blister model were fully viable and functionally competent (able to phagocytose microbes). Also, similar to the neutrophils obtained from the skin

chamber model, these neutrophils displayed a distinctly primed phenotype with regard to surface markers, e.g., upregulation of CR3 and shedding of L-selectin. Although simple to use and bear, the drawback of the skin blister technique lies in the low total cell count, which makes it more difficult to perform functional studies.

An inflammatory skin model based on intradermal LPS injections were recently characterized by Buters *et al* (207). The authors wanted to validate intradermal LPS for future "proof of pharmacology" studies involving anti-inflammatory drugs. Suction blisters were found to be convenient when it came to studying the inflammatory response and the results from their naïve control blisters correspond well to the results from the kinetic study in **paper I**. In a follow-up study, the clinical and cellular (but not cytokine) inflammatory response to intradermal LPS was shown to be suppressed by oral as well as topical corticosteroids, demonstrating that the LPS model could detect the effects of anti-inflammatory drugs (209). A similar model was reported by Motwani *et al*; intradermal UV-killed *Escherichia coli* triggered a well-tolerated, transient acute inflammatory reaction that was evaluated by suction blisters. Treatment with naproxen (a commonly used NSAID) provided a detectable anti-inflammatory effect (208).

SYNOVIAL FLUID NEUTROPHILS

The bones of a synovial joint are covered by hyaline cartilage and united by a fibrous joint capsule that encloses the joint cavity and provides structural support. The inner lining of the joint capsule, the synovium, is a specialized connective tissue built up by a thin layer of cells composed of two cell types, macrophage-like synoviocytes and fibroblast-like synoviocytes, and an underlying tissue including blood and lymphatic vessels. The synovium maintains the synovial fluid that lubricates the surfaces and provides nutrients to the cartilage (210).

The chronically inflamed synovium seen in inflammatory (non-infectious) rheumatic arthritis that will be discussed here is characterized by hyperplasia, increased vascularization, recruitment and activation of immune cells. However, pathogenic pathways differ between various rheumatic arthritis, as well as between different individuals and there are important differences in e.g., the degree of neo-angiogenesis and lining layer hypertrophy as well as the inflammatory infiltrate (211). Rheumatic arthritis comprises both autoimmune conditions, typically linked to the presence of autoreactive T-cells and/or autoantibodies, and autoinflammatory diseases, mainly driven by the innate immunity. However, rather than being separate entities, these conditions overlap and share characteristics and rheumatic diseases can be placed somewhere along a spectrum with classic autoinflammatory diseases (such as periodic fevers) in one end and classic autoimmune diseases (such as SLE) in the other end. The common chronic inflammatory arthritis RA is typically associated with autoantibodies against rheumatoid factor (RF) and/or cyclic citrullinated peptides (anti-CCP) and is generally considered an autoimmune disease, but the pathogenesis of RA is complex; different RA "pathotypes" have been suggested and some forms of RA rather display features of both autoimmunity and autoinflammation. In addition, there is a large variation in treatment responses in RA. Spondyloarthritis (SpA), including e.g., psoriatic arthritis and ankylosing spondylitis, like some forms of RA, can be placed somewhere in between autoimmunity and autoinflammation. Another common rheumatic arthritis, generally considered an autoinflammatory disease and that will be further discussed in the next chapter, is gout (6).

Paper II – neutrophil recruitment to inflamed joints

Neutrophils possess the capacity to cause damage to joints and are represented in the inflamed synovium early in RA disease progression (212-215). As the disease progresses, the cellular pattern changes so that the inflamed synovium consists mainly of macrophage- and invasive fibroblast-like synoviocytes, lymphocytes and osteoclasts. The chronically inflamed synovium constitutes an invasive tissue that destroys cartilage and bone (216), but in spite of the fact that the inflamed synovium is mostly infiltrated by synoviocytes and lymphocytes, neutrophils are the most abundant leukocytes in synovial fluid during disease flares of inflammatory arthritis.

In **paper II**, *in vivo* transmigrated neutrophils, derived from synovial fluid of patients with ongoing disease flares of different inflammatory arthritis, were found to often display no or very limited signs of priming. These cells often kept L-selectin and had undergone restricted upregulation of CR3 and were more similar to (resting) blood neutrophils than to, e.g., fully primed skin chamber neutrophils. In support of these findings, cell-free synovial fluid was shown to trigger chemotaxis *in vitro* without altering the receptor expression. These data demonstrate that L-selectin cleavage is not a prerequisite for neutrophils to leave the blood stream and that it is indeed possible for neutrophils to egress to inflamed tissues without undergoing pronounced priming. Furthermore, the synovial fluid neutrophils in our study contained IL-8 levels that were only marginally increased as compared to blood neutrophils from the same donor. This is in stark contrast to skin chamber neutrophils that contained significantly higher levels of IL-8 as compared to blood neutrophils from the same donor.

Taken together, these data indicate that transmigration can occur without cellular priming and that recruitment to inflamed joints might be different from transmigration to other inflamed tissues. It is likely that the principles of neutrophil recruitment cannot be entirely translated into all vascular beds and conditions, which is supported by observations on different recruitment mechanisms in the liver, lung, brain, lymph nodes and kidney (9). In the liver for example, it has been shown that integrins and not selectins are involved/required for recruitment of neutrophils (217, 218). In the brain, platelets have

been shown to play a critical role in neutrophil recruitment. Platelets were shown to adhere to inflamed brain capillaries and to help recruit neutrophils through their, in relation to brain endothelium, enormous production of P-selectins (219, 220).

It should be mentioned that other groups have reported contradictory results regarding neutrophil recruitment to inflamed joints. Emery *et al* (221) and Nurcombe *et al* (222) reported that synovial fluid neutrophils derived from patients with RA demonstrate a primed phenotype, but these studies, unlike ours, used separation steps including Ficoll-gradients, which could have affected the results. More recently, synovial fluid neutrophils derived from inflamed joints of children with juvenile idiopathic arthritis were shown to display a primed phenotype with upregulation of CR3 and shedding of L-selectin (223, 224), using a neutrophil preparation technique similar to ours, i.a., only centrifugation. All patients in these studies were children, without immunosuppressive treatment (except for NSAIDs), while in **paper II,** all patients were adults, most of which had immunosuppressive treatment, which could constitute a partial explanation for the different outcomes. Another difference between these studies is that only samples rich in neutrophils (with a neutrophil content >65%) were included in **paper II** and **III**.

In line with our findings in **paper II,** neutrophils derived from synovial fluid of different inflammatory arthritis were shown to produce various amounts of ROS in response to *ex vivo* stimulation with MSU crystals, while skin chamber derived neutrophils were all highly primed with respect to ROS production (**paper III**). The varying ROS responses likely reflect the heterogeneity among inflammatory arthritis. Our data however clearly show that it is possible for neutrophils to reach the synovial fluid without getting (fully) primed.

GOUT

Gout has been a clinical entity for millennia, referenced in writing by the Egyptians as early as 2600 BC. The name gout comes from the Latin *Gutta*, meaning "drop of liquid", referring to the belief that vicious humors went from the blood stream into the (previously asymptomatic) joint, causing the gouty attack (225). It was first in the mid-19th century that the Englishman Alfred B Garrod, sometimes referred to as the father of modern rheumatology, discovered that MSU crystals were central in gouty inflammation (226).

Gout is the most common arthritis worldwide, triggered by the deposition of needle-shaped MSU crystals in (and around) the joints, as a result of oversaturation of uric acid (urate) in the bloodstream (227, 228). Uric acid is the final product of purine metabolism and both increased intake and/or production, as well as impaired excretion of uric acid by the kidneys, may lead to hyperuricemia, defined as elevated urate levels above the solubility level of around 405 µmol/L (6.8 mg/dL). Hyperuricemia is a central risk factor for gout and other risk factors are linked to hyperuricemia, e.g., obesity, genetic factors, dietary factors, such as red meat and beer, and chronic kidney disease. Once described as the "disease of kings", gout has been perceived as closely linked to "rich food" and alcohol, but it is debated which of genes or lifestyle that contribute most to gout. In a recent meta-analysis, genetic factors were found to contribute substantially more to hyperuricemia than diet (229). Gout patients have an established increased risk of cardiovascular disease as well as a high burden of comorbidities, but evidence is mixed whether it is hyperuricemia/gout, or the comorbidities, that cause the increased risk of cardiovascular disease (227, 228).

The first joint to be affected in gout is usually the metatarsophalangeal joint, by Hippocrates referred to as "the podagra" (roughly translated "attack in the foot") (225). One reason for the early engagement of this joint might be its relatively reduced perfusion and therefore lower temperature which seems to bring down the saturation limit for uric acid. The inflamed joint is often particularly painful with maximal pain within the first day. Gout flares are self-limiting by nature and usually resolve within 10-14 days. The disease has an episodic nature, in which flares tend to become more frequent and affect additional joints, i.e., become polyarticular (or chronic) if hyperuricemia persists. Chronic gout also includes the presence of tophi; superficial, yellow-white deposits of MSU crystals seen around the joints (***Fig. 8***) (228). Despite their uninflamed features, tophi can become infected, cause pain and lead to decrease in function. Depending on their location, complications can also occur when tophi develops in, e.g., heart valves, spine or larynx, although these locations are unusual (230). Identification of MSU crystals in synovial fluid or in tophi by polarization microscopy allows for definitive diagnosis (231) and preventive urate-lowering treatment aims at achieve and maintain urate levels below 360 µmol/L (6 mg/dL), or below 300 µmol/L (5 mg/dL) in severe tophaceous gout. During flares, inflamed joints can be effectively treated using corticosteroids, NSAIDs or colchicine (232).

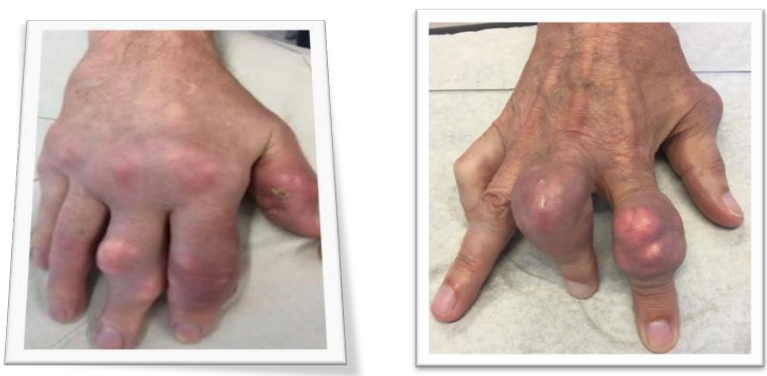


Figure 8. Two patients with chronic, tophaceous gout. *Photos are published with patient consent.*

Inflammasome activation in gout

The nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) are intracellular PRRs, expressed mainly in macrophages and neutrophils and critical in the inflammatory response in gout (15, 228). When macrophages ingest tissue deposited MSU crystals, an intracellular, multiprotein complex known as the NLR family pyrin domain containing 3 (NLRP3) inflammasome is assembled/activated, leading to activation of caspase-1 and subsequent proteolytic cleavage and activation of IL-1β. This proinflammatory cytokine is the best described inflammatory mediator in gout and the release of IL-1β triggers a massive inflammatory response with influx of neutrophils (228, 233).

As mentioned earlier, rheumatic diseases can be placed more towards autoimmunity or autoinflammation (6). Autoinflammatory diseases compose a heterogeneous group of systemic inflammatory diseases that are linked to antigen-independent activation of the innate immune response and not to autoantibodies or autoreactive T-cells (i.e., the adaptive immune response) (6, 7). The prototypic autoinflammatory disease is Familial Mediterranean fever (FMF), prevalent in countries of the eastern Mediterranean region and associated with mutations in the MEFV gene encoding the pyrin-inflammasome, characterized by recurrent flares of fever, serositis and synovitis (234). Gout exhibits both clinical and mechanistic overlap with autoinflammatory diseases and is associated with multiple gene variants that are linked to autoinflammatory conditions (235). The fact that gout has a known trigger distinguishes it from (most) other autoinflammatory conditions, but far from all individuals with hyperuricemia develop gout (236, 237), suggesting that additional inflammatory elements contribute to the pathogenesis of the disease. Both gout and autoinflammatory diseases normally exhibit a good treatment response to IL-1-inhibitors, supporting an important role of inflammasome activation in the pathogenesis of these diseases (238, 239).

Colchicine – an ancient drug still in use today

Colchicine is a plant extract from the autumn crocus (***Fig. 9***), described by the Egyptians as early as 1550 BC for its effect on joint pain and swelling, and used for many hundreds of years to treat gout (240, 241). Colchicine has a dark history of being a poison and is indeed extremely toxic in overdose. Today, although demonstrating a narrow therapeutic index due to early gastrointestinal intolerance, serious toxicity at prescribed doses is rare (242). Except from gout, colchicine is also used to treat, e.g., FMF, acute and relapsing pericarditis, and has become of high interest for cardiovascular research, being shown to reduce the risk of cardiovascular events in patients with cardiovascular disease (243, 244). Colchicine inhibits intracellular microtubule assembly and is preferentially accumulated in leukocytes (245). The mechanism(s) of action of colchicine is not fully understood, but it is believed to reduce inflammation by inhibiting migration of neutrophils and other leukocytes to the gouty inflamed site (246, 247) and to suppress activation of the NLRP3 inflammasome (247). Colchicine has also been shown to suppress MSU crystal induced neutrophil ROS production (248, 249) (**paper III)**, which will be further discussed in the next subchapter.

Figure 9. Autumn crocus, the plant source of colchicine*. Photo credit to the authors mother.*

NEUTROPHIL ACTIVATION IN GOUT

Inflammasome activation and subsequent release of IL-1β and other proinflammatory cytokines trigger recruitment of neutrophils to the gouty inflamed site (228). Neutrophils that encounter MSU crystals *in vitro* (at least try to) phagocytose the (big) crystals (250-254), produce ROS (164, 248, 250, 255-259) and form NETs (164, 252, 254, 258-261). It has been proposed that MSU crystals initially induce NET formation associated by release of proinflammatory mediators, but during high neutrophil densities (as a result of massive recruitment), these NETs form aggregates (resembling gouty tophi) and NET-associated serine proteases degrade proinflammatory cytokines and chemokines, favoring resolution of inflammation (258). It is possible that the uninflamed features of gouty tophi, and the reason why acute gouty attacks are self-limiting within 10-14 days, can be explained by these findings.

Paper III – MSU crystal triggered neutrophil responses

In **paper III**, blood neutrophils from healthy controls were challenged with MSU crystals *in vitro*, resulting in (a dose-dependent) production of ROS and NETs (***Fig. 10***).

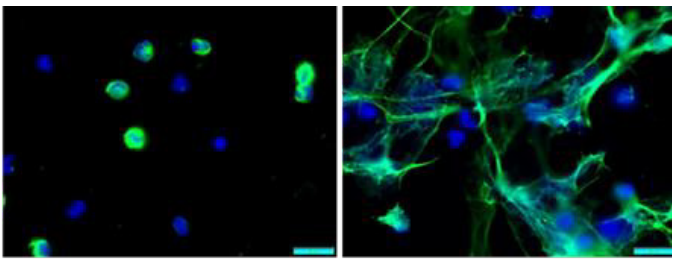


Figure 10. NET formation triggered by MSU crystals. *Microscopy image of unstimulated (left) and MSU crystal stimulated (right) neutrophils, stained for DNA (blue) and MPO (green). (Reprinted with permission.)*

MSU triggered ROS were shown to be strictly intracellular; two different assays used to detect extracellular ROS (O_2^- and H_2O_2 respectively), resulted in clear responses to PMA (the positive control) as expected, but not upon MSU crystal stimulation. To investigate if this MSU triggered intracellular ROS production origins from phagosomes, neutrophils were pretreated with a blocker of actin polymerization, making the neutrophils unable to phagocytose, before challenging them with the crystals. The result was a significant decrease in ROS production, implying that the oxidative response originates from neutrophils ingesting the crystals.

We then tested whether pretreatment of the cells with colchicine would affect MSU triggered neutrophil ROS production. The result was a significant decrease in ROS production, but interestingly, not linked to ingestion of the crystals, demonstrated by the fact that colchicine-treated cells were still able to fully phagocytose opsonized *Staphylococcus aureus*. One possible explanation for this would be that colchicine exerts its effect on (MSU crystal induced) neutrophil ROS production by interfering with the activation/assembly of the NADPH-oxidase. The effect that colchicine exerts on neutrophil ROS production seems, at least to some extent, be specific for MSU crystals since ROS production triggered by e.g., PMA (262, 263) (**paper III**) or fMLF (256, 262, 263) is not affected.

Several pathways involved in MSU triggered NET formation have been suggested. In line with Chatfield *et al* (164), but unlike others (258, 259), our data clearly demonstrate that MSU triggered NET formation takes place *independent* of ROS production. Neutrophils that were pretreated with inhibitors of the NADPH oxidase, as well as cells from a patient with CGD (unable to produce ROS), still formed NETs upon MSU crystal stimulation.

As discussed earlier, the ROS response to MSU crystals was shown to be highly primed in *in vivo* transmigrated neutrophils derived from skin chambers, while tissue neutrophils derived from inflamed (non-gouty) synovial fluid displayed various levels of priming with respect to ROS production and this

observation fits well with our earlier finding that synovial fluid neutrophils often display no or only mild signs of priming (**paper II).** Neither of our studied tissue neutrophils were primed with respect to MSU induced NET formation, demonstrating that the primed phenotype of tissue neutrophils does not apply for NET formation, at least not when triggered by MSU crystals, or PMA.

Paper IV – priming of blood neutrophils in gout patients

Neutrophils can be primed in the circulation and this have been shown in sepsis (11-13), as well as in different inflammatory conditions (118, 185, 189, 190). Vedder *et al* (264) recently demonstrated the presence of NETs, as detected by an MPO-DNA-ELISA, in the circulation of gout patients. Further, neutrophil activation markers in the circulation (the cytoplasmic protein calprotectin and peroxidase activity), but not NETs, were shown to associate with characteristics of active, polyarticular gout (264) and increased cardiovascular risk during long term follow-up (265). We wanted to further investigate neutrophil activation by MSU crystals and test whether neutrophils from gout patients react similarly as those from healthy controls.

In **paper IV** blood neutrophils from polyarticular gout patients appear to exhibit a reinforced intracellular ROS production, as compared to controls, both at baseline (without stimulation) and upon MSU crystal stimulation *in vitro*. One could speculate that small circulating MSU crystals in peripheral blood of gout patients (264, 266) prime the neutrophils and give rise to the enhanced background activity seen in our ROS experiments. An interesting upcoming experiment would be to add cytochalasin B to otherwise unstimulated gout neutrophils to see if that would confer a dip in baseline ROS production. If so, the idea that the enhanced background activity of gout neutrophils stems from phagocytosis of small circulating MSU crystals would be strengthened. Another explanation could be that proinflammatory cytokines in the bloodstream of gout patients (267) prime neutrophils to produce increased amounts of ROS. Frozen plasma from our study participants will be used to analyze the occurrence of soluble inflammatory mediators that might be responsible for the primed ROS response. The study is however small and since there is a substantial variability in neutrophil responses on an individual level, large cohorts are needed to fully adjust for these variations.

Despite the primed ROS response, surprisingly, the expression of cell surface markers L-selectin and CR3 were similar between neutrophils derived from gout patients and controls. *In vitro* priming with TNFα significantly primed neutrophils, assayed on basis of surface expression of L-selectin and CR3, from both groups in a very similar manner, again indicating that these blood cells are truly resting and that the priming machinery is intact. NET formation was also similar in both groups, both at baseline and upon stimulation with MSU crystals. The latter fits well with the results in **paper III**, where the ROS response, but not NET formation, was primed in tissue neutrophils.

As mentioned, gout patients have an established increased risk of cardiovascular disease and it has been described as a paradox that uric acid, a weak acid known to possess antioxidative properties, and that may offer neuroprotective advantages (268-271), correlates with conditions associated with oxidative stress such as cardiovascular disease. It has been proposed that uric acid may become pro-oxidant in certain situations, particularly when present at high levels (272-274). The role of uric acid in relation to neutrophil ROS production is unclear and soluble uric acid has been shown to prime (267), as well as neutralize (275), neutrophil ROS. In **paper III**, the presence of MSU crystal induced extracellular ROS could not be demonstrated, in fact the extracellular ROS signal (using isoluminol-enhanced CL) seemed to dip below background levels (cells treated with buffer). When the MSU crystal dose was triturated <0.1 μg/mL, the signal was equal to buffer treated samples. One possible explanation for this outcome might be that (high concentrations of) MSU crystals neutralize ROS. In **paper IV**, mean plasma urate level in the patient group was not significantly different ($p>0.05$) from the plasma urate in the control group and a clear connection between the levels of ROS and uric acid could not be demonstrated. It is possible that untreated patients, with higher levels of plasma urate in circulation, would generate different results.

CONCLUDING REMARKS

Inflammation is required for defense against microbial infections, but can also cause extensive damage to host cells and tissues and is implicated in a wide range of human diseases. A deeper level of the understanding of neutrophil function has been reached in recent years and neutrophils are seen both as potent effector cells and important regulators of inflammation. Combining clinical and basic research provides synergetic effects that helps understand inflammatory processes and the overall aim of this thesis was to increase the current knowledge of processes that participate in regulating inflammation, with the intention to bridge the gap between clinical and basic research.

Initially, we described and characterized a simple skin blister model that can be used to study transmigrated leukocytes and inflammatory responses (**paper I**). Using the described skin blister model, and the more widely used skin chamber model, we showed that transmigration to inflamed, but otherwise healthy tissue, in line with the leading dogma, induce neutrophil priming (**paper I** and **II**). However, recruitment to tissues does not always result in neutrophil priming as shown in **paper II**, where neutrophil transmigration to acutely inflamed joints of patients with different inflammatory diseases was shown to occur with no, or only mild, signs of priming. In addition, priming of neutrophils clearly does not mean that all cellular effector functions are affected similarly, for example ROS production can be primed while NET formation is not (**paper III** and **IV**).

Priming can also occur in the circulation, often linked to adverse outcomes. In gout, the most common arthritis worldwide, activation of circulating blood neutrophils has been reported to associate with an increased risk of cardiovascular events during follow-up. The main finding in **paper IV** was that blood neutrophils from gout patients display a primed intracellular ROS response, however not reflected in changes in receptor expression or increased NET formation. The cause and importance of this finding needs to be further studied.

The results that are presented in this thesis highlight the complexity of neutrophil priming and activation and the changes that neutrophils undergo during transmigration to different inflamed tissues. Increased understanding of neutrophil biology will likely help explain underlying causes for pathologies involving inflammatory components and hopefully, this will contribute to the development of new treatment strategies for inflammatory diseases in the future.

ACKNOWLEDGEMENT

Ett stort tack till alla er som på olika sätt bidragit till denna avhandling!

Speciellt tack till:

* Mina tre handledare - *Johan, Karin och Lena* – en stabil trio som kompletterat varandra väl och vars respektive styrkor på olika sätt har hjälp mig framåt. *Johan* – min huvudhandledare – har varit lätt att nå och gärna diskuterat forskning. Jag uppskattar också den utvecklande feedback jag fått på mitt skrivande. *Karin* – min bihandledare – kan det mesta som finns att kunna om neutrofiler och på labbet och har bland mycket annat gett mig ovärderlig hands-on handledning. *Lena* (last but not least) – också bihandledare - var den som gjorde att jag hamnade här från början. När jag var ny ST och jobbade ihop med Lena tänkte jag ”här får man ta tillfället i akt att haffa en bra handledare”! Om neutrofilforskning visste jag inte så mycket (och det var kanske tur det höll jag på att säga…)! Under dessa år har det hunnit rinna mycket vatten under broarna och det känns väldigt tillfredsställande att snart hålla den färdiga boken i handen. Stort tack till er som funnits med hela vägen!
* Alla patienter och försökspersoner som deltagit i studierna!
* Mina medförfattare och alla personer som jag har haft runt mig, först i Fagocytgruppen nere på Guldhedsgatan och senare uppe på Odontologen – ingen nämnd och ingen glömd - men ett särskilt stort tack till *Agnes och Felix*, mina doktorandkollegor de senaste åren!
* Min hemklinik - Reumatologi SU - en forskningsvänlig arbetsplats med trevliga kollegor som bidragit med klinisk kontext och gett mig den nödvändiga förutsättningen för mina doktorandstudier – nämligen forskningstid i schemat!
* *Katrín* – nu hemflyttad till Island - för uppmuntrande ord någonstans i mitten av småbarnsår, specialisttjänstgöring och ett laborativt doktorandprojekt; ”Man känner hur man kravlar omkring på botten, men när man kommer ut på andra sidan är man oövervinnelig” (eller nåt sånt… min fria tolkning såhär några år senare)!
* *Susanna* – som jag känt ända sedan 1991 då vi blev stäm-kompisar i musikskolans blåsorkester – tack för den fina omslagsbilden!
* Mina föräldrar och syskon med familjer för omtanke och support!
* Ett extra speciellt tack till mina svärföräldrar för ert ovärderliga engagemang i oss och barnen!
* Min familj - *Oleg, Ludvig och Viktor* – kanske inte så mycket för ert bidrag till avhandlingen (!) men för att ni finns, ni är mina ♡♡♡

This thesis was supported by grants from the:

* Gothenburg Society of Medicine
* King Gustav V Memorial Foundation
* Swedish Rheumatism Association
* Swedish Research Council
* Swedish state under the ALF-agreement

REFERENCES

1. Riedel S. Edward Jenner and the history of smallpox and vaccination. Proc (Bayl Univ Med Cent). 2005;18(1):21-5.

2. Kaufmann SH. Remembering Emil von Behring: from Tetanus Treatment to Antibody Cooperation with Phagocytes. mBio. 2017;8(1).

3. Chaplin DD. Overview of the immune response. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S3-23.

4. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. Allergy Asthma Clin Immunol. 2018;14(Suppl 2):49.

5. Plotkin S. History of vaccination. Proc Natl Acad Sci U S A. 2014;111(34):12283-7.

6. Szekanecz Z, McInnes IB, Schett G, Szamosi S, Benko S, Szucs G. Autoinflammation and autoimmunity across rheumatic and musculoskeletal diseases. Nat Rev Rheumatol. 2021;17(10):585-95.

7. Manthiram K, Zhou Q, Aksentijevich I, Kastner DL. The monogenic autoinflammatory diseases define new pathways in human innate immunity and inflammation. Nat Immunol. 2017;18(8):832-42.

8. Gilroy D, De Maeyer R. New insights into the resolution of inflammation. Semin Immunol. 2015;27(3):161-8.

9. Liew PX, Kubes P. The Neutrophil's Role During Health and Disease. Physiol Rev. 2019;99(2):1223-48.

10. Vogt KL, Summers C, Condliffe AM. The clinical consequences of neutrophil priming. Curr Opin Hematol. 2019;26(1):22-7.

11. Bass DA, Olbrantz P, Szejda P, Seeds MC, McCall CE. Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. J Immunol. 1986;136(3):860-6.

12. Patel JM, Sapey E, Parekh D, Scott A, Dosanjh D, Gao F, et al. Sepsis Induces a Dysregulated Neutrophil Phenotype That Is Associated with Increased Mortality. Mediators Inflamm. 2018;2018:4065362.

13. Whitmore LC, Hook JS, Philiph AR, Hilkin BM, Bing X, Ahn C, et al. A Common Genetic Variant in TLR1 Enhances Human Neutrophil Priming and Impacts Length of Intensive Care Stay in Pediatric Sepsis. J Immunol. 2016;196(3):1376-86.

14. Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 1989;54 Pt 1:1-13.

15. Li D, Wu M. Pattern recognition receptors in health and diseases. Signal Transduct Target Ther. 2021;6(1):291.

16. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388(6640):394-7.

17. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282(5396):2085-8.

18. O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol. 2013;13(6):453-60.

19. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 2018;233(9):6425-40.

20. Collin M, Bigley V. Human dendritic cell subsets: an update. Immunology. 2018;154(1):3-20.

21. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. Science. 2010;327(5966):656-61.

22. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013;496(7446):445-55.

23. Kumar V, Sharma A. Mast cells: emerging sentinel innate immune cells with diverse role in immunity. Mol Immunol. 2010;48(1-3):14-25.

24. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. Science. 2011;331(6013):44-9.

25. Morvan MG, Lanier LL. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer. 2016;16(1):7-19.

26. de Bont CM, Boelens WC, Pruijn GJM. NETosis, complement, and coagulation: a triangular relationship. Cell Mol Immunol. 2019;16(1):19-27.

27. Bennett KM, Rooijakkers SH, Gorham RD, Jr. Let's Tie the Knot: Marriage of Complement and Adaptive Immunity in Pathogen Evasion, for Better or Worse. Front Microbiol. 2017;8:89.

28. West EE, Kolev M, Kemper C. Complement and the Regulation of T Cell Responses. Annu Rev Immunol. 2018;36:309-38.

29. Brodszki N, Frazer-Abel A, Grumach AS, Kirschfink M, Litzman J, Perez E, et al. European Society for Immunodeficiencies (ESID) and European Reference Network on Rare Primary Immunodeficiency, Autoinflammatory and Autoimmune Diseases (ERN RITA) Complement Guideline: Deficiencies, Diagnosis, and Management. J Clin Immunol. 2020;40(4):576-91.

30. Reis ES, Mastellos DC, Hajishengallis G, Lambris JD. New insights into the immune functions of complement. Nat Rev Immunol. 2019;19(8):503-16.

31. van de Bovenkamp FS, Dijkstra DJ, van Kooten C, Gelderman KA, Trouw LA. Circulating C1q levels in health and disease, more than just a biomarker. Mol Immunol. 2021;140:206-16.

32. Fishelson Z. Complement C3: a molecular mosaic of binding sites. Mol Immunol. 1991;28(4-5):545-52.

33. Conigliaro P, Triggianese P, Ballanti E, Perricone C, Perricone R, Chimenti MS. Complement, infection, and autoimmunity. Curr Opin Rheumatol. 2019;31(5):532-41.

34. Weinstein A, Alexander RV, Zack DJ. A Review of Complement Activation in SLE. Curr Rheumatol Rep. 2021;23(3):16.

35. Aringer M, Costenbader K, Daikh D, Brinks R, Mosca M, Ramsey-Goldman R, et al. 2019 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Systemic Lupus Erythematosus. Arthritis Rheumatol. 2019;71(9):1400-12.

36. Fullerton JN, Gilroy DW. Resolution of inflammation: a new therapeutic frontier. Nat Rev Drug Discov. 2016;15(8):551-67.

37. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428-35.

38. Sinniah A, Yazid S, Flower RJ. From NSAIDs to Glucocorticoids and Beyond. Cells. 2021;10(12).

39. Borregaard N. Neutrophils, from marrow to microbes. Immunity. 2010;33(5):657-70.

40. Levi M, van der Poll T. Inflammation and coagulation. Crit Care Med. 2010;38(2 Suppl):S26-34.

41. Keragala CB, Draxler DF, McQuilten ZK, Medcalf RL. Haemostasis and innate immunity - a complementary relationship: A review of the intricate relationship between coagulation and complement pathways. Br J Haematol. 2018;180(6):782-98.

42. Reinke JM, Sorg H. C. Eur Surg Res. 2012;49(1):35-43.

43. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340(6):448-54.

44. Litao MK, Kamat D. Erythrocyte sedimentation rate and C-reactive protein: how best to use them in clinical practice. Pediatr Ann. 2014;43(10):417-20.

45. Tillett WS, Francis T. Serological Reactions in Pneumonia with a Non-Protein Somatic Fraction of Pneumococcus. J Exp Med. 1930;52(4):561-71.

46. Berman M, Ben-Ami R, Berliner S, Anouk M, Kaufman I, Broyde A, et al. The Effect of Tocilizumab on Inflammatory Markers in Patients Hospitalized with Serious Infections. Case Series and Review of Literature. Life (Basel). 2021;11(3).

47. Kooistra EJ, van Berkel M, van Kempen NF, van Latum CRM, Bruse N, Frenzel T, et al. Dexamethasone and tocilizumab treatment considerably reduces the value of C-reactive protein and procalcitonin to detect secondary bacterial infections in COVID-19 patients. Crit Care. 2021;25(1):281.

48. Smolen JS, Beaulieu A, Rubbert-Roth A, Ramos-Remus C, Rovensky J, Alecock E, et al. Effect of interleukin-6 receptor inhibition with tocilizumab in patients with

rheumatoid arthritis (OPTION study): a double-blind, placebo-controlled, randomised trial. Lancet. 2008;371(9617):987-97.

49. Kishimoto T, Kang S. IL-6 Revisited: From Rheumatoid Arthritis to CAR T Cell Therapy and COVID-19. Annu Rev Immunol. 2022;40:323-48.

50. Fajgenbaum DC, June CH. Cytokine Storm. N Engl J Med. 2020;383(23):2255-73.

51. Serhan CN, Chiang N, Dalli J, Levy BD. Lipid mediators in the resolution of inflammation. Cold Spring Harb Perspect Biol. 2014;7(2):a016311.

52. Sugimoto MA, Sousa LP, Pinho V, Perretti M, Teixeira MM. Resolution of Inflammation: What Controls Its Onset? Front Immunol. 2016;7:160.

53. Kourtzelis I, Hajishengallis G, Chavakis T. Phagocytosis of Apoptotic Cells in Resolution of Inflammation. Front Immunol. 2020;11:553.

54. Panigrahy D, Gilligan MM, Serhan CN, Kashfi K. Resolution of inflammation: An organizing principle in biology and medicine. Pharmacol Ther. 2021;227:107879.

55. Tecchio C, Cassatella MA. Neutrophil-derived chemokines on the road to immunity. Semin Immunol. 2016;28(2):119-28.

56. Farahi N, Loutsios C, Tregay N, Summers C, Lok LSC, Ruparelia P, et al. Radiolabelled leucocytes in human pulmonary disease. Br Med Bull. 2018;127(1):69-82.

57. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. Trends Immunol. 2010;31(8):318-24.

58. Hidalgo A, Chilvers ER, Summers C, Koenderman L. The Neutrophil Life Cycle. Trends Immunol. 2019;40(7):584-97.

59. Manz MG, Boettcher S. Emergency granulopoiesis. Nat Rev Immunol. 2014;14(5):302-14.

60. Spoor J, Farajifard H, Rezaei N. Congenital neutropenia and primary immunodeficiency diseases. Crit Rev Oncol Hematol. 2019;133:149-62.

61. Mehta HM, Malandra M, Corey SJ. G-CSF and GM-CSF in Neutropenia. J Immunol. 2015;195(4):1341-9.

62. Ella K, Csepanyi-Komi R, Kaldi K. Circadian regulation of human peripheral neutrophils. Brain Behav Immun. 2016;57:209-21.

63. Adrover JM, Del Fresno C, Crainiciuc G, Cuartero MI, Casanova-Acebes M, Weiss LA, et al. A Neutrophil Timer Coordinates Immune Defense and Vascular Protection. Immunity. 2019;50(2):390-402 e10.

64. Aroca-Crevillen A, Adrover JM, Hidalgo A. Circadian Features of Neutrophil Biology. Front Immunol. 2020;11:576.

65. Casanova-Acebes M, Pitaval C, Weiss LA, Nombela-Arrieta C, Chevre R, N AG, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell. 2013;153(5):1025-35.

66. Martin C, Burdon PC, Bridger G, Gutierrez-Ramos JC, Williams TJ, Rankin SM. Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. Immunity. 2003;19(4):583-93.

67. Tak T, Tesselaar K, Pillay J, Borghans JA, Koenderman L. What's your age again? Determination of human neutrophil half-lives revisited. J Leukoc Biol. 2013;94(4):595-601.

68. Christenson K, Bjorkman L, Karlsson J, Sundqvist M, Movitz C, Speert DP, et al. In vivo-transmigrated human neutrophils are resistant to antiapoptotic stimulation. J Leukoc Biol. 2011;90(6):1055-63.

69. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood. 1992;80(8):2012-20.

70. Greenlee-Wacker MC. Clearance of apoptotic neutrophils and resolution of inflammation. Immunol Rev. 2016;273(1):357-70.

71. de Oliveira S, Rosowski EE, Huttenlocher A. Neutrophil migration in infection and wound repair: going forward in reverse. Nat Rev Immunol. 2016;16(6):378-91.

72. Zindel J, Kubes P. DAMPs, PAMPs, and LAMPs in Immunity and Sterile Inflammation. Annu Rev Pathol. 2020;15:493-518.

73. Mathias JR, Perrin BJ, Liu TX, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. J Leukoc Biol. 2006;80(6):1281-8.

74. Buckley CD, Ross EA, McGettrick HM, Osborne CE, Haworth O, Schmutz C, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. J Leukoc Biol. 2006;79(2):303-11.

75. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. Nat Immunol. 2011;12(8):761-9.

76. Duffy D, Perrin H, Abadie V, Benhabiles N, Boissonnas A, Liard C, et al. Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells. Immunity. 2012;37(5):917-29.

77. Wang J, Hossain M, Thanabalasuriar A, Gunzer M, Meininger C, Kubes P. Visualizing the function and fate of neutrophils in sterile injury and repair. Science. 2017;358(6359):111-6.

78. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood. 1997;89(10):3503-21.

79. Sengelov H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J Immunol. 1995;154(8):4157-65.

80. Borregaard N, Heiple JM, Simons ER, Clark RA. Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. J Cell Biol. 1983;97(1):52-61.

81. Sengelov H, Kjeldsen L, Borregaard N. Control of exocytosis in early neutrophil activation. J Immunol. 1993;150(4):1535-43.

82. Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. Immunol Rev. 2016;273(1):11-28.

83. Pham CT. Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol. 2006;6(7):541-50.

84. Mohammad AJ. An update on the epidemiology of ANCA-associated vasculitis. Rheumatology (Oxford). 2020;59(Suppl 3):iii42-iii50.

85. Nakazawa D, Masuda S, Tomaru U, Ishizu A. Pathogenesis and therapeutic interventions for ANCA-associated vasculitis. Nat Rev Rheumatol. 2019;15(2):91-101.

86. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med. 2009;15(6):623-5.

87. Jayne DRW, Merkel PA, Schall TJ, Bekker P, Group AS. Avacopan for the Treatment of ANCA-Associated Vasculitis. N Engl J Med. 2021;384(7):599-609.

88. Silvestre-Roig C, Fridlender ZG, Glogauer M, Scapini P. Neutrophil Diversity in Health and Disease. Trends Immunol. 2019;40(7):565-83.

89. Blanco-Camarillo C, Aleman OR, Rosales C. Low-Density Neutrophils in Healthy Individuals Display a Mature Primed Phenotype. Front Immunol. 2021;12:672520.

90. Rosales C. Neutrophils at the crossroads of innate and adaptive immunity. J Leukoc Biol. 2020;108(1):377-96.

91. Hacbarth E, Kajdacsy-Balla A. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. Arthritis Rheum. 1986;29(11):1334-42.

92. Hassani M, Hellebrekers P, Chen N, van Aalst C, Bongers S, Hietbrink F, et al. On the origin of low-density neutrophils. J Leukoc Biol. 2020;107(5):809-18.

93. McKenna E, Mhaonaigh AU, Wubben R, Dwivedi A, Hurley T, Kelly LA, et al. Neutrophils: Need for Standardized Nomenclature. Front Immunol. 2021;12:602963.

94. Stockfelt M, Larsson G, Engstrom H, Puttonen H, Zetterberg H, Blennow K, et al. Activated low-density granulocytes in peripheral and intervillous blood and neutrophil inflammation in placentas from SLE pregnancies. Lupus Sci Med. 2021;8(1).

95. Veglia F, Sanseviero E, Gabrilovich DI. Myeloid-derived suppressor cells in the era of increasing myeloid cell diversity. Nat Rev Immunol. 2021;21(8):485-98.

96. Aarts CEM, Hiemstra IH, Beguin EP, Hoogendijk AJ, Bouchmal S, van Houdt M, et al. Activated neutrophils exert myeloid-derived suppressor cell activity damaging T cells beyond repair. Blood Adv. 2019;3(22):3562-74.

97. Quail DF, Amulic B, Aziz M, Barnes BJ, Eruslanov E, Fridlender ZG, et al. Neutrophil phenotypes and functions in cancer: A consensus statement. J Exp Med. 2022;219(6).

98. Lonardi S, Missale F, Calza S, Bugatti M, Vescovi R, Debora B, et al. Tumor-associated neutrophils (TANs) in human carcinoma-draining lymph nodes: a novel TAN compartment. Clin Transl Immunology. 2021;10(2):e1252.

99. Dahlstrand Rudin A, Amirbeagi F, Davidsson L, Khamzeh A, Thorbert Mros S, Thulin P, et al. The neutrophil subset defined by CD177 expression is preferentially recruited to gingival crevicular fluid in periodontitis. J Leukoc Biol. 2021;109(2):349-62.

100. Goldschmeding R, van Dalen CM, Faber N, Calafat J, Huizinga TW, van der Schoot CE, et al. Further characterization of the NB 1 antigen as a variably expressed 56-62 kD GPI-linked glycoprotein of plasma membranes and specific granules of neutrophils. Br J Haematol. 1992;81(3):336-45.

101. Hu N, Westra J, Huitema MG, Bijl M, Brouwer E, Stegeman CA, et al. Coexpression of CD177 and membrane proteinase 3 on neutrophils in antineutrophil cytoplasmic autoantibody-associated systemic vasculitis: anti-proteinase 3-mediated neutrophil activation is independent of the role of CD177-expressing neutrophils. Arthritis Rheum. 2009;60(5):1548-57.

102. Sachs UJ, Andrei-Selmer CL, Maniar A, Weiss T, Paddock C, Orlova VV, et al. The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1 (CD31). J Biol Chem. 2007;282(32):23603-12.

103. Bayat B, Werth S, Sachs UJ, Newman DK, Newman PJ, Santoso S. Neutrophil transmigration mediated by the neutrophil-specific antigen CD177 is influenced by the endothelial S536N dimorphism of platelet endothelial cell adhesion molecule-1. J Immunol. 2010;184(7):3889-96.

104. Clemmensen SN, Bohr CT, Rorvig S, Glenthoj A, Mora-Jensen H, Cramer EP, et al. Olfactomedin 4 defines a subset of human neutrophils. J Leukoc Biol. 2012;91(3):495-500.

105. Welin A, Amirbeagi F, Christenson K, Bjorkman L, Bjornsdottir H, Forsman H, et al. The human neutrophil subsets defined by the presence or absence of OLFM4 both transmigrate into tissue in vivo and give rise to distinct NETs in vitro. PLoS One. 2013;8(7):e69575.

106. Alder MN, Opoka AM, Lahni P, Hildeman DA, Wong HR. Olfactomedin-4 Is a Candidate Marker for a Pathogenic Neutrophil Subset in Septic Shock. Crit Care Med. 2017;45(4):e426-e32.

107. Kangelaris KN, Clemens R, Fang X, Jauregui A, Liu T, Vessel K, et al. A neutrophil subset defined by intracellular olfactomedin 4 is associated with mortality in sepsis. Am J Physiol Lung Cell Mol Physiol. 2021;320(5):L892-L902.

108. Lundquist H, Andersson H, Chew MS, Das J, Turkina MV, Welin A. The Olfactomedin-4-Defined Human Neutrophil Subsets Differ in Proteomic Profile in Healthy Individuals and Patients with Septic Shock. J Innate Immun. 2022:1-14.

109. Underhill DM, Gordon S, Imhof BA, Nunez G, Bousso P. Elie Metchnikoff (1845-1916): celebrating 100 years of cellular immunology and beyond. Nat Rev Immunol. 2016;16(10):651-6.

110. Rabinovitch M. Professional and non-professional phagocytes: an introduction. Trends Cell Biol. 1995;5(3):85-7.

111. Uribe-Querol E, Rosales C. Phagocytosis: Our Current Understanding of a Universal Biological Process. Front Immunol. 2020;11:1066.

112. Freeman SA, Grinstein S. Phagocytosis: receptors, signal integration, and the cytoskeleton. Immunol Rev. 2014;262(1):193-215.

113. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, et al. Specificity and affinity of human Fcgamma receptors and their polymorphic variants for human IgG subclasses. Blood. 2009;113(16):3716-25.

114. Dustin ML. Complement Receptors in Myeloid Cell Adhesion and Phagocytosis. Microbiol Spectr. 2016;4(6).

115. Li K, Underhill DM. C-Type Lectin Receptors in Phagocytosis. Curr Top Microbiol Immunol. 2020;429:1-18.

116. Segal AW, Dorling J, Coade S. Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. J Cell Biol. 1980;85(1):42-59.

117. Levin R, Grinstein S, Canton J. The life cycle of phagosomes: formation, maturation, and resolution. Immunol Rev. 2016;273(1):156-79.

118. Glennon-Alty L, Hackett AP, Chapman EA, Wright HL. Neutrophils and redox stress in the pathogenesis of autoimmune disease. Free Radic Biol Med. 2018;125:25-35.

119. Hoffmann MH, Griffiths HR. The dual role of Reactive Oxygen Species in autoimmune and inflammatory diseases: evidence from preclinical models. Free Radic Biol Med. 2018;125:62-71.

120. Winkelstein JA, Marino MC, Johnston RB, Jr., Boyle J, Curnutte J, Gallin JI, et al. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore). 2000;79(3):155-69.

121. de Oliveira-Junior EB, Zurro NB, Prando C, Cabral-Marques O, Pereira PV, Schimke LF, et al. Clinical and Genotypic Spectrum of Chronic Granulomatous Disease in 71 Latin American Patients: First Report from the LASID Registry. Pediatr Blood Cancer. 2015;62(12):2101-7.

122. Nauseef WM, Clark RA. Intersecting Stories of the Phagocyte NADPH Oxidase and Chronic Granulomatous Disease. Methods Mol Biol. 2019;1982:3-16.

123. Holland SM. Chronic granulomatous disease. Hematol Oncol Clin North Am. 2013;27(1):89-99, viii.

124. Nauseef WM. The phagocyte NOX2 NADPH oxidase in microbial killing and cell signaling. Curr Opin Immunol. 2019;60:130-40.

125. Belambri SA, Rolas L, Raad H, Hurtado-Nedelec M, Dang PM, El-Benna J. NADPH oxidase activation in neutrophils: Role of the phosphorylation of its subunits. Eur J Clin Invest. 2018;48 Suppl 2:e12951.

126. Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol. 2005;77(5):598-625.

127. Bylund J, Brown KL, Movitz C, Dahlgren C, Karlsson A. Intracellular generation of superoxide by the phagocyte NADPH oxidase: how, where, and what for? Free Radic Biol Med. 2010;49(12):1834-45.

128. Dahlgren C, Karlsson A, Bylund J. Intracellular Neutrophil Oxidants: From Laboratory Curiosity to Clinical Reality. J Immunol. 2019;202(11):3127-34.

129. Bjornsdottir H, Granfeldt D, Welin A, Bylund J, Karlsson A. Inhibition of phospholipase A(2) abrogates intracellular processing of NADPH-oxidase derived reactive oxygen species in human neutrophils. Exp Cell Res. 2013;319(5):761-74.

130. Bjornsdottir H, Welin A, Dahlgren C, Karlsson A, Bylund J. Quantification of heterotypic granule fusion in human neutrophils by imaging flow cytometry. Data Brief. 2016;6:386-93.

131. Warnatsch A, Tsourouktsoglou TD, Branzk N, Wang Q, Reincke S, Herbst S, et al. Reactive Oxygen Species Localization Programs Inflammation to Clear Microbes of Different Size. Immunity. 2017;46(3):421-32.

132. Boyle KB, Gyori D, Sindrilaru A, Scharffetter-Kochanek K, Taylor PR, Mocsai A, et al. Class IA phosphoinositide 3-kinase beta and delta regulate neutrophil oxidase activation in response to Aspergillus fumigatus hyphae. J Immunol. 2011;186(5):2978-89.

133. Bjorkman L, Dahlgren C, Karlsson A, Brown KL, Bylund J. Phagocyte-derived reactive oxygen species as suppressors of inflammatory disease. Arthritis Rheum. 2008;58(10):2931-5.

134. Hultqvist M, Olsson LM, Gelderman KA, Holmdahl R. The protective role of ROS in autoimmune disease. Trends Immunol. 2009;30(5):201-8.

135. Sareila O, Kelkka T, Pizzolla A, Hultqvist M, Holmdahl R. NOX2 complex-derived ROS as immune regulators. Antioxid Redox Signal. 2011;15(8):2197-208.

136. Sies H, Belousov VV, Chandel NS, Davies MJ, Jones DP, Mann GE, et al. Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology. Nat Rev Mol Cell Biol. 2022;23(7):499-515.

137. Bjornsdottir H, Welin A, Michaelsson E, Osla V, Berg S, Christenson K, et al. Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. Free Radic Biol Med. 2015;89:1024-35.

138. Takei H, Araki A, Watanabe H, Ichinose A, Sendo F. Rapid killing of human neutrophils by the potent activator phorbol 12-myristate 13-acetate (PMA) accompanied by changes different from typical apoptosis or necrosis. J Leukoc Biol. 1996;59(2):229-40.

139. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532-5.

140. Castanheira FVS, Kubes P. Neutrophils and NETs in modulating acute and chronic inflammation. Blood. 2019;133(20):2178-85.

141. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med. 2012;18(9):1386-93.

142. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin Invest. 2013.

143. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host Microbe. 2012;12(3):324-33.

144. Kolaczkowska E, Jenne CN, Surewaard BG, Thanabalasuriar A, Lee WY, Sanz MJ, et al. Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. Nat Commun. 2015;6:6673.

145. Tanaka K, Koike Y, Shimura T, Okigami M, Ide S, Toiyama Y, et al. In vivo characterization of neutrophil extracellular traps in various organs of a murine sepsis model. PLoS One. 2014;9(11):e111888.

146. von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med. 2012;209(4):819-35.

147. Saitoh T, Komano J, Saitoh Y, Misawa T, Takahama M, Kozaki T, et al. Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. Cell Host Microbe. 2012;12(1):109-16.

148. Branzk N, Lubojemska A, Hardison SE, Wang Q, Gutierrez MG, Brown GD, et al. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. Nat Immunol. 2014;15(11):1017-25.

149. Abi Abdallah DS, Denkers EY. Neutrophils cast extracellular traps in response to protozoan parasites. Front Immunol. 2012;3:382.

150. Behnen M, Leschczyk C, Moller S, Batel T, Klinger M, Solbach W, et al. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. J Immunol. 2014;193(4):1954-65.

151. Keshari RS, Jyoti A, Dubey M, Kothari N, Kohli M, Bogra J, et al. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. PLoS One. 2012;7(10):e48111.

152. Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. J Clin Invest. 2012;122(7):2661-71.

153. Wang M, Ishikawa T, Lai Y, Nallapothula D, Singh RR. Diverse Roles of NETosis in the Pathogenesis of Lupus. Front Immunol. 2022;13:895216.

154. Goel RR, Kaplan MJ. Deadliest catch: neutrophil extracellular traps in autoimmunity. Curr Opin Rheumatol. 2020;32(1):64-70.

155. Martinod K, Wagner DD. Thrombosis: tangled up in NETs. Blood. 2014;123(18):2768-76.

156. Thalin C, Hisada Y, Lundstrom S, Mackman N, Wallen H. Neutrophil Extracellular Traps: Villains and Targets in Arterial, Venous, and Cancer-Associated Thrombosis. Arterioscler Thromb Vasc Biol. 2019;39(9):1724-38.

157. Snoderly HT, Boone BA, Bennewitz MF. Neutrophil extracellular traps in breast cancer and beyond: current perspectives on NET stimuli, thrombosis and metastasis, and clinical utility for diagnosis and treatment. Breast Cancer Res. 2019;21(1):145.

158. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. J Cell Biol. 2007;176(2):231-41.

159. Boeltz S, Amini P, Anders HJ, Andrade F, Bilyy R, Chatfield S, et al. To NET or not to NET:current opinions and state of the science regarding the formation of neutrophil extracellular traps. Cell Death Differ. 2019;26(3):395-408.

160. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. J Immunol. 2010;185(12):7413-25.

161. Parker H, Dragunow M, Hampton MB, Kettle AJ, Winterbourn CC. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. J Leukoc Biol. 2012;92(4):841-9.

162. Byrd AS, O'Brien XM, Johnson CM, Lavigne LM, Reichner JS. An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to Candida albicans. J Immunol. 2013;190(8):4136-48.

163. Hosseinzadeh A, Thompson PR, Segal BH, Urban CF. Nicotine induces neutrophil extracellular traps. J Leukoc Biol. 2016;100(5):1105-12.

164. Chatfield SM, Grebe K, Whitehead LW, Rogers KL, Nebl T, Murphy JM, et al. Monosodium Urate Crystals Generate Nuclease-Resistant Neutrophil Extracellular Traps via a Distinct Molecular Pathway. J Immunol. 2018;200(5):1802-16.

165. Bjornsdottir H, Dahlstrand Rudin A, Klose FP, Elmwall J, Welin A, Stylianou M, et al. Phenol-Soluble Modulin alpha Peptide Toxins from Aggressive Staphylococcus aureus Induce Rapid Formation of Neutrophil Extracellular Traps through a Reactive Oxygen Species-Independent Pathway. Front Immunol. 2017;8:257.

166. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. Nat Med. 2016;22(2):146-53.

167. Douda DN, Khan MA, Grasemann H, Palaniyar N. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. Proc Natl Acad Sci U S A. 2015;112(9):2817-22.

168. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ. 2009;16(11):1438-44.

169. McIlroy DJ, Jarnicki AG, Au GG, Lott N, Smith DW, Hansbro PM, et al. Mitochondrial DNA neutrophil extracellular traps are formed after trauma and subsequent surgery. J Crit Care. 2014;29(6):1133 e1-5.

170. Wang H, Li T, Chen S, Gu Y, Ye S. Neutrophil Extracellular Trap Mitochondrial DNA and Its Autoantibody in Systemic Lupus Erythematosus and a Proof-of-Concept Trial of Metformin. Arthritis Rheumatol. 2015;67(12):3190-200.

171. Caielli S, Athale S, Domic B, Murat E, Chandra M, Banchereau R, et al. Oxidized mitochondrial nucleoids released by neutrophils drive type I interferon production in human lupus. J Exp Med. 2016;213(5):697-713.

172. Desai J, Mulay SR, Nakazawa D, Anders HJ. Matters of life and death. How neutrophils die or survive along NET release and is "NETosis" = necroptosis? Cell Mol Life Sci. 2016;73(11-12):2211-9.

173. Rosazza T, Warner J, Sollberger G. NET formation - mechanisms and how they relate to other cell death pathways. FEBS J. 2021;288(11):3334-50.

174. Singhal A, Kumar S. Neutrophil and remnant clearance in immunity and inflammation. Immunology. 2022;165(1):22-43.

175. Tan C, Aziz M, Wang P. The vitals of NETs. J Leukoc Biol. 2021;110(4):797-808.

176. Zarbock A, Ley K, McEver RP, Hidalgo A. Leukocyte ligands for endothelial selectins: specialized glycoconjugates that mediate rolling and signaling under flow. Blood. 2011;118(26):6743-51.

177. Ivetic A. A head-to-tail view of L-selectin and its impact on neutrophil behaviour. Cell Tissue Res. 2018;371(3):437-53.

178. Lamers C, Pluss CJ, Ricklin D. The Promiscuous Profile of Complement Receptor 3 in Ligand Binding, Immune Modulation, and Pathophysiology. Front Immunol. 2021;12:662164.

179. Metzemaekers M, Gouwy M, Proost P. Neutrophil chemoattractant receptors in health and disease: double-edged swords. Cell Mol Immunol. 2020;17(5):433-50.

180. Futosi K, Fodor S, Mocsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. Int Immunopharmacol. 2013;17(3):638-50.

181. Rajarathnam K, Schnoor M, Richardson RM, Rajagopal S. How do chemokines navigate neutrophils to the target site: Dissecting the structural mechanisms and signaling pathways. Cell Signal. 2019;54:69-80.

182. Kuhns DB, Gallin JI. Increased cell-associated IL-8 in human exudative and A23187-treated peripheral blood neutrophils. J Immunol. 1995;154(12):6556-62.

183. Weiss E, Kretschmer D. Formyl-Peptide Receptors in Infection, Inflammation, and Cancer. Trends Immunol. 2018;39(10):815-29.

184. Sheppard FR, Kelher MR, Moore EE, McLaughlin NJ, Banerjee A, Silliman CC. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. J Leukoc Biol. 2005;78(5):1025-42.

185. El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie JC, Gougerot-Pocidalo MA, Dang PM. Priming of the neutrophil respiratory burst: role in host defense and inflammation. Immunol Rev. 2016;273(1):180-93.

186. Vogt KL, Summers C, Chilvers ER, Condliffe AM. Priming and de-priming of neutrophil responses in vitro and in vivo. Eur J Clin Invest. 2018;48 Suppl 2:e12967.

187. Zarember KA, Kuhns DB. Editorial: will the real neutrophil please stand up? J Leukoc Biol. 2011;90(6):1039-41.

188. Brown KA, Brain SD, Pearson JD, Edgeworth JD, Lewis SM, Treacher DF. Neutrophils in development of multiple organ failure in sepsis. Lancet. 2006;368(9530):157-69.

189. Kundu S, Ghosh P, Datta S, Ghosh A, Chattopadhyay S, Chatterjee M. Oxidative stress as a potential biomarker for determining disease activity in patients with rheumatoid arthritis. Free Radic Res. 2012;46(12):1482-9.

190. Harper L, Cockwell P, Adu D, Savage CO. Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis. Kidney Int. 2001;59(5):1729-38.

191. Kuhns DB, Long Priel DA, Gallin JI. Loss of L-selectin (CD62L) on human neutrophils following exudation in vivo. Cell Immunol. 1995;164(2):306-10.

192. Hustinx WN, Van Kessel CP, Heezius E, Burgers S, Lammers JW, Hoepelman IM. Effects of granulocyte colony-stimulating factor (G-CSF) treatment on granulocyte function and receptor expression in patients with ventilator-dependent pneumonia. Clin Exp Immunol. 1998;112(2):334-40.

193. Hartl D, Krauss-Etschmann S, Koller B, Hordijk PL, Kuijpers TW, Hoffmann F, et al. Infiltrated neutrophils acquire novel chemokine receptor expression and chemokine responsiveness in chronic inflammatory lung diseases. J Immunol. 2008;181(11):8053-67.

194. Dahlgren C, Briheim G, Norman B, Stendahl O. Deactivation of leucocyte chemotaxis in vivo. Locomotion of cells isolated from a patient with meningococcal meningitis. Scand J Haematol. 1981;27(3):219-23.

195. Briheim G, Stendahl O, Dahlgren C. Factor-specific deactivation of leucocyte chemotaxis in vivo. Scand J Haematol. 1986;37(3):253-8.

196. Lukac J, Mravak-Stipetic M, Knezevic M, Vrcek J, Sistig S, Ledinsky M, et al. Phagocytic functions of salivary neutrophils in oral mucous membrane diseases. J Oral Pathol Med. 2003;32(5):271-4.

197. Margaroli C, Moncada-Giraldo D, Gulick DA, Dobosh B, Giacalone VD, Forrest OA, et al. Transcriptional firing represses bactericidal activity in cystic fibrosis airway neutrophils. Cell Rep Med. 2021;2(4):100239.

198. Makam M, Diaz D, Laval J, Gernez Y, Conrad CK, Dunn CE, et al. Activation of critical, host-induced, metabolic and stress pathways marks neutrophil entry into cystic fibrosis lungs. Proc Natl Acad Sci U S A. 2009;106(14):5779-83.

199. Tirouvanziam R, Gernez Y, Conrad CK, Moss RB, Schrijver I, Dunn CE, et al. Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. Proc Natl Acad Sci U S A. 2008;105(11):4335-9.

200. Liberek T, Chmielewski M, Lichodziejewska-Niemierko M, Lewandowski K, Rutkowski B. Transmigration of blood leukocytes into the peritoneal cavity is related to the upregulation of ICAM-1 (CD54) and Mac-1 (CD11b/CD18) adhesion molecules. Perit Dial Int. 2004;24(2):139-46.

201. Liu GN, Shi HZ, Xie ZH, Shen HH, Huang HQ, Deng JM, et al. Epithelial neutrophil-activating peptide-78 recruits neutrophils into pleural effusion. Eur Respir J. 2009;34(1):184-90.

202. Follin P, Dahlgren C. A skin chamber technique as a human model for studies of aseptic inflammatory reactions. Methods Mol Biol. 2007;412:333-46.

203. Follin P, Wymann MP, Dewald B, Ceska M, Dahlgren C. Human neutrophil migration into skin chambers is associated with production of NAP-1/IL8 and C5a. Eur J Haematol. 1991;47(1):71-6.

204. Follin P. Skin chamber technique for study of in vivo exudated human neutrophils. J Immunol Methods. 1999;232(1-2):55-65.

205. Follin P, Briheim G, Dahlgren C. Mechanisms in neutrophil priming: characterization of the oxidative response induced by formylmethionyl-leucyl-phenylalanine in human exudated cells. Scand J Immunol. 1991;34(3):317-22.

206. Sjobom U, Christenson K, Hellstrom A, Nilsson AK. Inflammatory Markers in Suction Blister Fluid: A Comparative Study Between Interstitial Fluid and Plasma. Front Immunol. 2020;11:597632.

207. Buters TP, Hameeteman PW, Jansen IME, van Hindevoort FC, Ten Voorde W, Florencia E, et al. Intradermal lipopolysaccharide challenge as an acute in vivo inflammatory model in healthy volunteers. Br J Clin Pharmacol. 2022;88(2):680-90.

208. Motwani MP, Flint JD, De Maeyer RP, Fullerton JN, Smith AM, Marks DJ, et al. Novel translational model of resolving inflammation triggered by UV-killed E. coli. J Pathol Clin Res. 2016;2(3):154-65.

209. Buters TP, Hameeteman PW, Jansen IME, van Hindevoort FC, Ten Voorde W, Grievink HW, et al. Clinical, Cellular, and Molecular Effects of Corticosteroids on the Response to Intradermal Lipopolysaccharide Administration in Healthy Volunteers. Clin Pharmacol Ther. 2022;111(4):964-71.

210. Smith MD. The normal synovium. Open Rheumatol J. 2011;5:100-6.

211. Small A, Wechalekar MD. Synovial biopsies in inflammatory arthritis: precision medicine in rheumatoid arthritis. Expert Rev Mol Diagn. 2020;20(3):315-25.

212. Mourao AF, Canhao H, Sousa E, Cascao R, da Costa JB, de Almeida LS, et al. From a neutrophilic synovial tissue infiltrate to a challenging case of rheumatoid arthritis. Acta Reumatol Port. 2010;35(2):228-31.

213. Edwards SW, Hallett MB. Seeing the wood for the trees: the forgotten role of neutrophils in rheumatoid arthritis. Immunol Today. 1997;18(7):320-4.

214. Palmer DG, Hogg N, Revell PA. Lymphocytes, polymorphonuclear leukocytes, macrophages and platelets in synovium involved by rheumatoid arthritis. A study with monoclonal antibodies. Pathology. 1986;18(4):431-7.

215. Tak PP, Smeets TJ, Daha MR, Kluin PM, Meijers KA, Brand R, et al. Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. Arthritis Rheum. 1997;40(2):217-25.

216. Bartok B, Firestein GS. Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. Immunol Rev. 2010;233(1):233-55.

217. Wong J, Johnston B, Lee SS, Bullard DC, Smith CW, Beaudet AL, et al. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. J Clin Invest. 1997;99(11):2782-90.

218. Lee WY, Kubes P. Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs. J Hepatol. 2008;48(3):504-12.

219. Ishikawa M, Cooper D, Russell J, Salter JW, Zhang JH, Nanda A, et al. Molecular determinants of the prothrombogenic and inflammatory phenotype assumed by the postischemic cerebral microcirculation. Stroke. 2003;34(7):1777-82.

220. Carvalho-Tavares J, Hickey MJ, Hutchison J, Michaud J, Sutcliffe IT, Kubes P. A role for platelets and endothelial selectins in tumor necrosis factor-alpha-induced leukocyte recruitment in the brain microvasculature. Circ Res. 2000;87(12):1141-8.

221. Emery P, Lopez AF, Burns GF, Vadas MA. Synovial fluid neutrophils of patients with rheumatoid arthritis have membrane antigen changes that reflect activation. Ann Rheum Dis. 1988;47(1):34-9.

222. Nurcombe HL, Bucknall RC, Edwards SW. Neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis: priming and activation in vivo. Ann Rheum Dis. 1991;50(3):147-53.

223. Arve-Butler S, Schmidt T, Mossberg A, Berthold E, Gullstrand B, Bengtsson AA, et al. Synovial fluid neutrophils in oligoarticular juvenile idiopathic arthritis have an altered phenotype and impaired effector functions. Arthritis Res Ther. 2021;23(1):109.

224. Metzemaekers M, Malengier-Devlies B, Yu K, Vandendriessche S, Yserbyt J, Matthys P, et al. Synovial Fluid Neutrophils From Patients With Juvenile Idiopathic Arthritis Display a Hyperactivated Phenotype. Arthritis Rheumatol. 2021;73(5):875-84.

225. Nuki G, Simkin PA. A concise history of gout and hyperuricemia and their treatment. Arthritis Res Ther. 2006;8 Suppl 1:S1.

226. Garrod AB. A Treatise on gout and rheumatic gout (rheumatoid arthritis): Longmans, Green; 1876.

227. Dehlin M, Jacobsson L, Roddy E. Global epidemiology of gout: prevalence, incidence, treatment patterns and risk factors. Nat Rev Rheumatol. 2020;16(7):380-90.

228. Dalbeth N, Gosling AL, Gaffo A, Abhishek A. Gout. Lancet. 2021;397(10287):1843-55.

229. Major TJ, Topless RK, Dalbeth N, Merriman TR. Evaluation of the diet wide contribution to serum urate levels: meta-analysis of population based cohorts. BMJ. 2018;363:k3951.

230. Sriranganathan MK, Vinik O, Bombardier C, Edwards CJ. Interventions for tophi in gout. Cochrane Database Syst Rev. 2014(10):CD010069.

231. Richette P, Doherty M, Pascual E, Barskova V, Becce F, Castaneda J, et al. 2018 updated European League Against Rheumatism evidence-based recommendations for the diagnosis of gout. Ann Rheum Dis. 2020;79(1):31-8.

232. Richette P, Doherty M, Pascual E, Barskova V, Becce F, Castaneda-Sanabria J, et al. 2016 updated EULAR evidence-based recommendations for the management of gout. Ann Rheum Dis. 2017;76(1):29-42.

233. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat Rev Immunol. 2019;19(8):477-89.

234. Skendros P, Papagoras C, Mitroulis I, Ritis K. Autoinflammation: Lessons from the study of familial Mediterranean fever. J Autoimmun. 2019;104:102305.

235. Bodofsky S, Merriman TR, Thomas TJ, Schlesinger N. Advances in our understanding of gout as an auto-inflammatory disease. Semin Arthritis Rheum. 2020;50(5):1089-100.

236. Kapetanovic MC, Nilsson P, Turesson C, Englund M, Dalbeth N, Jacobsson L. The risk of clinically diagnosed gout by serum urate levels: results from 30 years follow-up of the Malmo Preventive Project cohort in southern Sweden. Arthritis Res Ther. 2018;20(1):190.

237. Dalbeth N, Phipps-Green A, Frampton C, Neogi T, Taylor WJ, Merriman TR. Relationship between serum urate concentration and clinically evident incident gout: an individual participant data analysis. Ann Rheum Dis. 2018;77(7):1048-52.

238. Nigrovic PA, Lee PY, Hoffman HM. Monogenic autoinflammatory disorders: Conceptual overview, phenotype, and clinical approach. J Allergy Clin Immunol. 2020;146(5):925-37.

239. Campbell L, Raheem I, Malemud CJ, Askari AD. The Relationship between NALP3 and Autoinflammatory Syndromes. Int J Mol Sci. 2016;17(5).

240. Gracheva IA, Shchegravina ES, Schmalz HG, Beletskaya IP, Fedorov AY. Colchicine Alkaloids and Synthetic Analogues: Current Progress and Perspectives. J Med Chem. 2020;63(19):10618-51.

241. Deftereos SG, Beerkens FJ, Shah B, Giannopoulos G, Vrachatis DA, Giotaki SG, et al. Colchicine in Cardiovascular Disease: In-Depth Review. Circulation. 2022;145(1):61-78.

242. Tong DC, Wilson AM, Layland J. Colchicine in cardiovascular disease: an ancient drug with modern tricks. Heart. 2016;102(13):995-1002.

243. Tardif JC, Kouz S, Waters DD, Bertrand OF, Diaz R, Maggioni AP, et al. Efficacy and Safety of Low-Dose Colchicine after Myocardial Infarction. N Engl J Med. 2019;381(26):2497-505.

244. Slomski A. Colchicine Reduces Cardiovascular Events in Chronic Coronary Disease. JAMA. 2020;324(16):1599.

245. Chappey ON, Niel E, Wautier JL, Hung PP, Dervichian M, Cattan D, et al. Colchicine disposition in human leukocytes after single and multiple oral administration. Clin Pharmacol Ther. 1993;54(4):360-7.

246. Cronstein BN, Molad Y, Reibman J, Balakhane E, Levin RI, Weissmann G. Colchicine alters the quantitative and qualitative display of selectins on endothelial cells and neutrophils. J Clin Invest. 1995;96(2):994-1002.

247. Slobodnick A, Shah B, Krasnokutsky S, Pillinger MH. Update on colchicine, 2017. Rheumatology (Oxford). 2018;57(suppl_1):i4-i11.

248. Chia EW, Grainger R, Harper JL. Colchicine suppresses neutrophil superoxide production in a murine model of gouty arthritis: a rationale for use of low-dose colchicine. Br J Pharmacol. 2008;153(6):1288-95.

249. Roberge CJ, Gaudry M, Gilbert C, Malawista SE, de Medicis R, Lussier A, et al. Paradoxical effects of colchicine on the activation of human neutrophilis by chemotactic factors and inflammatory microcrystal. J Leukoc Biol. 1996;59(6):864-71.

250. Simchowitz L, Atkinson JP, Spilberg I. Stimulation of the respiratory burst in human neutrophils by crystal phagocytosis. Arthritis Rheum. 1982;25(2):181-8.

251. Rich AM, Giedd KN, Cristello P, Weissmann G. Granules are necessary for death of neutrophils after phagocytosis of crystalline monosodium urate. Inflammation. 1985;9(3):221-32.

252. Sil P, Hayes CP, Reaves BJ, Breen P, Quinn S, Sokolove J, et al. P2Y6 Receptor Antagonist MRS2578 Inhibits Neutrophil Activation and Aggregated Neutrophil Extracellular Trap Formation Induced by Gout-Associated Monosodium Urate Crystals. J Immunol. 2017;198(1):428-42.

253. Baggio C, Sfriso P, Cignarella A, Galozzi P, Scanu A, Mastrotto F, et al. Phagocytosis and inflammation in crystal-induced arthritis: a synovial fluid and in vitro study. Clin Exp Rheumatol. 2021;39(3):494-500.

254. Schorn C, Janko C, Latzko M, Chaurio R, Schett G, Herrmann M. Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. Front Immunol. 2012;3:277.

255. Abramson S, Hoffstein ST, Weissmann G. Superoxide anion generation by human neutrophils exposed to monosodium urate. Arthritis Rheum. 1982;25(2):174-80.

256. Gaudry M, Roberge CJ, de Medicis R, Lussier A, Poubelle PE, Naccache PH. Crystal-induced neutrophil activation. III. Inflammatory microcrystals induce a distinct pattern of tyrosine phosphorylation in human neutrophils. J Clin Invest. 1993;91(4):1649-55.

257. Naccache PH, Bourgoin S, Plante E, Roberge CJ, de Medicis R, Lussier A, et al. Crystal-induced neutrophil activation. II. Evidence for the activation of a phosphatidylcholine-specific phospholipase D. Arthritis Rheum. 1993;36(1):117-25.

258. Schauer C, Janko C, Munoz LE, Zhao Y, Kienhofer D, Frey B, et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. Nature medicine. 2014;20(5):511-7.

259. Schorn C, Janko C, Krenn V, Zhao Y, Munoz LE, Schett G, et al. Bonding the foe - NETting neutrophils immobilize the pro-inflammatory monosodium urate crystals. Front Immunol. 2012;3:376.

260. Mitroulis I, Kambas K, Chrysanthopoulou A, Skendros P, Apostolidou E, Kourtzelis I, et al. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PLoS One. 2011;6(12):e29318.

261. Sil P, Wicklum H, Surell C, Rada B. Macrophage-derived IL-1beta enhances monosodium urate crystal-triggered NET formation. Inflamm Res. 2017;66(3):227-37.

262. Minta JO, Williams MD. Interactions of antirheumatic drugs with the superoxide generation system of activated human polymorphonuclear leukocytes. J Rheumatol. 1986;13(3):498-504.

263. Vrba J, Modriansky M. N-formyl-Met-Leu-Phe-induced oxidative burst in DMSO-differentiated HL-60 cells requires active Hsp90, but not intact microtubules. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2004;148(2):141-4.

264. Vedder D, Gerritsen M, Duvvuri B, van Vollenhoven RF, Nurmohamed MT, Lood C. Neutrophil activation identifies patients with active polyarticular gout. Arthritis Res Ther. 2020;22(1):148.

265. Vedder D, Gerritsen M, Nurmohamed MT, van Vollenhoven RF, Lood C. A neutrophil signature is strongly associated with increased cardiovascular risk in gout. Rheumatology (Oxford). 2021;60(6):2783-90.

266. Pieterse E, Jeremic I, Czegley C, Weidner D, Biermann MH, Veissi S, et al. Blood-borne phagocytes internalize urate microaggregates and prevent intravascular NETosis by urate crystals. Sci Rep. 2016;6:38229.

267. Martin WJ, Grainger R, Harrison A, Harper JL. Differences in MSU-induced superoxide responses by neutrophils from gout subjects compared to healthy controls and a role for environmental inflammatory cytokines and hyperuricemia in neutrophil function and survival. J Rheumatol. 2010;37(6):1228-35.

268. Spitsin S, Koprowski H. Role of uric acid in Alzheimer's disease. J Alzheimers Dis. 2010;19(4):1337-8.

269. He Q, You Y, Yu L, Yao L, Lu H, Zhou X, et al. Uric acid levels in subjects with schizophrenia: A systematic review and meta-analysis. Psychiatry Res. 2020;292:113305.

270. Rentzos M, Nikolaou C, Anagnostouli M, Rombos A, Tsakanikas K, Economou M, et al. Serum uric acid and multiple sclerosis. Clin Neurol Neurosurg. 2006;108(6):527-31.

271. Ellmore TM, Suescun J, Castriotta RJ, Schiess MC. A Study of the Relationship Between Uric Acid and Substantia Nigra Brain Connectivity in Patients With REM Sleep Behavior Disorder and Parkinson's Disease. Front Neurol. 2020;11:815.

272. Sautin YY, Johnson RJ. Uric acid: the oxidant-antioxidant paradox. Nucleosides Nucleotides Nucleic Acids. 2008;27(6):608-19.

273. Lippi G, Montagnana M, Franchini M, Favaloro EJ, Targher G. The paradoxical relationship between serum uric acid and cardiovascular disease. Clin Chim Acta. 2008;392(1-2):1-7.

274. Keenan RT. The biology of urate. Semin Arthritis Rheum. 2020;50(3S):S2-S10.

275. Arai Y, Nishinaka Y, Arai T, Morita M, Mizugishi K, Adachi S, et al. Uric acid induces NADPH oxidase-independent neutrophil extracellular trap formation. Biochem Biophys Res Commun. 2014;443(2):556-61.