Neutrophil function in health and disease

– role of intracellular radicals and galectin-3 as regulators of inflammation

Martina Sundqvist

UNIVERSITY OF GOTHENBURG

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

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Abstract

The focus for this PhD project has been to investigate neutrophil functions in different (inflammatory) settings with specific focus on phagocyte-derived intracellular reactive oxygen species (ROS) as well as neutrophil interaction with the inflammatory mediator galectin-3.

Neutrophils, the most abundant leukocyte in human blood, have traditionally been viewed upon mainly as professional phagocytes, being able to degrade invading microbes, and thus essential in the defence against infection. However, studies including this thesis, suggest that neutrophils are important players also in sterile inflammatory conditions.

Neutrophils are versatile cells, able to change their appearance and functions in relation to time and localization. Their phenotype can vary from being resting, preactivated/primed or fully activated. The primed neutrophils display increased receptors on their cell surfaces resulting in that they can respond to a variety of stimuli, e.g., the β-galactoside binding lectin galectin-3. ROS produced by neutrophils are primarily thought of as toxic metabolites produced to degrade invading microbes, however, neutrophils can also produce intracellular ROS (icROS) in the absence of microbial uptake and a decrease in these icROS has been correlated to inflammation.

Paper I demonstrates that cord blood neutrophils from term neonates delivered by elective Caesarean section display a primed phenotype, responding to galectin-3, in contrast to adult blood neutrophils. This primed phenotype is accentuated by vaginal delivery. Paper II investigates a pediatric autoinflammatory syndrome, periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA), and demonstrates that three key aspects of neutrophil function, namely apoptosis, priming, and icROS production, are all altered in this disease, most prominently during febrile attacks. Paper III demonstrates that phagocytes from patients with chronic granulomatous disease (CGD), devoid of antimicrobial ROS production, display increased levels of mitochondrial-derived ROS. CGD patients are hyper-susceptible to infections, but also suffer from sterile inflammatory conditions. Paper III suggests that mitochondrial ROS might drive the sterile inflammatory manifestations in CGD. In paper IV, neutrophil interactions with galectin-3 have been studied, and the main results show that a truncated fragment of galectin-3 can inhibit galectin-3 induced activity. Further, a novel type of interaction between galectin-3 and the truncated form, when binding to the cell surface, is presented.

In conclusion, investigation of neutrophils from different settings of health and disease has been utilized to increase our detailed knowledge regarding basic functions in these cells, in addition to providing new information on severe inflammatory syndromes that contribute to the overall understanding of inflammatory diseases.

List of publications

This thesis is based on the following papers referred to in the text by their Roman numerals:

I Sundqvist M*, Osla V*, Jacobsson B, Rudin A, Sävman K, and Karlsson A
Cord blood neutrophils display a galectin-3 responsive phenotype accentuated by vaginal delivery
*BMC Pediatrics (2013) 13:128 *joint first authorship*

II Sundqvist M, Wekell P, Osla V, Bylund J, Christenson K, Sävman K, Foell D, Cabral D, Fasth A, Berg S, Brown KL, and Karlsson A
Increased intracellular reactive oxygen radical production in neutrophils during febrile episodes of PFAPA syndrome
Arthritis and Rheumatism (2013) 65(11):2971-83

III Brown KL*, Sundqvist M*, Christenson K, Björnsdottir H, Osla V, Karlsson A, Dahlgren C, Speert DP, Fasth A, and Bylund J
Elevated mitochondrial reactive oxygen species promote cellular redox imbalance and inflammation in chronic granulomatous disease
*Submitted Manuscript *joint first authorship*

IV Sundqvist M, Welin A, Osla V, Nilsson U, Leffler H, Bylund J, and Karlsson A
Type C-self association of galectin-3 on neutrophil cell surfaces; role of the carbohydrate recognition domain in regulating cell function
In Manuscript

The following paper is also referred to in the text:

Appendix Brown KL, Wekell P, Osla V, Sundqvist M, Sävman K, Fasth A, Karlsson A, and Berg S
Profile of blood cells and inflammatory mediators in periodic fever, apthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome
BMC Pediatrics (2010) 10:65

Contents

Populärvetenskaplig sammanfattning

Målet med arbetet som presenteras i denna avhandling har varit att studera den vanligaste av alla vita blodkroppar, neutrofilen, och dess funktioner vid hälsa jämfört med vid olika inflammatoriska tillstånd.

Vi människor är utrustade med ett komplext immunförsvar för att skydda oss mot angrepp av mikroorganismer, t.ex. bakterier eller virus. Som följd av ett sådant angrepp uppstår en försvarsreaktion, inflammationsreaktionen, som i vävnaden karakteriseras av rodnad, värme, svullnad, smärta och eventuell funktionsnedsättning, och vars slutliga mål är att eliminera den invaderande mikroorganismen varpå inflammationen avslutas. Inflammation kan också uppstå utan inblandning av mikroorganismer, t.ex. vid en vävnadsskada då trasiga celler behöver avlägsnas från kroppen. Vissa människors immunförsvar är felprogrammerat så att de vita blodkropparna skapar inflammation utan synlig anledning eller uppfattar kroppsegna strukturer som hot vilka ska elimineras. Dessa tillstånd kallas för autoinflammatoriska respektive autoimmuna sjukdomar. Sådana tillstånd blir ofta långvariga, kroniska, varpå inflammationen kan blir skadlig för kroppen.

Vita blodkroppar utgör en viktig del av vårt immunförsvar. De vanligaste vita blodkropparna kallas för neutrofiler. De kan äta upp, fagocytera, och avdöda mikroorganismer med hjälp av olika toxiska substanser som finns inuti neutrofilen. Neutrofiler spelar på så sätt en central roll i infektionsförsvaret, men samma mekanismer som neutrofilerna använder för att döda mikroorganismer kan också orsaka skada på kroppens egen vävnad, t.ex. genom produktion av toxiska syremetaboliter, s.k. reaktiva syreradikaler (ROS, förkortat efter engelskans; Reactive [reaktiva] Oxygen [syre] Species [radikaler]). Den största mängden ROS som produceras i neutrofilen bildas med hjälp av ett enzym, NADPH-oxidaset, som aktiveras i syfte att avdöda mikroorganismer. Dock kan NADPH-oxidaset också producera ROS i avsaknad av mikroorganismer, en effekt som man tror är en del av cellens interna funktionsreglering. Även andra källor, t.ex. den energiproducerande mitokondrien som bildar ROS som en bi-produkt, kan bidra med intracellulär ROS-produktion. I blodbanan hos en frisk vuxen är neutrofilerna i ett vilande tillstånd tills de på grund av t.ex. en infektion blir kallade till vävnaden av inflammationssignaler. Både inflammationssignalerna och mikroorganismerna gör att neutrofilerna ändras från vilande till aktiverade celler (också kallade primade celler), vilka bland annat karakteriseras av ett ökat uttryck av receptorer på cellytan. Sådana receptorer finns lagrade i intracellulära förråd, granule, i den vilande neutrofilen. Då den primade neutrofilen börjar uttrycka dessa receptorer på cellytan får de möjlighet att interagera med olika ämnen (både kroppsegna och mikrobiella) vilket t.ex. ökar förflyttningen av cellerna in i vävnaden och hjälper till vid fagocytos av mikroben. Receptorbinding kan också initiera produktion av ROS. En kroppsegen substans som har möjlighet att framkalla ROS-produktion från NADPH-oxidaset i primade, men inte vilande neutrofiler är galektin-3. De vilande neutrofilerna saknar de receptorer som behövs på sin yta, medan de primade cellerna har gott om sådana receptorer. Galektin-3 är ett socker-bindande protein som binder till laktosinnehållande sockerstrukturer på dessa receptorer. Den del av galektin-3-molekylen

som binder till receptorn kallas CRD (förkortat efter engelskans Carbohydrate [kolhydrat] Recognition [igenkännande] Domain [domän]).

I *arbete I* har vi undersökt neutrofiler i navelsträngsblod från nyfödda bebisar förlösta vaginalt eller med planerat kejsarsnitt. Då nyfödda bebisar lätt drabbas av infektioner är det mycket viktigt att deras neutrofiler fungerar som de ska. Data i *arbete I* visar att neutrofiler i blod hos nyfödda bebisar som förlösts med planerat kejsarsnitt kan svara på galektin-3, d.v.s. de nyföddas neutrofiler är primade jämfört med neutrofiler från vuxna, vilka inte svarar då de stimuleras med galektin-3. Detta tyder på att neutrofiler är primade i bebisars blod innan de föds, möjligen som en förberedelse för förlossning och ett liv i världen utanför. Neutrofiler från bebisar som förlösts vaginalt producerade mer galektin-3-framkallad ROS jämfört med celler från planerat kejsarsnitt. Detta är troligtvis associerat med den påfrestning som en vaginal förlossning innebär.

I *arbete II* har neutrofilfunktionen hos barn med den autoinflammatoriska sjukdomen PFAPA (Periodic Fever [periodisk feber], Aphthous stomatitis [aftös stomatit ~ blåsor i munnen], Pharyngitis [faryngit ~ svalginflammation], and cervical Adenitis [cervikal adenit ~ lymfkörtelinflammation i käkvinklarna]) undersökts. PFAPA karakteriseras av episoder med hög feber och inflammation framför allt i halsregionen. Dessa febrila och inflammatoriska episoder återkommer regelbundet ungefär var tredje till var femte vecka och däremellan är patienterna friska. Resultaten i *arbete II* visar att neutrofiler under en febril PFAPA-episod har en ökad livslängd, en ökad produktion av ROS (från NADPH-oxidaset i frånvaro av mikroorganismer) och är mer aktiverade jämfört med neutrofiler under den icke febrila PFAPA perioden eller jämfört med neutrofiler från barn med feber av annan orsak än PFAPA. Då PFAPA är en vanlig sjukdom vars orsaker inte är färdigutredda, kan dessa fynd bidra till ökad förståelse för orsaken bakom sjukdomen och bidra till förbättrad diagnostik och behandling.

I *arbete III* har neutrofilfunktionen hos patienter med den sjukdomen CGD (Chronic [kronisk] Granulomatous [granulomatös ~ en avgränsad ansamling av inflammatoriska celler] Disease [sjukdom]) undersökts. Patienter med CGD har en immundefekt som beror av genetiska mutationer i NADPH-oxidaset och därför kan deras neutrofiler inte producera ROS för att avdöda bakterier. Detta leder till att patienter med CGD drabbas av svåra och ibland dödliga infektioner. Då CGD-patienterna är ”friska”, d.v.s. då de inte lider av infektioner, har de av okänd anledning olika inflammatoriska symptom t.ex. inflammatorisk tarmsjukdom. Data i *arbete III* visar att CGD-neutrofiler producerar ökade mängder ROS från mitokondrierna och att dessa ROS kan trigga cellerna att producera inflammatoriska signalämnen, cytokiner. Ökad produktion av ROS från mitokondrier skulle således kunna vara den bakomliggande orsaken till inflammationspåslaget hos CGD-patienter i frånvaro av infektion. Resultaten ger flera ingångar till hur reglering av inflammationsprocessen via ROS skulle kunna undersökas.

I *arbete IV* har interaktion mellan galektin-3 och neutrofiler studerats med syfte att fördjupa vår kunskap om hur galektin-3 påverkar cellerna. Resultaten visar att utöver att galektin-3 framkallar ROS-produktion i primade neutrofiler så kan primade neutrofiler klyva galektin-3 till CRD, vilken i sin tur kan blockera galektin-3-effekten. Dessa data skulle kunna tyda på att i kroppen så binder primade neutrofiler galektin-3 och produce-

rar ROS, sedan klyver de primade neutrofilerna galektin-3 till CRD vilket gör att neutrofilerna inte kan svara på ytterligare stimulering med galektin-3, d.v.s. neutrofiler har ett inbyggt försvarssystem för att skydda kroppsegna strukturer från överproduktion av galektin-3-framkallad ROS-produktion. Vidare har en ny typ av bindning mellan galektin-3, CRD och celler definierats. Hur denna binding påverkar cellernas funktion är ännu inte klarlagt.

Sammanfattningsvis har studierna som presenteras i min avhandling och dess delarbeten avseende neutrofiler från olika sammanhang, både vid friska tillstånd och vid sjukdomstillstånd, bidragit till att öka vår kunskap om grundläggande funktioner hos neutrofiler och deras interaktion med molekyler, celler och vävnader. Vidare har studierna genererat ny lärdom om svåra inflammatoriska syndrom, insikter som kan komma att medverka till en ökad förståelse för inflammatoriska sjukdomar generellt.

Abbreviations

ANCA	anti-neutrophil cytoplasmic autoantibody
AR	autosomal recessive
CAPS	cryopyrin associated periodic syndromes
CGD	chronic granulomatous disease
CINCA	chronic infantile neurological cutaneous articular syndrome
CL	chemiluminescence
COX-2	cyclooxygenase-2
CRD	carbohydrate recognition domain
CRP	C-reactive protein
CS	Caesarean section
DAMPs	danger-associated molecular patterns
DIRA	deficiency in IL1 receptor antagonist
ecROS	extracellular ROS
ER	endoplasmatic reticulum
ESR	erythrocyte sedimentation rate
FCAS	familial cold autoinflammatory syndrome
FMF	familial Mediterranean fever
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
HIDS	hyperimmunoglobulinemia D with periodic fever syndrome
IFNγ	interferon γ
IL	interleukin
IL1Ra	IL1 receptor antagonist
IP10	interferon γ induced protein 10
JIA	juvenile idiopathic arthrithis
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
MKD	mevalonate kinase deficiency
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mtROS	mitochondrial ROS
MWS	Muckle-Wells syndrome
NADPH	nicotinamide adenine dinucleotide phosphate
NCF	neutrophil cytosolic factor
NETs	neutrophil extracellular traps
NF-κB	nuclear factor-κB
NLRP3	NOD-like receptor family, pyrin domain containing 3
NLRs	NOD like receptors
NOD	nucleotide-binding oligomerization domain
NOMID	neonatal-onset multisystem inflammatory disease
NSAIDs	non-steroidal antiinflammatory drugs
PAMPs	pathogen-associated molecular patterns
PFAPA	periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis
PFS	periodic fever syndromes
phox	phagocyte oxidase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocytes
PRRs	pattern recognition receptors
ROS	reactive oxygen species
SAA	serum amyloid A
SAPHO	synovitis, acne, pustulosis, hyperostosis and osteitis
SOD	superoxide dismutase
TLRs	toll like receptors
TNFα	tumor necrosis factor α
TNFR1	TNF receptor 1
TRAPS	TNF receptor associated periodic syndrome

Introduction

The ability of the human body to maintain internal balance, homeostasis, regardless of encounters with internal or external challenges, is a prerequisite to maintain health. Our body is constantly exposed to threats, e.g., microbes, physical injury, or irritants, which if not handled properly can cause infections and/or permanent damage. Inflammation is part of the complex bodily response to these hazards, aiming to protect our bodies by eliminating the threat and initiate a healing process. In most cases, inflammation is a passing state, tightly regulated by our body. However, for different reasons, the human body sometimes perceives itself as harmful, leading to that the immune system starts attacking the own tissue, resulting in long-lasting conditions called autoimmune or autoinflammatory diseases.

The human immune system consists of the innate and the adaptive immunity, both built up by cellular and humoral components involved in a complex interplay. The innate immune system forms our bodies' first line of defence against harm, acting quickly and aiming at eliminating the threat and/or at delaying the spread of, e.g., microbes until the slower acting, more specific adaptive immune system is mobilized. For different reasons the immune system can become "over active" and induce unwanted inflammation that becomes harmful for the host, leading to disorders termed either autoinflammatory or autoimmune diseases. In the autoinflammatory diseases, innate immune cells become activated, resulting in unrestrained inflammation and subsequent tissue damage, whereas in the autoimmune diseases, cells of the adaptive immune system starts recognizing endogenous tissue as harmful, leading to activation of these cells and production of autoantibodies (antibodies directed towards endogenous tissues) which start attacking the tissue and thereby cause tissue damage.

This thesis deals with the innate immune system, and more specifically with the most abundant white blood cell, the neutrophil. Neutrophils play a central role in the defence toward infection as well as in inflammatory pathology, since the same mechanisms that are responsible for the killing of microbes also may damage endogenous, healthy tissue.

The aim of my PhD project has been to elucidate variability in basic neutrophil functions by comparing cells from healthy subjects to cells from individuals with an inflammatory condition. Specific emphasis has been on the neutrophil interaction with the sugar-binding protein galectin-3, which has been proposed as a novel inflammatory mediator, and on the production of reactive oxygen species (ROS) in intracellular compartments of the neutrophil.

The human immune defence

Innate and adaptive immunity

The human immune system comprises the innate and the adaptive immunity, both built up by cellular and humoral components. The innate immune system is present from birth and considered more primitive and less specific than the adaptive immune system. It is largely dependent of cells of myeloid origin, including mononuclear leukocytes (monocytes and macrophages) and polymorphonuclear leukocytes (PMN; eosinophils, basophils and neutrophils). Neutrophils and monocytes/macrophages are professional phagocytes, i.e., they have the ability to ingest and eliminate harmful particles, microbes, and dead or dying cells, effector functions that together with other components of the innate immune system form our first line of defence. Components of the innate immune system thus either eliminate the threat or perform damage control until the slower-acting, but more fine-tuned, adaptive immune response is mobilized [1]. The adaptive immune system includes cells of lymphoid origin e.g., T-lymphocytes and B-lymphocytes, and has the ability to create immunological memory after an initial response to a certain threat, which leads to a quicker response at subsequent encounters. Of note, there is no situation at which the innate or adaptive immune systems are singularly active, as these systems overlap and cooperate to protect our body [2].

Inflammation

As a response to challenge, both of non-infectious (aseptic) and infectious origin, our body reacts with inflammation. The inflammatory process is driven by inflammatory mediators, produced and released by injured or infected cells, and is defined by its resulting clinical symptoms, known as the cardinal signs of inflammation; redness, increased temperature (heat), swelling, pain, and loss of function [3, 4].

Acute inflammation

Acute inflammation is a rapid process, driven by innate immune leukocytes present in the tissue (e.g., tissue-resident macrophages and mast cells) that express receptors termed pattern recognition receptors (PRRs). The PRRs recognize pathogen-associated molecular patterns (PAMPs) present on or released from microbes, and danger-associated molecular patterns (DAMPs), e.g., nuclear or cytosolic proteins released by damaged cells, i.e., intracellular structures harmless once inside the cell but harmfull when exposed to the extracellular milieu. This recognition leads to activation of the innate immune leukocytes that start to release proinflammatory mediators (e.g., cytokines and chemokines) responsible for the clinical signs of inflammation [5, 6]. The released inflammatory mediators induce (i) vasodilation, which results in increased blood flow causing redness and local increase in temperature, (ii) increased permeability in the blood vessels, leading to leakage of plasma proteins and fluid into the tissue, causing swelling, pain, and loss of function, and (iii) increased receptor exposure on the endothelial cells in the vessels, fostering interactions with circulating leukocytes (mainly neutrophils) which then migrate into the affected tissue through chemotaxis. Chemotaxis is a process by which the neutrophils move towards increasing concentrations of specific signalling molecules (chemoattractants)

released from the inflamed site and recognized by receptors on the neutrophil cell-surface, allowing the cell to find the focus of infection/inflammation [7]. Acute inflammation is normally regulated locally, but in cases when the local damage/infection is severe enough, systemic signs of acute inflammation appear (described under "Clinical signs of inflammation" below). The acute inflammatory response is tightly regulated also temporally and is typically resolved fairly quickly. An active antiinflammatory reaction starts a few hours after initiation of the acute inflammation process. This reaction, together with removal of the initial trigger (e.g., after the microbe that caused the inflammation has been cleared), leads to a resolution of the inflammation without further complications, i.e., acute inflammation is positive for the host resulting in clearance of, e.g., invading microbes.

Regulation and resolution of inflammation

The process of inflammation is essential to effectively protect us against potentially lethal infections, but if not regulated properly it can cause severe harm to surronding tissues. Leukocytes are able to detect, and respond to both PAMPs and DAMPs by a variety of PRRs that are either expressed on the cell surface (e.g., toll like receptors [TLRs]), or at intracellular sites where they may bind internalized or cytosolic structures that warrant a response (e.g., viral nucleic acids). Prominent examples of cytosolic PRRs are the nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) that survey the intracellular environment for infection, noxious substances, and metabolic perturbations [8]. Stimulation of both TLR and NLR can lead to activation of the transcription factor nuclear factor-κB (NF-κB) responsible for the production of a number of cytokines, e.g., interleukin (IL) 6, IL8, and tumor necrosis factor α (TNFα). Activation of some of the NLRs can lead to activation of inflammasomes, multiprotein complexes containing the receptor itself that are expressed in myeloid cells and that convert the protease procaspase-1 to active caspase-1. Caspase-1 promotes maturation of the inactive precursor forms of the proinflammatory cytokines IL1α, IL1β and IL18 into active forms that are released to the extracellular milieu [9, 10]. The most extensively studied NLR with regard to inflammasome formation is NOD-like receptor family, pyrin domain 3 (NLRP3, also known as CIAS1, NALP3 and cryopyrin) and gain of function mutations in the gene encoding NLRP3 is associated with many autoinflammatory syndromes [11].

Binding of PAMPs and DAMPs to PRRs trigger the initiation of acute inflammation, mediated by the production of proinflammatory cytokines and chemokines, as well as arachidonic acid-derived proinflammatory mediators (prostaglandins and leukotrienes). Once acute inflammation is established and neutrophils have reached the inflammatory focus in the tissues, the eventual resolution phase is initiated. This includes a switch in the arachidonic acid metabolism from proinflammatory mediators to antiinflammatory mediators, e.g., lipoxins. The production of lipoxins coincides with the synthesis of other antiinflammatory mediators called resolvins and protectins, derived from omega-3 fatty acids [12, 13]. Another mechanism involved in the termination of acute inflammation is the strictly controlled process of programmed cell death, apoptosis (described in more detail under "Neutrophil longevity" below) at the inflamed site and subsequent clearance of dead cells by, e.g., tissue resident macrophages. Proper removal of apoptotic neutrophils from an inflammatory site is important since macrophage phagocytosis of apoptotic cells will result in the release of antiinflammatory cytokines (e.g., IL10 and transforming growth factor β1 [TGFβ1]), which further promote resolution of the inflammation [14].

Once the threat that initiated the inflammatory reaction has been eliminated and no trigger is left to sustain the inflammatory response, the antiinflammatory mediators come in majority and complete the resolution of the inflammation.

Destructive inflammation

For different reasons the acute inflammatory process is not always properly resolved, leading to a destructive, prolonged, and sometimes chronic inflammation. The exact cause for an inflammation to become chronic is not fully understood. However, if, the immune system misinterprets entities of the own body as harmful, these entities will rarely be completely removed and thus continue to trigger unwanted inflammation. Such chronic conditions are termed autoimmune syndromes and are mostly driven by cells of the adaptive immune system. If cells from the innate immune system in the absence of apparent trigger become activated and induce "unwanted" inflammation, such ongoing inflammatory conditions are called autoinflammatory syndromes [11, 15]. During certain chronic inflammatory conditions, a basal, low-grade and on-going inflammation can evolve into a more acute-like inflammation, only to later return back to a low-grade inflammation. Such a cyclical disease feature is seen, e.g., in the autoinflammatory periodic fever syndromes (PFS; described in more detail under "Periodic fever syndromes" below).

Clinical signs of inflammation

Most minor everyday cases of acute inflammation in local tissues are too insignificant to induce any systemic responses, but more severe inflammatory conditions are often accompanied by systemic changes that can be used as clinical biomarkers for, e.g., inflammatory syndromes. Clinical signs of inflammation include increased serum levels of acute phase proteins, synthesised predominantly by the liver as a response to proinflammatory cytokines. Two prominent examples of acute phase proteins used for clinical diagnosis of inflammation are serum amyloid A (SAA) and C-reactive protein (CRP). The specific function of SAA is unclear. It has been shown that SAA can act as an opsonin, i.e., facilitate the phagocytic uptake of microbes [16], however, with regard to other inflammatory activities SAA is shown to be completely inert [17]. CRP, on the other hand, has more well-defined functions, e.g., it can activate the complement system and (like SAA) function as an opsonin by facilitating the phagocytic uptake of both microbes and apoptotic cells (reviewed in [18]). Another sign of systemic inflammation is increased erythrocyte sedimentation rate (ESR), attributed mainly to alterations in plasma proteins that induce erythrocyte aggregation. During inflammatory conditions, increased numbers of leukocytes are often found in the circulation (leukocytosis), most probably to help resolve the inflammation by degrading microbes and/or damaged cells. Fever, defined as a body temperature above the normal level of ~ 37°C is another feature that can bee seen during inflammatory processes [19]. The fever-inducing substances are termed pyrogens and comprise prostaglandins, microbial molecules such as lipopolysaccharide (LPS) and cytokines such as IL1α, IL1β, TNFα, TNFβ and IL6, which are increased as part of an inflammatory process. The pyrogens are typically induced by infections, but are also able to induce fever in the absence of infection [20-22]. They circulate in the blood and bind receptors on structures surrounding the hypothalamus, which contains the thermoregulatory centre of the body. This leads to induction of cyclooxygenase 2 (COX-2) that induces the synthesis of prostaglandin E_2 (PGE_2). Increased levels of PGE_2 in the brain stimulate release of neurotransmitters that raise the set point in the thermoregulatory

centre, leading to neuronal signals to the cortex to conserve body heat. This is achieved by, e.g., vasoconstriction of peripheral blood vessels (leading to that we start to feel cold) and shivers (a way of our body to use muscle movement to produce more heat). The resulting increase in body temperature is maintained until the PGE_2 levels are reduced, i.e., when the inflammation subsides or treatment with antipyretics (e.g., drugs containing COX-2 inhibitors, e.g., non-steroidal antiinflammatory drugs (NSAIDs) is introduced [23].

Measuring serum levels of CRP, SAA, ESR, leukocyte numbers, and body temperature can thus give indications of an on-going inflammatory process, however, these parameters do not necessarily distinguish if the inflammation is of an infectious origin or not. A complementing factor that increases during bacterial infections is the serum protein pro-calcitonin [24]. In the appendix to this thesis, the levels of procalcitonin have been used to distinguish patients with the autoinflammatory PFS periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) syndrome from patients with infectious diseases of bacterial origin [25].

Neutrophils

Neutrophils are the most abundant leukocytes in human blood and belong together with eosinophils and basophils to the leukocyte subgroup of PMN, also called granulocytes. The name PMN derives from the multilobulated shape of the nuclei of these cells (the neutrophil nucleus is often divided into 2 – 5 lobes), while the name granulocytes derive from the presence of large quantities of cytoplasmic granules (small membrane-enclosed organelles). These two features contribute to the distinction between PMN and mononuclear cells (monocytes and lymphocytes), the latter having unlobulated nuclei and containing fewer granules. In common parlance, the terms PMN or granulocytes often refer to only neutrophils, most probably because the neutrophils constitute by far the main part of this leukocytes subgroup.

Neutrophils circulate in the human blood at a concentration of 1.7 - 7.5 x 10^9 cells/L, and constitute around 50 - 75 % of all circulating leukocytes. In healthy humans, neutrophils are distributed almost equally between a circulating and a marginating pool. The circulating pool comprises the neutrophils that are floating in the blood and thus are obtained in a blood sample. The marginating pool includes the neutrophils that are loosely attached to the endothelium at sites where the blood flow is relatively low and is not included in the measured concentration of the amount of neutrophils in a blood sample. Neutrophils continuously exchange between the circulating and marginating pools [26, 27].

During inflammatory conditions, the neutrophil concentration can increase several-fold, indicating that the fundamental role of neutrophils is to contribute to a functioning immune defence. That neutrophils are essential for human health is confirmed also by the fact that certain genetic mutations causing a marked decrease in neutrophil numbers (0 – 0.5 x 10^9 neutrophils/L) result in immunodeficiency, characterized by recurrent severe infections [28, 29].

Neutrophil development

Neutrophils, as well as all other blood cells, are derived from haematopoietic stem cells present in the bone marrow, where different signalling molecules direct and regulate the stem cells to differentiate into a number of different cell types. The formation of myeloid cells (PMN and monocytes) is called myelopoiesis and is above all driven by the signalling molecules granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and, macrophage colony-stimulating factor (M-CSF). As implicated by the acronym, G-CSF is the most important factor for neutrophil production (≈ 2 x 10^{11} neutrophils / day) and maturation [27, 30, 31]. Neutrophil maturation, or granulopoiesis, is a 12 - 14 day process during which the neutrophils pass different maturation stages that are associated with the formation of granules of different kinds. The lysosome-like azurophilic (primary), specific (secondary), and gelatinase (tertiary) granules are formed through budding off from the endoplasmatic reticulum (ER)/Golgi network, while the secretory vesicles are formed through endocytosis during the late stages of maturation [32-34]. The mature neutrophil is finally released from the bone marrow to the blood, a process in part dependent on the presence of ligands that act

on the chemokine receptors CXCR4 (supporting retention) and CXCR2 (supporting release) expressed on the neutrophil plasma membrane [35].

Neutrophil granules

As mentioned above, neutrophils contain at least four types of distinct granules/intracellular vesicles that are used for the execution of all the different functions that the cell will have to perform during its lifetime. These vesicles are formed at different stages during granulopoiesis, with the largest granules, the azurophil granules, being formed first, followed by the specific granules, the gelatinase granules, and finally the small secretory vesicles. The granules are filled with different sets of soluble molecules as well as exclusive combinations of membrane-localized receptors. The sorting into different granules is achieved by the process of "sorting-by-timing", suggesting that a granule is filled with the proteins that are synthezised solely at the same time (during cell maturation) as the granule is formed [36].

The azurophil granules are formed first, during the myeloblast and promyelocytic stage of neutrophil maturation. These granules are traditionally defined as peroxidase-positive granules as they contain large amounts of myeloperoxidase (MPO). Apart from MPO, the azurophil granules also contain lysosomal enzymes and proteases used for degradation of phagocytised material. This granule is primarily mobilized to a closed phagosome (described in more detail under "Phagocytosis" below), into which it empties its antimicrobial substances.

During the next stages of neutrophil maturation, the myelocyte and metamyelocyte stages, the specific granules are formed. These granules contain high concentrations of antimicrobial substances (e.g., lactoferrin), proteases, phagocytic receptors, and cytochrome b_{558}, the membrane-component of the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase) responsible for production of ROS. The specific granule, as the azurophil granule, fuses primarily with a phagosome, showering an infectious prey in antimicrobial substances, including ROS [7, 32, 34, 37-39].

Thirdly, during the band cell stage, the gelatinase granules are formed. In similarity to the specific granules the gelatinase granules also contain high concentrations of antimicrobial substances. However, the gelatinase granules also contain hydrolytic proteases (e.g., gelatinase B, also known as matrix metalloproteinase [MMP] 9), used to degrade the extracellular matrix during extravasation in the tissue, as well as chemotactic and adhesive receptors that are needed in the movement from the blood to the site of inflammation.

Finally, neutrophils contain an easily mobilized organelle called the secretory vesicle, formed through endocytosis during the final stages of neutrophil maturation in the bone marrow. The secretory vesicles thus contain plasma proteins such as albumin, and a variety of plasma membrane receptors that are thought to mediate primary adhesion to the endothelium and chemotaxis over the vessel wall into the extravascular space [7, 34, 38, 39].

By a process called degranulation, the granules are mobilized to and fuse with the plasma membrane, emptying the granule contents into the extracellular milieu (exocytosis) or into the membrane-enclosed phagosome (forming a phagolysosome). To the plasma

membrane, the granules are mobilized in opposite order to how they are formed, i.e., secretory vesicles formed last are mobilized first, whereas azurophilic granules, formed first are mobilized last (if at all) [40]. Incorporation of granule membrane receptors in the plasma membrane makes the cell more prone to react to stimulation, a mechanism designated priming (described in more detail under "Priming of neutrophils" below). Once granules have mobilized they cannot be re-generated, thus degranulation is a one-way road to cellular alteration [34].

Phagocytosis

Neutrophils, together with monocytes, macrophages, dendritic cells, and mast cells belong to the leukocyte group of professional phagocytes, i.e., they have the ability to engulf and degrade microbes and cell debris and thus play a central role during acute inflammatory responses by their ability to eradicate threats. Phagocytosis is an active process in which receptors (e.g., Fc- and complement receptors) on the neutrophil cell-surface recognize, e.g., an antibody- or complement-opsonized microbe, and attaches to it. Following protrusion of the plasma membrane to gradually surround the prey, the particle becomes internalized into a vacuole, the phagosome. The phagosome then fuses with azurophil, specific and gelatinase granules, forming a phagolysosome, and the granular antimicrobial substances are emptied onto the microbe to degrade/eliminate the ingested particle [41].

During phagocytosis, the neutrophil consumption of molecular oxygen (O_2) increases. The electron transport system responsible for the O_2 consumption is called the NADPH-oxidase, which has become incorporated in the membrane of the phagolysosome during the fusion between the phagosome and the specific and gelatinase granules [42, 43]. Upon activation of the NADPH-oxidase, superoxide anion (O_2^-), a short-lived bactericidal ROS, is formed in the phagolysosome where it may spontaneously dismutate into hydrogen peroxide (H_2O_2). The presence of MPO in the same compartment (delivered by fusion with the azurophil granules), will lead to transformation of O_2^- and H_2O_2 into other ROS, including hypochlorus acid (HOCl), which are even more toxic and potent antimicrobial substances [34, 41, 44].

Production of reactive oxygen species (ROS)

Radicals are chemically reactive molecules mainly derived from oxygen (ROS) or nitrogen (reactive nitrogen species [RNS]). The majority of ROS produced by neutrophils is derived from the NOX2-containing NADPH-oxidase, which is in focus in all papers of this thesis [45, 46]. However, ROS can also be derived by several other cellular sources (reviewed in [47-50]) including the mitochondria [51]. Mitochondria-derived ROS has been studied in paper III of this thesis.

As described above, ROS formed by the phagocyte NADPH-oxidase are essential for protection against invading microbes, but these and ROS from other sources may also have a variety of other functions. Clearly, ROS are ubiquitous entities present in all cells (not just phagocytes) and in line with their ability to react with most common biomolecules they are known to control a variety of cell-signalling pathways by different redox reactions. With regards to inflammation, the best examples of ROS-controlled cell-signalling pathways are activation of the inflammasome (resulting in the maturation and

release of inflammasome related cytokines IL1α, IL1β and IL18 [52-54]), and activation of the transcription factor NF-κB, resulting in production and release of proinflammatory cytokines [55]. The widespread use of ROS for the control of common cell-signalling events indicate that the cellular balance between oxidants and antioxidants, the so-called redox balance, needs to be tightly controlled. Our bodies are supplied with a wide variety of antioxidants that neutralize oxidants and all cells express potent antioxidants such as superoxide dismutase (SOD), catalase, glutathione peroxidase and thioredoxin [56]. It is likely that small perturbations in redox balance may serve to control cell-signalling, whereas severe imbalances (in favour of the oxidants) is referred to as oxidative stress and can be deleterious to cells and surrounding tissues.

NADPH-oxidase derived ROS

The NADPH-oxidase is a complex enzyme system consisting of both membrane bound and cytosolic components. The membrane bound component is a heterodimeric flavo-hemoprotein termed cytochrome b, which consists of two subunits: $p22^{phox}$ (the suffix phox stands for phagocyte oxidase) and $gp91^{phox}$ (also known as NOX2). Cytochrome b is localised in the plasma membrane, the membranes of secretory vesicles, and the specific and gelatinase granule membranes [34, 57, 58]. In itself cytochrome b is a dormant system, but upon cellular activation the cytosolic components of the NADPH-oxidase; $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, translocate to cytochrome b in the membrane, a process regulated by the small G-protein Rac2, creating a functional electron transport system [59]. The active NADPH-oxidase transfers electrons from NADPH in the cytosol, over the membrane, to molecular oxygen (O_2) on the other (intragranular, phagosomal or extracellular) side, resulting in the formation of O_2^- that rapidly dissociates spontaneously to H_2O_2 (Figure 1).

In the phagosome, the primary ROS (O_2^- and H_2O_2) can be transformed into more toxic metabolites such as HOCl (formed by an MPO-catalyzed reaction with chloride), and peroxynitrate ($ONOO^-$, formed upon reaction with nitric oxide) that efficiently can eliminate microbes. The exact reactions whereby ROS kill microbes in the phagosome are not entirely clear, but phagosomal ROS can react with microbial biomolecules in a number of destructive ways, e.g., peroxidation of membrane lipids, and chlorination, decarboxylation or deamination of amino acids [60].

Chronic Granulomatous Disease (CGD)

Chronic granulomatous disease (CGD) is a rare genetic disorder that affects subunits in the NADPH-oxidase [61-64]. As described earlier, the phagocytic NADPH-oxidase induced ROS are crucial for the killing of microbes, and mutations in the genes encoding the NADPH-oxidase subunits can compromise the ability to produce ROS. Thereof, patients suffering from CGD are highly susceptible to particular strains of fungi and bacteria that are resistant to non-oxidative killing of immune cells [61, 62, 65].

CGD is caused by defects in the genes encoding any of the five subunits ($gp91^{phox}$, $p22^{phox}$ $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) of the NADPH-oxidase. The most common (affecting approximately 65 % of the CGD patients [66]) and also most severe form of CGD is called X-linked CGD, which results from mutations in the *cytochrome b_{558} beta polypeptide* (*CYBB*) gene encoding the $gp91^{phox}$ subunit of the NADPH-oxidase. As the gene is located on the X chromosome, the mutation is inherited in an X-linked recessive manner

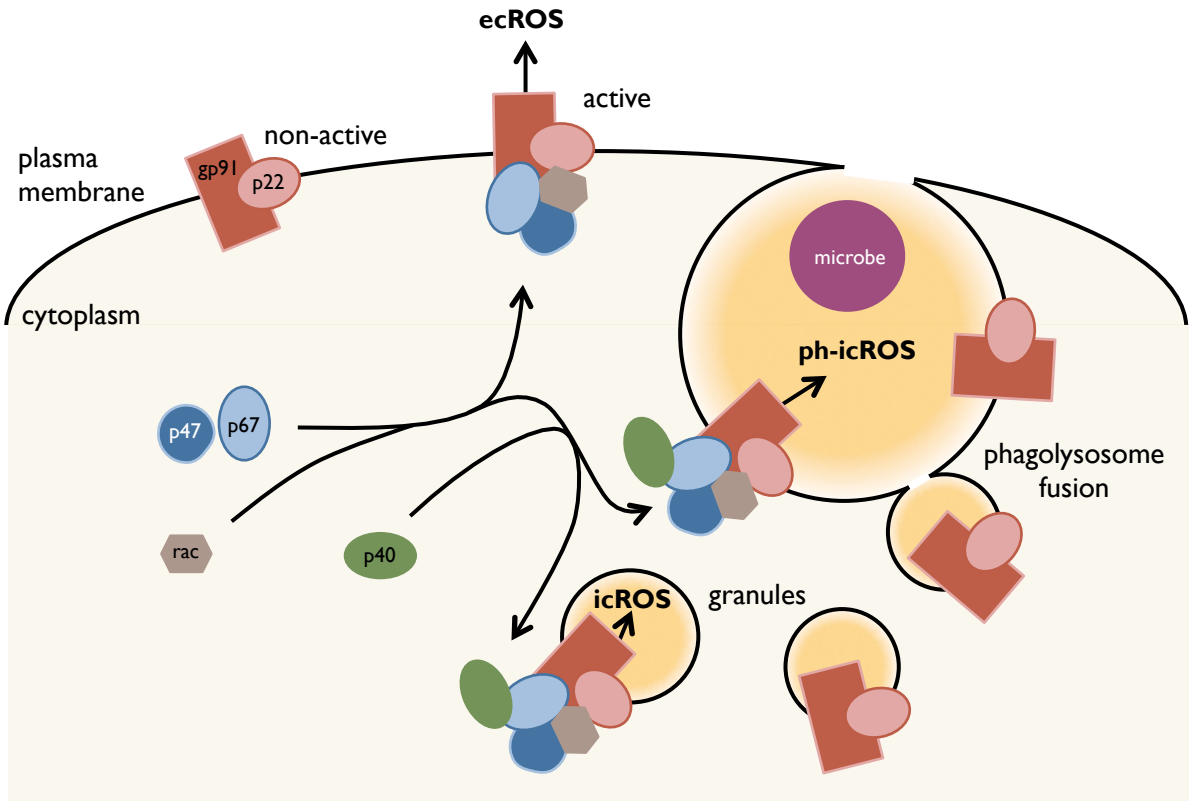

Figure 1. The NADPH-oxidase is activated at different sites in the neutrophil. Two of the NADPH-oxidase components, gp91^{phox} and p22 ^{phox} (also referred to as cytochrome b) are located in the plasma membrane and in the membrane of specific granules and gelatinase granules. Upon activation, the cytosolic components of the NADPH-oxidase, p67 ^{phox}, p47 ^{phox} and p40 ^{phox} as well as the cofactor Rac assemble with cytochrome b forming a functional NADPH-oxidase capable of producing reactive oxygen species (ROS). Assembly of the oxidase can occur at the plasma membrane, resulting in release of extracellular ROS (ecROS), and/or at granule membranes resulting in ROS produced inside the phagosome/phagolysosome (ph-icROS) or inside granules (icROS). The cytosolic subunit p40 ^{phox} has been suggested to be required for production of icROS, but to be dispensable for production of ecROS [71]. This figure is adapted from Bylund *et al* [43].

primarily affecting males [65, 67]. In most cases of X-linked CGD, the NADPH-oxidase derived ROS is completely abrogated [64], possibly accounting for the exceptional severity of the disease. As the opportunistic infections afflicting these patients most often are life threatening, hematopoietic stem cell transplantation is the preferable treatment for patients with X-linked CGD [68]. Mutations in the other subunits of the NADPH-oxidase are inherited in an autosomal recessive (AR) manner, affecting both sexes alike, and often lead to a slightly less severe disease as compared to the X-linked CGD. Less than 5 % of the patients with CGD have mutations in the *cytochrome b_{558} alpha polypeptide* (*CYBA*) gene encoding p22^{phox}, the subunit that together with gp91^{phox} constitute the the membrane bound cytochrome b [66]. It has been shown that lack of either p22^{phox} or gp91^{phox} leads to lack of cytochrome b expression in the membrane, indicating that these subunits are dependent on each other for proper expression [69]. With regard to the cytosolic factors of the NADPH-oxidase, less then 5 % of the patients with CGD have mutations in *neutrophil cytosolic factor* (*NCF*) *2* encoding p67^{phox}, and approximately 30 % of the patients with CGD have muations in *NCF1*, encoding p47^{phox}. The exact reason for why the X-linked CGD is more severe than the AR CGDs is not fully understood, but, e.g., p47^{phox} mutations retain small parts of the ROS production [65, 70], possibly contributing significantly to the antimicrobial defence. So far, only one patient with mu-

tations in *NCF4* encoding p40^{phox} has been described [71] (discussed under "The NADPH-oxidase is localised at two distinct sites in the neutrophils" below).

Apart from severe infections, patients with CGD also suffer from debilitating inflammatory conditions [63], indicating that NADPH-oxidase derived ROS not only play a crucial role as antimicrobial agents, but also functions as signalling molecules involved in inflammatory processes, an aspect of CGD examined in paper III of this thesis. The inflammatory features of CGD resemble those of autoinflammatory disorders, i.e., chronic sterile inflammation caused by cells of the innate immune system.

The NADPH-oxidase is localised at two distinct sites in neutrophils

The membrane-bound part of the NADPH-oxidase, cytochrome b, is localised at two distinct sites in the neutrophil, in the plasma membrane (≈ 5 %) and in granule membranes (the specific and gelatinase granules, ≈ 95 %) [57]. Assembly of the active NADPH-oxidase at the plasma membrane generates extracellularly released ROS (ecROS). The function of ecROS *in vivo* is not totally clarified, but they are proposed to attack extracellular microbes that are too large to be phagocytosed, i.e., they display antimicrobial properties [72, 73]. Phagocyte-derived ecROS have also been shown to function as signalling molecules, suppressing adaptive immune cells of importance for anti-cancer immunity (NK cells and T-cells) [74]. Hence, in this setting ecROS-signalling is bad for the host and pharmaceutics aimed at lowering ecROS levels are used to treat, e.g., certain leukemias [75]. In rodent models of arthritis, ecROS has similarly been shown to dampen the activity of autoreactive T-cells, but with the opposite (positive) outcome; rats with mutation in the gene coding for p47^{phox} (i.e., rats with CGD) display more severe arthrithis as compared to wild type rats [76, 77]. These two examples show that ecROS are important for regulation of immune responses also in non-infectuous settings.

The granule localised NADPH-oxidase generates ROS released intracellularly (icROS). Commonly, the NADPH-oxidase derived icROS is thought of as the antimicrobial ROS produced in the phagosome, however, ROS can also be produced inside the cell in the absence of phagosome formation, most probably inside the NADPH-oxidase containing granules [43, 44, 78-80]. Non-phagosomal icROS has been somewhat controversial, but this function recently gained increased support as data emerged describing a novel form of CGD with mutations in the gene encoding the p40^{phox} subunit of the NADPH-oxidase. This form of CGD resulted in neutrophils with intact ecROS production but severely tampered icROS production, both after challenge with a phagocytic prey and after challenge with a non-phagocytic stimulus, i.e., the latter most probably originating from NADPH-oxidase derived ROS in intracellular granules. The patient displayed signs of inflammation including eczema, aphthous ulcers, and granulomatous colitis, correlating the decreased icROS production to inflammation [71]. Such a correlation has been implicated also in another study where neutrophils from a patient with the autoinflammatory disease synovitis, acne, pustulosis, hyperostosis and osteitis (SAPHO) syndrome displayed a specific decrease in icROS production whilst ecROS production was intact [81]. Interestingly, in paper II of this thesis, the opposite, i.e., a specific increase in non-phagosomal icROS production is for the first time correlated with inflammation. The data show that neutrophils from patients with the autoinflammatory

PFS PFAPA produce increased icROS as compared to control neutrophils (discussed in more detail under "Neutrophil production of ROS in PFAPA" below). Hence, well-regulated icROS production in the absence of phagosome formation seems crucial for proper control of inflammatiory processes.

Mitochondrial ROS

Almost all eurokaryotic cells contain mitochondria, membrane enclosed structures that often are called the cellular power plants as they through cellular respiration produce adenosine triphosphate (ATP), the main source of energy used in cell metabolism. The number of mitochondria differs widely between different cell types, and the amount of mitochondria in a certain cell is not constant but varies depending on how much energy that is needed; e.g., muscle cells contain large amounts of mitochondria whereas myeloid cells contain less. During cellular respiration and production of ATP, O_2 is converted to H_2O. However, the cellular respiratory chain involves several steps where ROS can be produced and these ROS are generally thought of as toxic by-products. In non-phagocytic cells mitochondria are the primary source of cellular oxidants, however, in phagocytes the NADPH-oxidase is the major producer of ROS. Most of the mitochondrial ROS (mtROS) are produced inside the mitochondria, however, it has been described that mtROS can be transferred from the mitochondria to the cytoplasm through voltage-dependent ion channels [82]. For protection against damaging effects of mtROS, mitochondria are equipped with specific antioxidants, including a mitochondrial SOD termed MnSOD (also known as SOD2). The importance of proper protection against mtROS is demonstrated by that MnSOD knockout mice display with perinatal lethality [83] and that increased levels of mtROS can induce progression of cancer as well as neuronal damage (reviewed in [84]).

With regards to inflammation, mtROS are involved in multiple pathways and has been shown to be of importance for autoinflammatory diseases. Monocytes obtained from patients with the autoinflammatory PFS TNF receptor associated periodic syndrome (TRAPS) display increased mtROS, correlating to an enhanced responsiveness to LPS-induced production of proinflammatory cytokines [85]. It has also been demonstrated that mtROS can activate the NLRP3-inflammasome and thus induce the maturation and release of the inflammasome-related cytokines IL1α, IL1β, and IL18 [52]. These studies have mainly focused on monocytes, however also neutrophils contain a few mitochondria [51] and are able to produce mtROS as demonstrated in paper III of this thesis. The results show that CGD phagocytes, i.e., monocytes and neutrophils devoid of NADPH-oxidase derived ROS, display increased basal levels of mtROS. As described above, CGD patients are hyper-susceptible to infections, but also suffer from various inflammatory conditions and our data thus suggest that mtROS might be a driving force behind the inflammatory manifestations in CGD.

Studying neutrophil production of ROS

The neutrophil NADPH-oxidase derived ecROS and icROS can be detected using a luminol/isoluminol-amplified chemiluminescence (CL) system, which is the system primarily used in this thesis (papers I-IV). The readout for the CL system is energy in the form of light, released by the dyes luminol and isoluminol after being excited by ROS (primarily O_2^-), making it a highly sensitive system with the possibility to follow the

kinetics of the ROS response [44]. Neutrophils can be activated to produce ROS by a number of stimuli including chemoattractants, cytokines, lectins, phorbol esters, as well as particulate stimuli such as microorganisms, and to detect these ROS the CL system is dependent on a peroxidase to catalyse the reaction. For detection of icROS production, the neutrophil MPO is relied on for this purpose. However, for detection of ecROS production a peroxidase needs to be added, e.g., horseradish peroxidase (HRP), as secretion of MPO from the cells is limited [40]. Other than addition of a peroxidase, the ecROS production is measured using isoluminol, which is cell-impermeable, whereas icROS production is measured in the presence of luminol, which is cell permeable. For an isolated measurement of icROS production, extracellular cell-impermeable scavengers (SOD [catalyses the reduction of O_2^- to H_2O_2] and catalase [catalyses the reduction of H_2O_2 to H_2O]) are added [44, 70]. As the luminol-amplified CL system used to measure icROS production is dependent on endogenous MPO, it should be taken into account that alterations in icROS could reflect alterations in MPO, i.e., an increase in icROS production could be due to an increase in MPO, whereas a decrease in icROS production might be explained by decreased or lack of MPO (the latter evidenced by that patients with a deficiency in MPO produce low levels of icROS production when measured by luminol-amplified CL [86]). Presence of endogenous MPO can be analysed using enzyme-linked immunosorbent assay (ELISA) as used in paper II of this thesis.

The production of mtROS can be measured by staining cells with the fluorogenic probe MitoSOX Red, used in paper II and III of this thesis. MitoSOX Red is a live-cell, permeable, positively charged compound that selectively targets the mitochondria where it accumulates as a function of mitochondrial membrane potential. Upon interaction with ROS, MitoSOX Red becomes oxidized into a highly fluorescent compound that can be detected by flow cytometry and/or microscopy. Microscopical detection of MitoSOX Red stained cells allows for analysis of a relatively limited number of cells, whereas the use of flow cytometry has the advantage of analysing a large number of cells, making quantifications and comparisons of mtROS production between different cell-types or between different treatments possible [87, 88]. It should be taken into account that differences in mtROS could be due to different amounts of mitochondria (as the amount of mitochondria in a certain cell is not constant) or altered polarization of the mitochondrial membranes (as, e.g., depolarization of mitochondrial membranes can be associated with increased mtROS production). There are different probes available for analysing this by flow cytometry, e.g., Mitotracker Green (measures mitochondrial mass) and tetramethylrhodamine ethyl ester (TMRE, measures mitochondrial membrane potential), both used in paper III of this thesis.

There are several fluorogenic probes available for detection of ROS production by flow cytometry (and microscopy), however, the exact source from where the ROS are derived using these probes is not entirely clear. A commonly used fluorogenic probe is dihydrorhodamine-123 (DHR123) which is a non-fluorescent, live-cell, permeable dye that upon interaction with ROS becomes oxidized to the highly fluorescent compound rhodamine-123. In paper III of this thesis, the fluorogenic probe 2',7'-dichlorofluorescein diacetate (DCFDA) has been used. DCFDA is also a live-cell, permeable dye that once inside the cell becomes deacetylated by cellular esterases to a non-fluorescent compound that is trapped in the cell. Upon reaction with ROS, this compound becomes oxidized into the

highly fluorescent 2',7'-dichlorofluorescein (DCF), which can be detected by flow cytometry. A main drawback using fluorogenic probes for detection of ROS as compared to CL is that it is difficult to record the kinetics of a ROS response. Also, as mentioned above, it is not clear which source the detected ROS are derived from, or which particular species of oxygen radical that directly reacts with the fluorogenic probes to generate fluorescence [89]. Theoretically, the dyes should, if analysed by flow cytometry, mainly detect icROS, but extracellular H_2O_2 may also return into the cell, over the plasma membrane and react with the probe intracellularly. The latter problem can however be solved by adding extracellular catalase during DCFDA staining (unpublished observation). As demonstrated in paper III of this thesis, CGD phagocytes display increased basal levels (in the absence of stimuli) of DCFDA fluorescence as compared to wild type phagocytes even though the CGD cells lack a functional NADPH-oxidase. The ROS responsible for the DCFDA fluorescence in CGD phagocytes must thus be derived from another source than the NADPH-oxidase.

There are several other ways to detect both NADPH-oxidase derived ROS (reviewed in [70, 73, 90]) and mtROS (reviewed in [91, 92]), however those methods fall beyond the scope of this thesis.

Priming of neutrophils

In healthy humans, neutrophils circulate in a quiescent state in the blood stream waiting to be recruited to the tissue upon microbial invasion or tissue damage. The term extravasation refers to the process whereby neutrophils leave the blood vessels, pass through the endothelium, and move through the extravascular tissue towards the inflammatory focus. During extravasation the neutrophils undergo several functional changes, from a dormant phenotype in the blood stream into a more excitable phenotype in the tissue [40, 93-96]. This process in which neutrophils alter phenotype is referred to as priming, and results in that cellular responses to subsequent stimulation are enhanced. One important aspect of the priming process is the exocytosis of mobilisable granules supplying the plasma membrane with additional receptors. Consequently, the primed neutrophil is hyper-responsive to receptor-binding ligands on (opsonized) invading microbes, or produced as a result of inflammation. Although priming is typically associated with neutrophils in tissues, primed neutrophils can also be found in circulation during certain severe infectious or aseptic inflammatory conditions, e.g., in the circulation of patients with systemic inflammatory response syndrome (SIRS) [97, 98], anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitis [99], multiple sclerosis (MS) [100] or the autoinflammatory PFS familial Mediterranean fever (FMF) [101]. Most likely, high levels of proinflammatory cytokines in the blood of these patients induce priming of the neutrophils, which may contribute to disease progression by destroying host tissue. In this thesis, two situations in which neutrophils display a primed phenotype in circulation have been identified; paper I, showing that circulating neutrophils from term neonates are primed as compared to adult peripheral blood neutrophils, and paper II, showing that neutrophils during PFAPA flares are primed in circulation. Whether the primed neutrophils are a cause of the inflammatory symptoms associated with these circumstances or *vice versa* is discussed below.

Transmigration from blood to tissue renders neutrophils primed

The transmigration process is initiated by that tissue resident macrophages release signalling molecules as a response to DAMPs and/or PAMPs present in the tissue as a cause of injury or microbial invasion. These mediators activate the endothelial cells in nearby vasculature to express selectins and integrins on their luminal cell surface, promoting binding of circulating neutrophils, specifically interacting with receptors that are constitutively expressed on the dormant neutrophil cell surface (e.g., CD62L also referred to as L-selectin). This initial contact between the endothelium and the neutrophil results in that the leukocyte slows down and starts to roll along the vessel wall [7, 102, 103].

Interaction with the endothelium activates intracellular signalling pathways in the neutrophil, inducing cytoskeletal rearrangements and granule mobilization (degranulation of secretory vesicles and gelatinase granules) to the cell surface. Degranulation leads, among other things, to upregulation of new adhesion receptors (e.g., the integrin CD11b), which bind to specific ligands (e.g., ICAM-1 and -2) on the endothelial cells, concomitant with that CD62L is shed from the surface. This result in that the rolling stops and that the neutrophil becomes firmly adhered to the endothelium. Of note, the shedding of CD62L is dependent on activation of surface-localized proteases (e.g., the metalloproteinase ADAM-17), and is thus not directly dependent on degranulation [104]. Both CD62L shedding and receptor upregulation (e.g., CD11b) through degranulation are features associated with a primed neutrophil phenotype.

When the neutrophil has stopped moving with the circulating blood, an integrin-dependent transendothelial migration (diapedesis) into the tissue takes place. Once in the tissue the neutrophils start moving along a chemotactic gradient, towards increasing concentrations of chemoattractants released at the site of tissue damage. The chemotactic movement is dependent on cytoskeletal reorganization for directional movement, integrin-dependent interactions between the neutrophils and the extracellular matrix, recirculation of chemotactic receptors, and proteases released from gelatinase granules that break down extracellular matrix, facilitating migration to the site of tissue damage. Hence, during the chemotactic movement the neutrophil becomes increasingly primed, gaining capacity to respond to a variety of stimuli due to increased receptor exposure on the cell-surface [105-107] (Figure 2).

Studying neutrophil priming

Neutrophils can be experimentally primed both *in vitro* and *in vivo*. *In vitro*, priming can be induced by pretreating the cells with a priming agent, e.g., LPS [108], used in paper I of this thesis or TNFα used in paper II and IV of this thesis. *In vivo* primed neutrophils can be obtained, e.g., by using an aseptic skin chamber model, as described in detail by Follin and Dahlgren [109]. Briefly explained, this model involves the creation of skin blisters on the forearm of healthy volunteers. The blister roofs are removed, and the skin lesions are covered with chambers filled with autologous serum. After 20 hours, neutrophils can be collected from the chambers for analysis. As control, peripheral blood neutrophils isolated from the same donor are used. Several studies have described different priming features displayed by neutrophils derived from this model [93, 94, 110, 111], features that can be attained by *in vitro* priming with LPS or TNFα [40, 94] and also displayed by neutrophils obtained from pus [95] and saliva [96]. The priming status of a

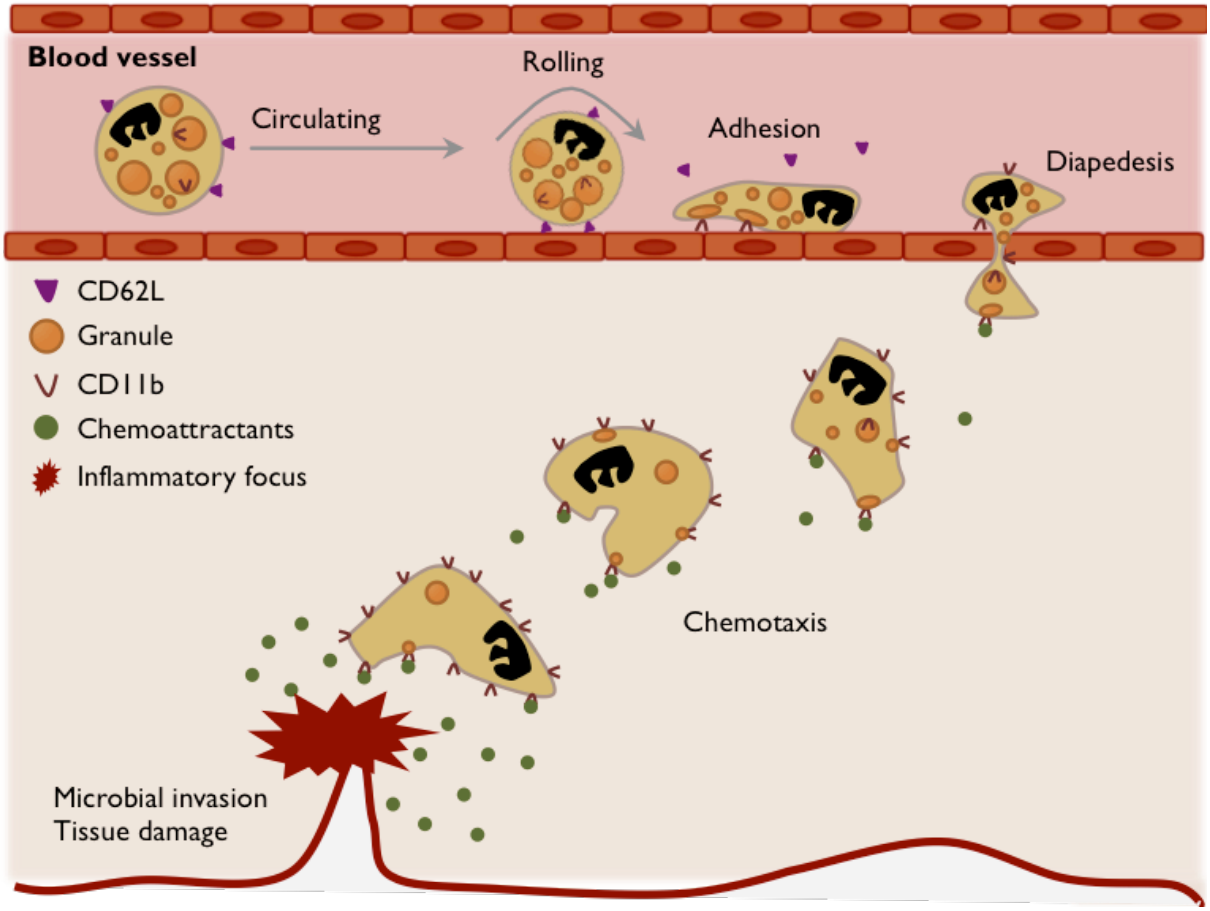

Figure 2. Transmigration from blood to tissue renders neutrophils primed. In response to, e.g., tissue damage or microbial invasion tissue resident cells produce inflammatory signals that activate the endothelial cells to express increased surface receptors that are used by neutrophils to transmigrate to the site of inflammation. Firstly, neutrophils attach the endothelial cells through CD62L and starts to roll along the endothelial cell lining. This and the inflammatory signals from the tissue induce rearrangement and activation of neutrophil of surface receptors, e.g., cleavage of CD62L and upregulation of CD11b. CD11b interaction with the endothelial cells mediates a firm adhesion of the neutrophils to the vessel wall. The cells then cross the endothelium by diapedesis and move towards increasing concentrations of chemoattractants released from the inflamed site. This process is associated with priming of neutrophils, which in part can be defined by the change in surface receptors, i.e., from dormant cells in the blood stream (expressing CD62L) to primed neutrophils in the tissue (with cleaved CD62L and upregulated CD11b).

neutrophil population can be analysed by measuring the expression of surface molecules, e.g., CD62L (shed from primed neutrophils) and CD11b (increased on primed neutrophils as a result of degranulation) [40, 110]. This can easily be performed by immunostaining neutrophils with antibodies directed towards these molecules and analysis by flow cytometry. Of note, neutrophils can become slightly primed during the isolation procedure [112, 113], thus, when analysing the priming status of neutrophils in circulation of, e.g., patients with inflammatory diseases, it is preferable to do the receptor expression analysis in a whole blood assay system, as used in paper II of this thesis.

Assessing the surface expression of different molecules present or absent on the cell surface is a commonly used method to analyse the priming status of neutrophils, however, it only measures the appearance of the neutrophils and does not show whether the neutrophils are functionally different compared to unprimed cells. A forthright way to investigate a functional difference between unprimed and primed neutrophils is to stimulate them with

the β-galactoside binding lectin galectin-3 (described in more detail under "Galectin-3 – a regulator of inflammation" below), as unprimed neutrophils are inert to this lectin whereas primed neutrophils respond with production of NADPH-oxidase derived ROS [94, 108, 114, 115]. This clear on/off priming feature has, in combination with analysing surface expression of priming-associated molecules, been used in paper I and II of this thesis. In fact, data in paper II suggest that galectin-3-induced ROS production is a more sensitive marker than receptor upregulation when measuring the degree of priming.

Neutrophil longevity

Neutrophils are generally viewed as short-lived leukocytes with a half-life in the circulation of approximately 8 hours in healthy humans [27, 116, 117]. One study has recently challenged this view, instead proposing a neutrophil lifespan of approximately 5 days [118], however the accuracy of the methods used in this study have been questioned [119, 120]. Regardless of time in circulation, the neutrophils will gradually age, and in the absence of microbial invasion or tissue damage, the older neutrophils will undergo programmed cell death, apoptosis [121] and be cleared from the blood stream by uptake in the liver or the spleen [122], or even by return to the bone marrow [123]. However, during inflammatory conditions, the longevity (as well as the number) of neutrophils increases several-fold [27], most likely due to interactions with cytokines and/or microbial components with antiapoptotic properties [124-127],

Neutrophil apoptosis

Programmed cell death, apoptosis, is a regulated destruction of cells, a process during which the neutrophils maintain plasma membrane integrity and, by a number of features, signals that it is ready to be cleared. Safe and rapid phagocytic clearance of apoptotic neutrophils by, e.g., macrophages, is of outmost importance since apoptotic neutrophils otherwise will lose their membrane integrity over time and become necrotic, resulting in release of toxic neutrophil contents and DAMPs that can harm surrounding tissue and risk to prolong inflammation [128]. Thus, being short-lived cells with a high turnover rate, neutrophils are destined to die by spontaneous apoptosis. Both spontaneous and stimulus-induced (e.g., through intrinsic factors, such as internal cell damage, or extrinsic factors, such as signals from other cells) apoptosis include the degradation of internal structures, leading to a number of morphological changes. Nuclear condensation and disintegration of the cytoskeleton inside an intact plasma membrane are prominent signs [129, 130]. Yet another feature is the loss of plasma membrane and mitochondrial membrane potential [131], resulting in redistribution of surface components. The phospholipid phosphatidylserine, which in a viable cell is localized on the inner leaflet of the plasma membrane, then becomes exposed on the outer leaflet where it functions as an apoptotic "eat me" signal to surrounding phagocytes [132, 133].

Neutrophil necrosis

As described above, apoptotic neutrophils will become necrotic over time if not properly cleared. Necrotic neutrophils are defined by leaky plasma membranes, leading to release of toxic molecules, e.g., proteolytic enzymes can pass freely through the membrane and cause harm to surrounding tissues. The breakage of cells will also result in release of DAMPs (e.g., the cytosolic S100 proteins) and exposure of these to surrounding cells trigger pro-

inflammatory responses. Hence, necrosis of neutrophils is a proinflammatory event, in contrast to clearance of apoptotic neutrophils, which is an antiinflammatory event.

During the last decade, another version of neutrophil cell death has been in focus, a process in which neutrophils release neutrophil extracellular traps (NETs), extracellular structures composed of the cell's own DNA, clad with histones and intracellular proteins. NETs were originally suggested to bind and kill extracellular microbes and thereby prevent their spreading [116, 134]. Whether NETs actually kill micobes, and whether neutrophils actually die after releasing NETs is currently a debated issue [116]. However, like necrosis, release of NETs (NETosis) can be viewed as a proinflammatory event, as it results in release/exposure of DAMPs that will activate surrounding cells.

Studying neutrophil cell death

The exposure of phosphatidylserine on the surface of the apoptotic neutrophil can be used to analyse the presence of apoptotic neutrophils, as used in paper II of this thesis. By simultaneously staining cells with a fluorescent probe, Annexin V, binding to phosphatidylserine and a cell-impermeable DNA stain (e.g., 7-Aminoactinomycin D, 7-AAD, that only leaks into necrotic neutrophils and stains the DNA) the proportion of viable neutrophils (negative for both Annexin V and 7-AAD), apoptotic neutrophils (positive for Annexin V and negative for 7-AAD), and necrotic neutrophils (positive for both Annexin V and 7-AAD) can be analysed by flow cytometry [135].

Neutrophil apoptosis can be manipulated by treatment with pro- or antiapoptotic stimuli. A proapoptotic stimulus is Fas ligand (FasL), binding to a neutrophil cell surface death receptor, CD95 (Fas). FasL is expressed as a membrane protein on different cells or as a soluble protein, which upon interaction with CD95 induces neutrophil apoptosis [132, 136]. *In vitro,* neutrophils can be incubated with a monoclonal anti-CD95 antibody, which induces apoptosis by cross-linking CD95. To induce antiapoptotic effects *in vitro* neutrophils can be treated by G-CSF [125], IL1β [126], or different PAMPs (e.g., LPS [124] and lipoteichoic acid [127]),

Neonatal neutrophils

The neutrophil functions described above are attributed to neutrophils obtained from adult donors. In paper I of this thesis, basic neutrophil functions were instead investigated in newborn babies, with focus on priming-related features.

During birth and the neonatal period, neonates are at increased risk of acquiring infections due to undeveloped immune mechanisms, both in the adaptive (e.g., lack of antibodies [137, 138] and antigen-specific T-cells [139]) and innate (e.g., low levels of complement components [140]) immune systems. It has previously been shown that neonatal neutrophils behave abnormally as compared to adult cells with regard to basic functions such as apoptosis and chemotaxis (reviewed in [141, 142]). In the context of priming, term neonatal cord blood neutrophils display an increased NADPH-oxidase derived oxidative response to the chemoattractant N-formylmethionyl-leucyl-phenylalanine (fMLF) as compared to adult cells, provided that the neonate was delivered vaginally [143]. Without labor, i.e., after elective Caesarean section (CS), the neonatal neutrophils did not show this enhanced response [144].

In paper I of this thesis, the focus of investigation has been on neonatal neutrophil responses to galectin-3. As described above, unprimed neutrophils are inert to this lectin whereas primed neutrophils respond with production of NADPH-oxidase derived ROS. Interestingly, in contrast to fMLF-induced ROS production, cord blood neutrophils obtained after elective CS responded to galectin-3 with production of NADPH-oxidase derived ROS, i.e., they were primed as compared to peripheral adult neutrophils, which were inert to galectin-3 in the absence of *in vitro* priming. No difference was found between levels of CD62L on the neutrophil cell surface between adult and elective CS neutrophils. However, CS neutrophils produced elevated levels of IL8 and increased levels of galectin-3 was found in plasma when compared to adults. Comparing the elective CS cord blood neutrophils to neonatal neutrophils obtained from cord blood after vaginal delivery, the latter displayed an even stronger galectin-3-induced NADPH-oxidase derived ROS response, indicating that vaginal delivery renders neonatal neutrophils increasingly primed. The more pronounced primed phenotype in cord blood neutrohils obtained after vaginal delivery was also evident by the finding that these cells more easily shed CD62L from their surface in response to *in vitro* priming. The increased production of IL8 and increased levels of galectin-3 in plasma found in cord blood were, however, independent of mode of delivery as compared to adults (the role for increased levels of plasma galectin-3 in neonates is discussed under "Galectin-3 in plasma – a novel marker of disease" below).

These novel results show that term neonatal neutrophils in the absence of labor display a primed phenotype as compared to adult neutrophils. The primed features are further accentuated after vaginal delivery. The altered neutrophil phenotype in neonates appearing already before labor may be a preparation for the encounter with microbial (maternal and commensal flora) and inflammatory challenges that occur during and after delivery. The increased priming of neutrophils during vaginal delivery may then be in response of the increased infectious challenge under these conditions. Speculatively, the primed neutrophil state in neonates may also help to compensate for the limited function of other parts of the immature immune system.

Galectin-3 – a regulator of inflammation

Specificity in biological interactions cannot be achieved solely by variations in protein code (genes) but is also granted through post-translational modifications of the amino acid sequence, such as addition of sugar moeities (glycans, carbohydrates). Glycans are both abundant and diverse, and are used as modification to increase biological complexity and thus, higher specificity in biological functions. Carbohydrates are part of almost every biological process, and play important roles in many diseases. Almost all cells carry carbohydrates on their cell-surfaces in the form of glycoproteins, glycolipids, and proteoglycans, and the extracellular matrix and bodily fluids contain large amounts of soluble glycoproteins [145].

Most carbohydrates in the cell are linked to proteins or lipids during maturation in the ER/Golgi, and depending on which glycan that is first attached, the glycoconjugates are grouped into different classes. The protein-linked glycans are divided in two groups; N-glycans and O-glycans. In N-glycans the monosaccharide N-acetylglucoseamine (GlcNAc) is attached to asparagine through an amino-glycosidic bond while in O-glycans the monosaccharide N-acetylgalactoseamine (GalNAc) is linked to the hydroxyl group of serine or threonine. The N-glycans can be divided into three groups; high-mannose type, complex type, and hybrid type, all sharing a common core structure but differing in the sugar-chains that are added to this core. Complex and hybrid type N-glycans most often contain N-acetylated lactoseamine (LacNac, Galβ1-4GlcNAc), the core carbohydrate structure that is bound by carbohydrate-binding proteins (lectins) called galectins. The LacNAc can be elongated with tandem repeats of the same sugars, giving a chain of LacNAc, polyLacNAc, onto which terminal fucose, sialic acid, Gal, GalNAc or sulphate is added. Also O-glycans can contain polyLacNAc structures that potentially can be bound by the galectins.

The galectins (also called S-type lectins) are present in fungi, plants and the animal kingdom. To date, 15 mammalian galectins (galectin-1 to -15) have been identified. The galectins are members of a protein family defined by their sequence similarities in the evolutionarily conserved 135 amino acid carbohydrate recognition domain (CRD) with affinity for β-galactosides. The proteins are both found intracellularly and in extracellular compartments, indicating that they may play both regulatory roles within the cell, but also have extracellular functions [146-149]. Endogenous galectins are synthesized on cytosolic ribosomes and are realeased as non-glycosylated proteins into the cytosol. From there they can be targeted to the nucleus or other subcellular sites or be secreted. As galectins lack a signalling sequence necessary for direction into the ER/Golgi network they are not released extracellularly by the classic secretion pathway involving transport vesicles. Instead, secretion of galectins to the extracellular milieu is suggested to occur through an alternative, non-classical secretion pathway (reviewed in [150]) as seen also for proteins such as IL1β and fibroblast growth factors [151].

According to the structural differences and similarities among the galectins, they have been classified into three subgroups; (i) the prototype galectins (galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15), containing one CRD which can occur as a monomer or as dimers, (ii) the tandem repeat galectins (galectin-4, -6, -8, -9 and -12), containing two distinct CRDs connected by a short linker region, thus being bivalent as monomers, and (iii) chimera type galectins (galectin-3), containing one CRD and one non-lectin N-terminal domain, which can occur as a monomer, as dimers, or as higher order oligomers [149, 152] (Figure 3).

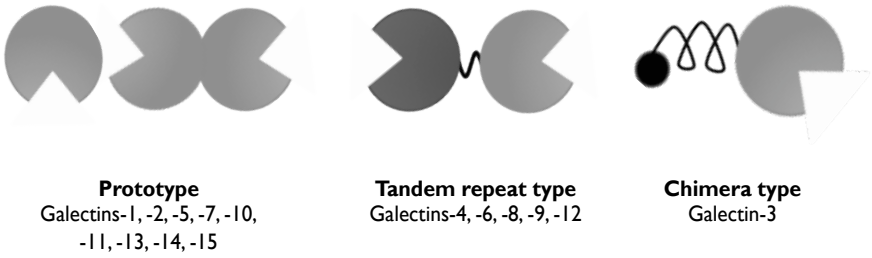

Figure 3. Based on structural differences the galectins are classified into three subgroups. All members of the galectin family share sequence similarities in the carbohydrate recognition domains (CRDs, grey) and dependent on structural similarities and differences the galectins are classified into three subgroups; prototype, composed of one CRD that may form homodimers, tandem repeat type, composed of two distinct CRDs linked together by a polypeptide chain, and chimera type, composed of one CRD linked to a non-lectin N-terminal by a collagen-like domain. This figure is adapted from Leffler *et al* [149].

The galectin carbohydrate recognition domain (CRD)

The galectin CRD structure is highly conserved from an evolutionary perspective; even when comparing the degree of conservation between human and mouse there is approximately 80 % amino acid identity. The CRD is composed of two slightly bent anti-parallel β-sheets with one sheet forming a convex side (consisting of five strands, F1-F5) and the other sheet forming a concave side (consisting of six strands, S1-S6). On the concave side of CRD there is a groove in which the primary binding site for β-galactosides (including lactose, Galβ1-4Glc) is localised. This groove is divided into five sites; A, B, C, D, and E, where sites C-D construct the core binding part for the β-galactoside. Site C is the most conserved site between the different galectins and binds the actual galactose of the β-galactoside. Site D, is less conserved among the galectins and defines a specific saccharide affinity in a distinctive galectin, i.e., different galectins have different affinity for different saccharides. Apart from binding the β-galactoside core structure in site C and D, the CRD can also bind other saccharides in sites A, B, and E, which further defines the fine-tuned specificity of each galectin [149].

Galectin-3

As the only chimera type galectin, galectin-3 (also known as L-29, Mac-2, εbp, L-34, or CPB-35) is unique within the family, comprising a C-terminal CRD linked to a non-lectin N-terminal domain consisting of 18 amino acids linked to a proline-, tyrosine-, and glycine-rich, collagen-like domain with several homologous repeats [149, 153-155]. Galectin-3 was first defined as a surface marker on macrophages [156] but has since then been found to be expressed in many cell types, including activated epithelial cells [157], lymphocytes, dendritic cells, monocytes/macrophages, mast cells, eosinophils and neutrophils (reviewed in [158]). The collagen-like N-terminal domain of galectin-3 can be proteolytically cleaved off by proteases including elastase [159], MMP2 (also referred to as gelatinase A), MMP9 (also referred to as gelatinase B) [160], MMP7 (also referred to as matrilysin-1) [161], bacterial collagenase [153, 162, 163]} and *Leishmania* surface protease leishmanolysin [164], leaving the CRD intact. Neutrophil granules contain elastase, collagenase, and MMPs [38], and subsequently primed neutrophils can cleave galectin-3 into CRD-containing fragments lacking (part of) the N-terminal domain [159], findings corroborated in paper IV of this thesis.

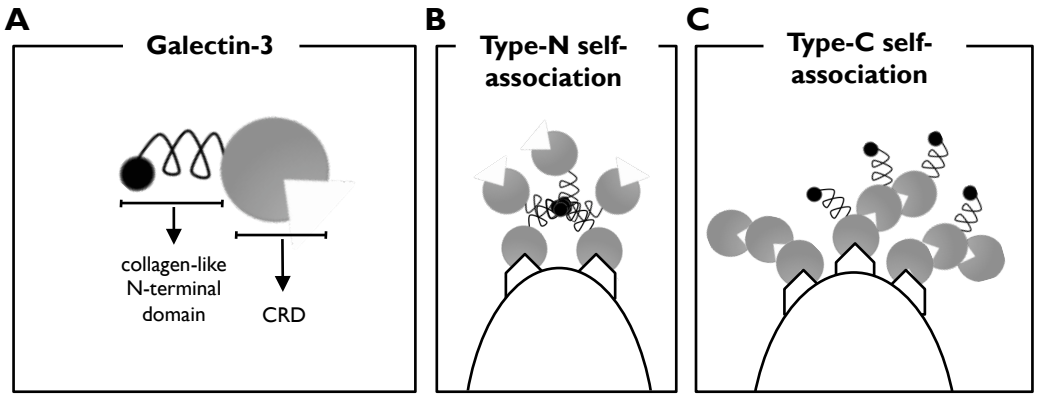

Figure 4. Association between galectin-3 molecules can occur either through the N-terminal domain or the carbohydrate recognition domain (CRD). (A) Galectin-3 is composed of a CRD linked to a collagen-like N-termin domain. (B) Upon binding to its ligand (e.g., a membrane glycoconjugate that contains β-galactosides), galectin-3 can form dimers and higher order oligomers through interactions involving the N-terminal domain, a type of oligomerisation termed type-N self-association. (C) Upon ligand binding, galectin-3 and/or CRD can also form oligomers through interactions involving the CRDs, so-called type-C self-association. This figure is in part adapted form Lepur *et al* [169].

Intermolecular galectin-3 interactions

In solution, both galectin-3 and CRD occurs as monomers [165]. However, upon binding to β-galactoside-containing ligands the lectin starts to oligomerize with other galectin-3 molecules, an association believed to be mediated through interactions involving the N-terminal domain. Such oligomerization may induce (i) cross-linking of surface glycoproteins leading to receptor clustering, which is described to be essential for most galectin-3 induced cellular activities, (ii) cross-linking of galectin-3 molecules bound to the surface of different cells, leading to aggregation of cells, and (iii) formation of multivalent galectin-3 complexes on cell surfaces, often referred to as a galectin-3 lattices [166].

In addition to N-terminal dependent aggregation, there are a few studies describing that galectin-3 can form oligomers also through interactions only involving the CRD [167,

168]. This type of association was recently described in more detail, and the term type-C self-association was launched to distinguish it from the oligomerisation of galectin-3 molecules through the N-terminal domain (type-N self-association) [169]. In type-C self-association, CRD or galectin-3 binds to another galectin-3/CRD molecule through a site other than the β-galactoside recognition site to form dimers and higher order oligomers (Figure 4). In paper IV of this thesis the role of type-C self-association between galectin-3/CRD molecules with regard to neutrophil function has been investigated.

Galectin-3 as a modulator of inflammation

Galectin-3 has been described to be involved in a multitude of biological events, both inside and outside the cell, and carries out an impressive magnitude of functions with regard to inflammatory processes. *In vivo* studies of galectin-3 null mice, as well as mice treated with galectin-3 have been important for understanding the complexity of galectin-3 induced mechanisms in infection and inflammation. Studies of galectin-3 null mice have revealed that these mice display decreased recruitment of phagocytic cells in response to non-infectious [170, 171] and infectious [172] inflammatory agents. However, under standard laboratory conditions these mice display no apparent phenotype and no morphological abnormalities [170], indicating that galectin-3 is not of importance for proper development but for proper immune defence. Further studies of galectin-3 null mice have suggested a proinflammatory role for galectin-3 in allergic airway inflammation [173], atopic dermatitis [174], atherosclerosis [175] and antigen-induced athrithis [176]. However, in infection-induced inflammatory processeses, such as infections with *Streptococcus pneumonia* [177] or *Toxoplasma gondii* [178] galectin-3 has been demonstrated to play a protective role. Data on galectin-3 null mice thus implies that galectin-3 plays a regulatory role during both infection-induced and sterile inflammatory processes.

In vitro studies of the interaction between innate immune cells and galectin-3 show that the lectin has numerous and diverse effects. Galectin-3 promotes neutrophil and monocyte adhesion to laminin, fibronectin and endothelial cells [179-181], indicating that the lectin is important for proper transmigration of phagocytes to inflamed tissue. Further, galectin-3 induces an oxidative burst in monocytes [182] and in primed, but not resting neutrophils (paper I, II and IV of this thesis and [94, 108, 114, 115]).

With regards to galectin-3 induced ROS production, this lectin (together with galectin-1 [183] and galectin-8 [184]) are unique NADPH-oxidase agonists, as they are the only known endogenous agonists acting through a receptor (tentatively identified as CD66a and/or CD66b [185]) that can induce NADPH-oxidase activation both at the plasma membrane (ecROS production) and at intracellular granule membranes (icROS production). Galectin-3 can also function as an opsonin by potentiating phagocytosis and clearance of microbes by neutrophils [177, 186] and red blood cells [187] as well as apoptotic neutrophils [188] by macrophages.

Galectin-3 acts as an inflammatory modulator by influencing cytokine production, e.g., suppressing IL12 production in dendritic cells [178], suppressing IL5 production in eosinophils [189], potentiating LPS-induced production of IL1β in monocytes [190], stimulating IL2 production in T-cells [191], as well as stimulating IL8 production in neutrophils [159].

When it comes to cell longevity, addition of extracellular galectin-3 has been shown to induce apoptosis in T-cells *in vitro*. However, T- and B-cell lines overexpressing intracellular galectin-3 display resistance to proapoptotic stimulation (reviewed in [158]), indicating a dual role for galectin-3 with regards to lymphocyte apoptosis, being proapoptotic once extracellular and antiapoptotic when intracellular.

Galectin-3 in plasma – a novel marker of disease

Galectin-3 is increasingly being used as a serum marker for inflammation, infection, and different pathologies. The galectin-3 concentration in healthy human plasma averages at 12 ng/ml [192, 193], but can increase several-fold during disease [194, 195]. Research on the role of galectin-3 in cancer is presently expanding, and the serum/plasma levels of galectin-3 has been shown not only to be increased in different types of cancer, but also to further increase with tumor aggresiveness and metastasis [196-198]. In patients with heart failure, the serum/plasma levels of galectin-3 are proposed as a novel marker for disease development and progression [199, 200].

In diseases where an inflammatory component is evident as part of the pathology, serum/plasma levels of galectin-3 have been shown to be enhanced. Prominent examples include autoimmune disorders such as systemic lupus erythematosus (SLE) [201], Bechet's disease [202], rheumatoid arthritis [203] and juvenile idiopathic arthrithis (JIA) [204], as well as (auto)inflammatory disorders such as Crohn's disease and ulcerative colitis [205]. Serum concentrations of galectin-3 have also been demonstrated to be elevated in patients with obesity and type 2 diabetes [206]. Interestingly, a recent study demonstrated plasma levels of galectin-3 to be increased during bacterial or fungal sepsis, but not during viral infections nor during autoinflammatory syndromes (gout as well as unspecified diseases) as compared to healthy controls [207]. In paper II of this thesis it is demonstrated that the galectin-3 levels in plasma from patients with the autoinflammatory PFS PFAPA were unaltered, even during the strongly proinflammatory febrile phase. The possibility to exploit this absence in galectin-3 increase as a marker to differentiate PFAPA from other PFS is interesting and merits further studies. Today, the only biomarker described to potentially distinguish PFAPA from other PFS is interferon γ (IFNγ)-induced protein 10 (IP10 [also known as CXCL10], as demonstrated in the appendix to this thesis and corroborated by three other studies [208-210]).

Data in paper I of this thesis demonstrate that plasma obtained from cord blood of term neonates contain increased levels of galectin-3 as compared to adult plasma. The increase was independent of the inflammatory stimulation that a vaginal delivery provides, as cord blood obtained from neonates delivered by vaginal delivery displayed similar galectin-3 amounts as neonates delivered by elective CS. The role of increased galectin-3 in infants as compared to adults can only be speculated upon. Neonates show reduced levels of complement components and complement activation [140, 211]), and possibly, the galectin-3 ability to function as an opsonin [177, 186-188], resembling the function of an activated complement system, could play a compensatory role, providing antimicrobial defence and immunomodulation.

Galectin-3 – neutrophil interactions *in vivo* - A hypothetical model

In episodes of physiological inflammation, i.e., with limited degree of tissue damage and/or infection, where the acute inflammatory response eventually subsides and is resolved, neutrophils may interact with galectin-3 at several time-points. In circulation, galectin-3 promotes the attachment of neutrophils to endothelial cells, thereby facilitating transmigration to extravascular tissue, a process associated with priming of the neutrophils by, e.g., granule mobilization [179-181]. Once in the tissue, approaching the inflammatory focus, the primed neutrophils should be highly responsive to galectin-3 (produced by macrophages [158]) demonstrated to be present at clearly detectable levels in tissues and bodily fluids [203, 212-214]. The encounter with substantial amounts of galectin-3 in the tissue could induce NADPH-oxidase activation in the primed neutrophils, both released to the extracellular mileu, with the risk of damaging surrounding tissue, as well as intracellularly, contributing to regulation of the inflammatory process [215].

In parallel to inducing a neutrophil oxidative burst, the galectin-3 is truncated into CRD by proteases, e.g., from the primed neutrophil [159]. As CRD has been suggested to function as a dominant negative molecule leading to inhibition of galectin-3 induced effects, this may abrogate the galectin-3-induced neutrophil oxidative response (as demonstrated in paper IV of this thesis), protecting the tissue from unneccesary damage by extracellular ROS or to cessation of the radical-induced intracellular signal transduction of which very little is known (Figure 5).

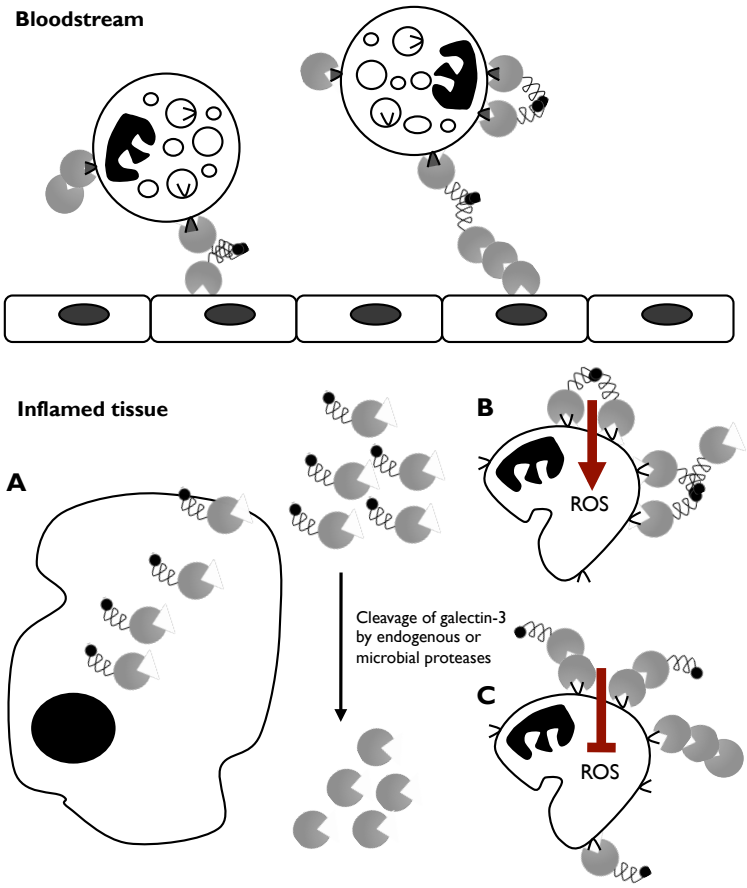

Figure 5. Hypothetical model of galectin-3 interactions with neutrophils in vivo. In blood, a certain amount of both galectin-3 and CRD are attached to the dormant neutrophils. Once the neutrophils become alerted to the tissue (due to tissue damage or microbial invasion) the attached lectins most probably promotes adherence to the endothelial cells by type-N (and type-C) self-association. After transmigration to the tissue, the now primed neutrophils may encounter high concentrations of galectin-3 released by, e.g., macrophages (A) and respond with production of NADPH-oxidase derived ROS (B). In parallel to inducing ROS production, galectin-3 becomes cleaved into CRD by, e.g., proteases released by activated cells and/or microbes. The CRD molecules also bind to neutrophils, but without inducing production of ROS. Instead, neutrophils covered with CRD becomes inert to further galectin-3 induced ROS production (C), perhaps as an endogenous embedded defensive system to protect surrounding tissue from unnecessary damage by extracellular ROS or to cease the ROS induced intracellular signal transduction of which very little is known.

We do not know much about the role of galectin-3 in case of a fullblown infection or a severe aseptic inflammation. Major increases in galectin-3 has been seen in some diseases [201-207] possibly leading to enhanced proinflammatory effects through neutrophil activation, but investigations on the relative abundance of full length galectin-3 and CRD at inflamed sites are lacking. However, pathologically inflamed bronchoalveolar lavage (BAL) fluid from chronic obstructive pulmonary disease (COPD) patients contain significantly less galectin-3 than that from healthy controls [214], suggesting that either galectin-3 production is downregulated in alveolar macrophages and fibroblasts, or a proteolytic of galectin-3 into CRD could be associated with inflammatory pathology. The cleavage of galectin-3 by microbial proteases may be a virulence factor used to terminate the antimicrobial oxidative response in the neutrophils and thereby prolonging an infection. Regardless of mechanism involved, the levels and ratios of galectin-3 and CRD should be further investigated as possible markers for inflammatory diseases.

Autoinflammation

Simplified, autoinflammatory diseases are inflammatory disorders caused by dysregulation of receptors and pathways of the innate immune system. The first definition of the term autoinflammation [216] was closely linked to the identification of causative mutations in the two monogenic PFS FMF [217, 218] and TRAPS [216]. At this point autoinflammatory conditions were defined as "conditions characterized by seemingly unprovoked episodes of inflammation, without high titres of autoantibodies or antigen-specific T-cells" [11]. Since then, several other definitions have been suggested including "clinical disorders marked by abnormally increased inflammation, mediated predominantly by cells and molecules of the innate immune system, with a significant host predisposition" [219] and recently "diseases with clinical signs of inflammation, associated with elevated levels of acute-phase reactants, which are attributable to dysfunction of the innate immune system, genetically determined or triggered by an endogenous factor" [220]. The understanding of the pathophysiology of autoinflammatory diseases and their connection with innate immunity has been closely connected to the improved understanding of the pathophysiology behind the cryopyrin associated periodic syndromes (CAPS, sometimes referred to as inflammasomopathies) with mutation in the NOD receptor NLRP3. Disease-causing mutations in the NLRP3 gene lead to overactivation of the NLRP3 inflammasome, which cleaves pro-IL1α, pro-IL1β and pro-IL18 into biologically active and secreted forms [221, 222]. Overactivation or activation (by endogenous triggers) of the inflammasome is implicated in several autoinflammatory diseases other than CAPS [11].

In contrast to the autoinflammatory disorders, autoimmune disorders are caused by dysfunctions in the adaptive immune system, inducing self-directed sterile inflammation resulting in tissue damage. The autoimmune diseases are characterized by presence of autoantibodies in sera/plasma [11, 15]. There is however no clear demarcation between autoimmune and autoinflammatory diseases and it has been proposed that most diseases can be represented on a spectrum with monogenic autoimmune and autoinflammatory disorders as endpoints (Figure 6).

Patients with autoinflammatory disease most often have lifelong recurrent episodes of inflammation, associated with fever in combination with symptoms such as malaise, arthralgia, abdominal pain, and skin rash. Clinically, the symptoms in patients with autoinflammatory conditions need to be distinguished from infections, malignancies and autoimmune disorders. The re-appearance of flares and the often disease specific combination of signs and symptom are important clues to proper diagnosis. However, in a large proportion of patients with PFS, specific clinical or genetic diagnosis cannot be made [11, 223]. If not diagnosed and adequately treated, patients with autoinflammatory disease may develop amyloid A (AA) amyloidosis, a condition in which the soluble acute phase reactant SAA accumulates and aggregates in tissues/organs. The AA amyloidosis is a serious condition, which can lead to end-stage organ failure if not diagnosed and treated in time [224].

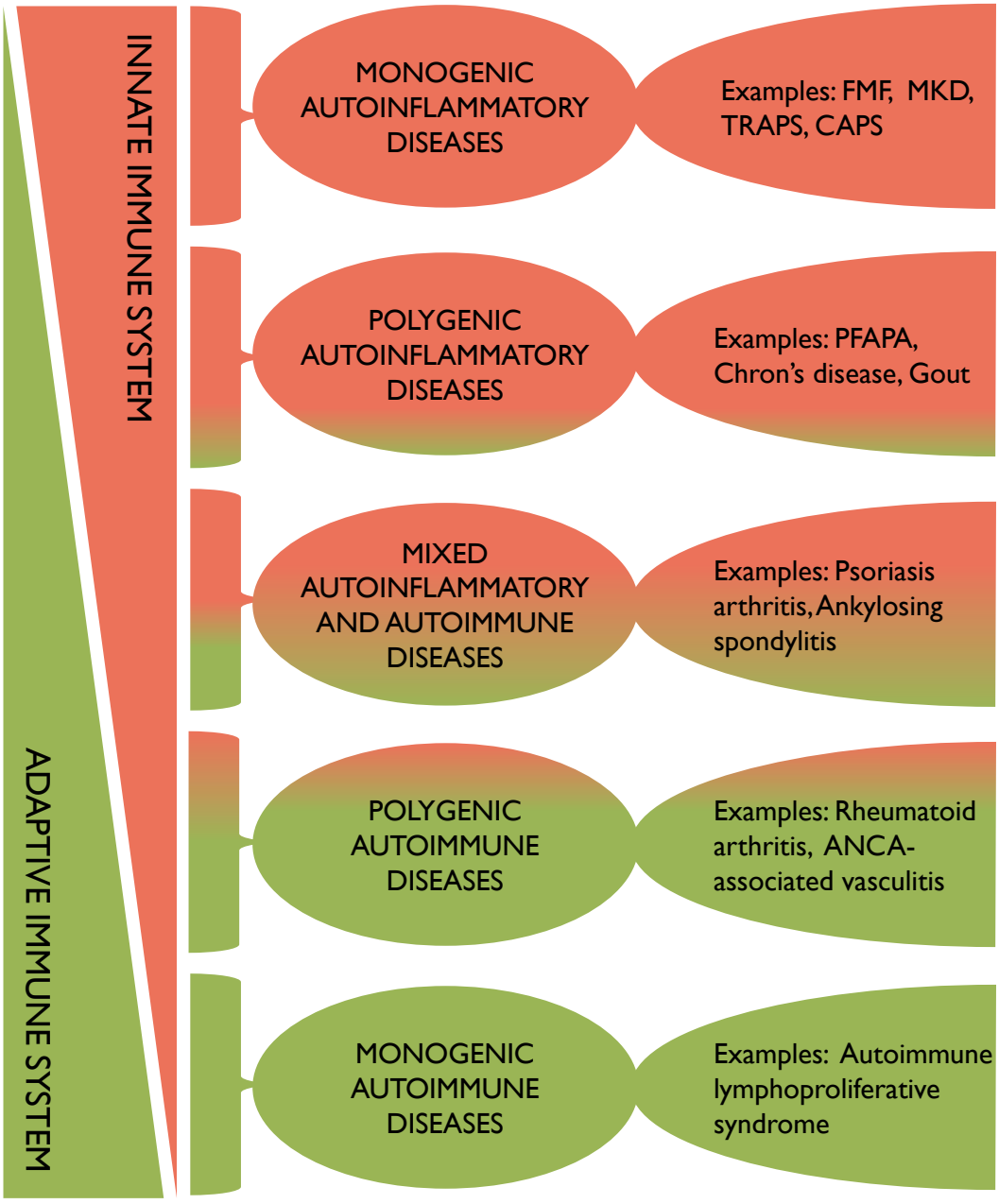

Figure 6. Proposed classification of autoinflammatory and autoimmune diseases. Malfunctions in the immune system may lead to autoinflammatory and autoimmune diseases, i.e., "unwanted" inflammation due to over-activation of the immune system. There is no clear demarcation between autoimmune and autoinflammatory diseases and it has been proposed that most diseases can be represented in a spectrum with monogenic autoimmune and autoinflammatory disorders as endpoints. This figure is in part adapted form McGonagle *et al* [15].

Periodic fever syndromes (PFS)

The PFS are a heterogeneous group of autoinflammatory disorders that typically manifest during childhood as recurrent inflammatory and febrile episodes (flares). The monogenic PFS, in which the genetics have been elucidated include FMF [217, 218], TRAPS [216], mevalonate kinase deficiency (MKD, also referred to hyperimmunoglobulinemia D with periodic fever syndrome [HIDS] [225]), CAPS (which include three phenotypes; familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) [226] and neonatal-onset multisystem inflammatory disease (NOMID, also known as chronic infantile neurological cutaneous articular syndrome [CINCA]) [11, 227]). The characteristics of these syndromes are described in more detail below, however cells isolated from these patients secrete increased amounts of IL1β, indicating that the mutations are directly or indirectly linked to the inflammasome, hence, most of these patients respond well to IL1 blockade [11, 220, 228-230].

FMF is the most commmon monogenic PFS with approximately 100 000 patients in the world today [231-233]. The largest proportion of patients have an origin in the eastern Mediterranean basin. Patients with FMF get recurrent attacks of fever with a duration of 12 - 72 hours, associated with abdominal pain or other manifestations such as chest pain and acute arthritis. In addition, many patients have rheumatic problems (e.g. chronic arthritis, and/or myalgia). FMF is inherited as an AR disease in typical cases, and all identified mutations are located in the Mediterranean Fever (*MEFV*) gene, which encodes pyrin [11, 217, 218, 234], a protein that has recently been discovered to form a NLRP3-independent inflammasome [235]. A significant proportion of patients are heterozygote and some patients lack mutation [236]. In these cases the patients have to be diagnosed solely on clinical grounds supported by clinical criteria [234]. The first choice of treatment for patients with FMF is colcichine, however a small proportion of patients do not respond to colcichine treatment and in these IL1 blockade can be an effective alternative [230].

The other monogenic PFS are rare diseases. Patients with TRAPS succumb to reccurent episodes of fever, migratory erythema, myalgia and conjunctivitis lasting for > 7 days [11, 229, 230, 234]. TRAPS is inherited as an autosomal dominant disease, caused by mutations in the gene *TNFRSF1A*, which encodes TNF receptor 1 (TNFR1) [216]. The mutation leads to misfolding of TNFR1, which then aggregates, prohibiting traffic through the ER/Golgi network to the plasma membrane. Trapping of TNFR1 in the ER triggers stress signals that activate proinflammatory cytokine production including IL1β [11, 228, 237]. Patients with TRAPS most often respond well to IL1 blockade or TNF inhibition [230]. Patients with MKD suffer from recurrent episodes of headache, fever, lymphadenopathy, abdominal pain and skin rash lasting for 3 – 7 days. The disease is inherited in an AR manner and the responsible gene (*MVK*) encodes the enzyme mevalonate kinase (MK), involved in the isoprenoid pathway. Patients with MKD may benefit from IL1 blockade or TNF inhibition [11, 229, 230]. The CAPS patients display symptoms such as fever, urticarial-like skin rash, conjunctivitis and various degrees of joint pain and neurological involvement. As described above, CAPS include three phenotypes (from mild to severe); FCAS, which present as recurrent cold-induced flares lasting < 24 hours, MWS, were patients succumb to recurrent flares lasting for 1 - 3 days (some

patients also develop hearing loss due to chronic inflammation of the inner ear) and NOMID characterised by continuous symptoms that apart from those described above include asepetic meningitis, mental impairment, malformations and arthropathy which present already during the first weeks of life. CAPS are due to autosomal dominant inherited (FCAS and MWS) or sporadic mutations (NOMID) in the gene *NLRP3*, thus, IL1 blockade is the standard treatment for all patients with CAPS [11, 229, 230, 234].

Interestingly, a novel autoinflammatory disease has been described where mutations are found in the IL1 receptor gene (*IL1RN*), termed deficiency in IL1 receptor antagonist (DIRA) [238, 239]. Patients with DIRA suffer from multifocal osteomyelitis, periostitis and pustulosis, however, fever is often low grade or can be absent [230]. IL1 receptor antagonist (IL1Ra) is an important endogenous IL-1 inhibitor controlling the many immunomodulatory effects induced by IL1 [240], i.e., cells from patients with DIRA (lacking IL1Ra) are hyper-responsive to IL1. Also patients with DIRA benefit from IL1 blockade, illustrating that the balance betwen endogenous inhibition and production of this cytokine is of great importance. Endogenous over-production of IL1 results in disease (e.g., CAPS) and endogenous lack of inhibition of IL1 results in disease (DIRA).

The most common autoinflammatory PFS in children outside the eastern Mediterranean basin is the non-mendelian polygenic disease PFAPA syndrome [11] which has been the focus of paper II and the appendix to this thesis.

Periodic fever, Aphthous stomatitis, Pharyngitis and cervical Adenitis (PFAPA)

PFAPA is an autoinflammatory PFS of unknown etiology that primarily affects pre-school children. It is regarded as a non-mendelian polygenic disease, however, clinical observations have revealed that in a few of the patients one of the parents or another relative have had symptoms similar to PFAPA in childhood [11, 241]. The incidence of PFAPA has been estimated to 2.3 / 10 000 children up to five years of age [242] and it is said that every pediatrician is likely to encounter at least one case of PFAPA during his/her career [243].

Clinical features and diagnosis of PFAPA

PFAPA was originally described in 1987 [244] and the disease acronym together with the disease criteria was coined two years later [245]. Five clinical criteria needs to be fulfilled for diagnosis and these include (i) onset of disease in early childhood (< 5 years of age), (ii) regulatory recurring episodes of fever lasting for approximately 3 - 5 days associated with a) symptoms of at least one of the clinical signs: aphthous stomatitis and/or pharyngitis and/or cervical lymphadenitis, in the absence of upper respiratory tract infection, and b) elevated acute phase markers such as erythrocyte sedimentation rate (ESR) and/or leukocytosis, (iii) completely asymptomatic clinical phenotype between febrile episodes (usually 3 - 5 weeks), benign long term course and normal growth and development, (iv) exclusion of cyclic neutropenia (an autosomal dominant inherited disease characterized by periodically reccurring episodes of neutropenia [recurring approximately every third week and lasting 3 – 5 days] which cause infections, usually in the respiratory tract, that induce fever), and (v) exclusion of other PFS, autoimmune disorders, chronic infections and immunodeficiencies. Another prominent feature of the PFAPA attacks is the often striking clockwork periodicity with which they occur [11, 246, 247].

Treatment of PFAPA

The recurrent flares in PFAPA syndrome have a major impact not only on the patient's life but also on the daily life of the entire family, and symptom relief is thus an important goal in the caring for these patients. NSAIDs are the most effective agents to reduce the symptoms during the febrile episodes and can be combined with paracetamol [247]. Also steroids are used in some countries but its use is restrictive in Sweden due to the potential side effects. However, steroids are highly effective to treat an episode as they abort the symptoms within hours in most patients (and this rapid response to steroids is sometimes included as a diagnostic tool), yet it tends to reduce the length of the symptom-free intervals [11, 248]. One study has indicated that IL1 blockade (Anakinra) can be used to treat PFAPA flares, however, the effectiveness of this treatment is hard to elucidate as only one dose of what is normally a two-dose per day regime was given [208]. As may be expected (due to the short half-life of Anakinra), one dose did not fully resolve the flare in the patients (3 out of 5 PFAPA patients responded).

So far, the only treatment that has been correlated with complete remission of PFAPA episodes is tonsillectomy, resulting in resolution of the disease in most patients [249-251]. The mechanisms behind this effect are not known; histology of tonsils from PFAPA patients do not show signs of chronic inflammation [252]. Speculatively, the tonsils might contain something that triggers the flares in predisposed patients. The trigger could be of microbial origin, and if so, of viral rather than bacterial origin as the patients do not show increased levels of procalcitonin (upregulated in bacterial, but not viral infections [24], appendix to this thesis) or galectin-3 (upregulated in bacterial and fungal but not viral infections [207], paper II of this thesis). The question whether PFAPA is an infectious disease or an immune dysregulation thus remains open. However, the duration of PFAPA for years without progression, and absence of clustering due to season or in geographic areas as well as lack of second cases in siblings or other close contacts, speak against infection unless exposure to an unusual environmental factor is required [253].

As described above, not all PFAPA patients benefit from tonsillectomy, and in general the PFAPA attacks cease (irrespective of treatment options) in average around 4 - 5 years after the first attack [246, 254]. The parents and physicians must therefore weigh the risks and consequences of surgery (e.g., hospitalizations, pain, postoperative time away from school/nursery) against the alternative of a period with PFAPA episodes potentially requiring regular medication and time away from school/nursery until the disease ceases by itself. However, even though it "is said" that the disease ceases a couple of years after the first attack, many of the patients continue to have attacks several years after "recovery", although of a milder kind, and most probably thereby not always reported [255].

PFAPA biomarkers

Most scientific reports regarding PFAPA are clinical descriptions of patients with laboratory findings obtained during febrile episodes, demonstrating leukocytosis (with neutrophilia), elevated ESR [246, 248, 254], and elevated levels of the acute phase reactants CRP [256] and SAA [234], i.e., factors indicative of an acute inflammatory reaction. Since PFAPA is a common and poorly understood recurrent fever syndrome in children there is a need for better insight into the disease pathophysiology. As the PFAPA diagnosis in part is based on exclusion of other PFS, infections, and cyclic neutropenia, biomarkers differentiating PFAPA from the other disorders would be very useful in clinical practice.

All monogenic PFS seems to be more or less IL1β-driven, shown by increased production of IL1β [11, 220, 228-230] and clinical response to IL1 blockade [11, 229, 230]. Indications that PFAPA is an IL1β-driven disease are scarce, with so far only one study that suggest a clinical response to IL1 blockade in a few patients [208]. Indeed, this study demonstrated increased levels of IL1β mRNA, however no increased levels of IL1β protein was found, corresponding to the results described in the appendix to this thesis as well as a study by Kolly *et al* [209]. The lack of IL1β in circulation could be due to the difficulty to measure this cytokine in serum (reviewed in [257]). Nevertheless, isolated PBMC from febrile PFAPA patients do produce large amounts of IL1β in response to LPS demonstrated in the appendix to this thesis [25] and by Kolly *et al* [209]. Taken together, the actual role of IL1β in PFAPA pathogenesis is left an unsettled issue that needs to be further evaluated.

As demonstrated in the appendix to this thesis, the serum concentration of IP10, a chemoattractant for activated T-lymphocytes [258], is increased during febrile episodes of PFAPA. This finding has been verified by three other studies [208-210] in which one showed the increase to be specific for PFAPA flares as compared to flares of other PFS [208], and another described the levels to be increased also during the afebrile intervals [210].

A specific group of proteins, the S100 proteins, including the phagocyte-specific S100A8/A9 and S100A12 (also referred to as calgranulins), have been suggested as markers for autoinflammatory diseases [259]. S100A8/A9 and S100A12 are cytoplasmic DAMPs that are released from innate immune cells. Exactly how this secretion occurs is unknown, but studies have suggested both active release by non-classical secretion pathways and passive release by necrosis/NET formation [259]. Once released from activated or damaged phagocytes to the extracellular environment, S100A8/A9 proteins can activate monocytes to produce cytokines, including IL1β [260]. In a study by Kolly *et al* it was demonstrated that S100A8/A9 and S100A12 are elevated in serum from febrile PFAPA patients [209], and we corroborate this finding in paper II of this thesis, extending it to show that neutrophils in the circulation of febrile PFAPA patients are primed, suggesting that neutrophil activation and subsequent secretion of S100 proteins may be an initiating factor for monocyte production of proinflammatory cytokines such as IL1β. However, the levels of S100A8/A9 and S100A12 in febrile PFAPA patients were not different from those in control patients with fever, indicating that elevations of these proteins correlate with fever rather than with PFAPA or autoinflammation.

In the search for specific PFAPA biomarkers, data in paper III of this thesis show that the circulating levels of galectin-3 are not increased in PFAPA, which could have been expected due to the strong inflammatory onset during PFAPA flares, as shown for other (auto)inflammatory diseases such as Crohn's disease and ulcerative colitis [205]. Whether absence of increased galectin-3 levels could be used to distinguish PFAPA from other PFS (possibly showing increased levels) remains to be investigated.

PFAPA neutrophils

Neutrophils have, although being the most abundant leukocytes in circulation, gained little focus in the autoinflammatory field, even though autoinflammatory diseases per definition are driven by cells of the innate immune system. The focus has instead been on monocytes/macrophages, which indeed produce vast amounts of proinflammatory cytokines that can drive disease. In the study by Stojanov *et al* [208] it was demonstrated that whole blood gene expression patterns could distinguish PFAPA flares from other PFS flares, indicating that the environment in the blood during a flare is different in PFAPA compared to other PFS. This led to our hypothesis that circulating PMN, which are increased during PFAPA flares (appendix and [208, 209]), and are highly responsive to their environment, would have a unique phenotype in PFAPA. In paper II of this thesis, basic functionality of neutrophils in PFAPA patients have been investigated, both during the afebrile period and in the beginning of a febrile episode, in comparison to afebrile and febrile pediatric controls.

Neutrophil apoptosis in PFAPA

The results obtained in paper II demonstrate that both spontaneous and stimulus-modified apoptosis in PFAPA neutrophils are altered. Rates of spontaneous apoptosis increase during afebrile periods and decrease during flares. Speculatively, the low rates of apoptosis during flares, in association with increased neutrophil counts, likely ensure that sufficient numbers of leukocytes are available for maintenance of the inflammatory response, while a propensity to undergo apoptosis during the afebrile phase may encourage the clearance of excess leukocytes and potentially assist in termination of the inflammatory process. Reduced apoptosis has been associated with inflammation in severe sepsis, possibly as a strategy to ensure the presence of enough phagocytes to fight invading pathogens [261, 262]. However, the febrile controls used in the study, which had fever due to low grade infection or aseptic inflammation, displayed no signs of decreased neutrophil apoptosis, suggesting that fever *per se* is not associated with changes in apoptotic rates.

The most likely mechanism behind the decreased neutrophil apoptosis during PFAPA flares is the presence of antiapoptotic factors in serum, and IL1β would be a prominent candidate. IL1β has been shown to display antiapoptotic properties [124, 263, 264] and increased levels of IL1β mRNA (however not IL1β protein) have been demonstrated during PFAPA flares [208]. Increased IL1β levels in plasma have been detected in patients with genetic variations in the inflammasome, correlating with decreased rates of neutrophil apoptosis [263]. However, treatment with Anakinra did not reverse this antiapoptotic effect [263], indicating that IL1β was not the driving force in apoptotic regulation of neutrophils. While the report by Stojanov *et al* suggest a clinical response to Anakinra in treating PFAPA flares, providing evidence that IL1β may be involved in PFAPA pathogenesis, it seems unlikely that IL1β is solely responsible for the lowered rates of spontaneous apoptosis in PMN during PFAPA flares. Other proinflammatory cytokines that are increased during PFAPA flares are known to regulate apoptosis, e.g., G-CSF and IL18 [125, 208, 265], which are key candidates to investigate in future studies.

Neutrophil production of ROS in PFAPA

Data presented in paper II of this thesis for the first time demonstrate that a propensity to produce elevated levels of (nonphagosomal) intracellular NADPH-oxidase derived ROS, can be correlated to inflammation. Neutrophils from febrile PFAPA patients displayed increased icROS production when stimulated as compared to febrile controls and afebrile PFAPA patients, which showed no alteration in this parameter. As described above, and in contrast to the findings in PFAPA, a specific decrease or deficiency in neutrophil icROS production has been linked with the inflammatory conditions in SAPHO syndrome [81] and a novel form of CGD [71]. It thus seems as if a balanced production of NADPH-oxidase derived icROS is a prerequisite for regulation of cellular inflammatory pathways, where deviation from normality can result in inflammatory disease.

The fact that PFAPA neutrophils are primed in circulation during fever flares (see "Neutrophil priming in PFAPA" below) could potentially affect the production of icROS, especially if induced by a receptor-dependent agonist that binds to mobilizable receptors. However, the data in paper II were obtained using the receptor-independent agonist phorbol 12-myristate 13-acetate (PMA) that directly stimulates protein kinase C (PKC), excluding receptor mobilization as explanation for the enhanced icROS production.

Instead, modulations in signal transduction pathways that activate the NADPH-oxidase specifically in granule (and not plasma) membranes may be at play in PFAPA. Signalling that leads to icROS and ecROS production differ regarding the involvement of PKC isozymes, intracellular calcium, phospholipase D, and cytoskeletal stabilization [43]. The phosphatidylinositide composition also differs in the respective membranes in which the oxidase assembles. Whether the increase in icROS is mechanistically associated with increased cytokine production, can only be speculated upon. The two parameters may relate in a causal manner, but may also be induced in parallel by upstream alterations in inflammatory regulation molecules, features that need to be investigated in future studies.

Neutrophil priming in PFAPA

The priming status of PFAPA neutrophils was also investigated, and the results demonstrate that during a PFAPA flare patients display primed neutrophils in circulation, shown by increased CD11b expression and responsiveness to galectin-3. Also during FMF flares, neutrophils have been demonstrated to express increased CD11b [101]. In line with the decreased rates of spontaneous apoptosis, the induction of priming in circulation suggests that the cells have been exposed to proinflammatory cytokines/chemokines triggering mobilization of granules and upregulation of granule-localized receptors. Interestingly, increased expression of CD11b was seen both in febrile PFAPA and febrile control neutrophils, while the galectin-3 responsiveness showed up only in the febrile PFAPA neutrophils, suggesting that CD11b exposure appears in a less primed phenoype while galectin-3-responsiveness demands a slightly more primed phenotype, seen only during a PFAPA flare and not in febrile controls. Another explanation could be that the priming of the galectin-3 response is influenced also by other mechanisms in addition to degranulation and receptor upregulation. Galectin-3-responsiveness may thus potentially be a differentiating marker for PFAPA flares in comparison to fever of other cause.

Final comments

As described, ROS are crucial in our defence against microbes, evidenced by that patients with CGD (lacking NADPH-oxidase derived ROS) suffer from severe infections. However, CGD patients also suffer from inflammatory conditions, and cells from these patients produce elevated levels of proinflammatory cytokines, suggesting that ROS dampen proinflammatory signalling, i.e., a lack of NADPH-oxidase derived ROS is associated with inflammation (paper III). Excitingly, in this thesis also the opposite is described, as neutrophils obtained from patients with PFAPA produce elevated levels of NADPH-oxidase derived icROS during a disease flare, i.e., also elevated levels of ROS can be associated with inflammation (paper II). How does this work? Is it simply so that alterations in ROS (NADPH-oxidase derived) result in a redox imbalance and that this imbalance triggers inflammatory signalling?

In terms of cell biology, redox reactions are most often discussed in the context of "cellular oxidative stress", i.e., an altered homeostasis caused by augmented production of ROS or reduced antioxidative defence, which can cause damage both on a cellular, tissue, and host level. Only recently it became evident that redox reactions are imperative for controlled signal transduction events that support desirable biological outcomes. In particular, studies have illuminated an imperative role of ROS in promoting inflammatory cytokine production. One would have guessed that if anything, CGD phagocytes would display decreased oxidative stress. However, CGD phagocytes surprisingly show clear signs of being under oxidative stress including increased expression of cellular antioxidants (paper III). Clearly, cellular ROS other than those derived from the NADPH-oxidase must be at play and data in paper III suggests mtROS as plausible candidates to explain the redox imbalance of CGD cells. This could mean that part of the pathology for a disease considered to be due to "lack of ROS" is in fact due to "excess ROS", which is a challenging thought for the established scientific community. Exactly how lack of NADPH-oxidase derived ROS could lead to increased mtROS in CGD cells is hard to say, but given the extremely complex interactions that control cellular redox balance, the concept is not implausible. A thought-provoking idea would be to treat the inflammatory symptoms in CGD patients with antioxidants. There are specific mitochondria-directed antioxidants available, however, my experience working with these substances *in vitro* is that they rarely function as expected. Similar to CGD leukocytes, leukocytes from PFAPA patients produce elevated levels of cytokines and their neutrophils also display increased mtROS as compared to neutrophils from healthy controls (paper II). It might well be that mtROS contribute to driving inflammation also in PFAPA and that antioxidant treatment could be an option also for these patients. As PFAPA neutrophils display increased NADPH-oxidase derived icROS during flares, i.e., the complete opposite of CGD neutrophils, it is utterly intriguing how "lack of icROS" and "excess icROS" both lead to increased mtROS and hyper-inflammation.

The traditional way in which neutrophils become primed is through transmigration from blood to a site of inflammation in the tissue, i.e., a spatially restricted setting in which the hyper-responsive, primed neutrophils are localised. Presence of primed neutrophils in circulation due to severe infections and/or inflammatory conditions most likely contribute to inflammatory pathology, e.g., by that these cells display augmented binding to the

blood vessels, which in turn can lead to formation of neutrophil aggregates releasing antimicrobial substances that can harm the surrounding tissue. In this thesis, two settings in which neutrophils display signs of priming in circulation are presented; the cord blood of term neonates (paper I) and the flares of PFAPA (paper II). During flares of PFAPA, the neutrophils that most likely have become primed due to the high levels of proinflammatory mediators present in the blood of these patients probably contribute to the inflammatory pathology. However, in term neonates, born with an immature immune system, and thereby susceptible to infections, primed neutrophils in the blood might compensate for other immune mechanisms that the neonate is lacking, i.e., this feature could be protective for the host. If neutrophils are being continually primed in circulation over time periods longer than the turn-over rate, one might wonder how and when this feature changes from being protective during the neonatal period (our hypothesis) to being hostile during later stages of life. It would be interesting to perform a follow-up study of the time-frame during which the neonates display primed neutrophils in circulation (hours/days/weeks/months after birth).

As described, galectin-3 levels are clearly detectable in plasma of healthy humans, hence it is not that surprising that circulating leukocytes have galectin-3 attached to their cell surface in circulation (paper IV). It would be interesting to investigate if the level of leukocyte-attached galectin-3 is increased during pathologies where the levels are increased in plasma. As both bacterial and neutrophil-related proteases can cleave galectin-3 into CRD, one might also wonder how the ratios between full-length and truncated lectin would appear in a disease where neutrophils are primed in circulation and/or where invasive microbes are present. One could speculate that in sterile inflammation, where endogenous proteases (e.g., released by primed neutrophils) cleave galectin-3 into CRD, presence of CRD that attaches the neutrophil surface is in favour for the host by inhibiting "over-production" of galectin-3 induced NADPH-oxidase derived ROS (paper IV). However in infection-associated inflammation it might be that microbial proteases cleave galectin-3 into CRD as a virulence factor to escape the antimicrobial affects of galectin-3 induced NADPH-oxidase derived ROS.

The work presented in this thesis demonstrates alterations in basic neutrophils functions with main focus on ROS production, priming and interactions with galectin-3 during different settings of health and disease. It is well known that the numbers of neutrophils increases in blood during most infectious and/or inflammatory conditions, however that the phenotype of these blood neutrophils also might change is less explored. As neutrophils are the most abundant cells in blood, small changes in their phenotype could have a major impact on the outcome of a disease.

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References

1. Beutler, B. (2004) Innate immunity: an overview. Mol. Immunol. 40, 845-59.
2. Hoebe, K., Janssen, E., Beutler, B. (2004) The interface between innate and adaptive immunity. Nature immunology 5, 971-4.
3. Celsus, A. C. De Medicina. Self published A.D. 25.
4. Tracy, R. P. (2006) The five cardinal signs of inflammation: Calor, Dolor, Rubor, Tumor ... and Penuria (Apologies to Aulus Cornelius Celsus, De medicina, c. A.D. 25). J. Gerontol. A. Biol. Sci. Med. Sci. 61, 1051-2.
5. Bianchi, M. E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukoc. Biol. 81, 1-5.
6. Matzinger, P. (1994) Tolerance, danger, and the extended family. Annu. Rev. Immunol. 12, 991-1045.
7. Borregaard, N. (2010) Neutrophils, from marrow to microbes. Immunity 33, 657-70.
8. Inohara, N. and Nunez, G. (2003) NODs: intracellular proteins involved in inflammation and apoptosis. Nature reviews. Immunology 3, 371-82.
9. Schroder, K. and Tschopp, J. (2010) The inflammasomes. Cell 140, 821-32.
10. Lamkanfi, M. and Dixit, V. M. (2012) Inflammasomes and their roles in health and disease. Annu. Rev. Cell Dev. Biol. 28, 137-61.
11. Masters, S. L., Simon, A., Aksentijevich, I., Kastner, D. L. (2009) Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease. Annu. Rev. Immunol. 27, 621-68.
12. Serhan, C. N. and Savill, J. (2005) Resolution of inflammation: the beginning programs the end. Nature immunology 6, 1191-7.
13. Zhang, M. J. and Spite, M. (2012) Resolvins: anti-inflammatory and proresolving mediators derived from omega-3 polyunsaturated fatty acids. Annu. Rev. Nutr. 32, 203-27.
14. Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R., Girkontaite, I. (1997) Immunosuppressive effects of apoptotic cells. Nature 390, 350-1.
15. McGonagle, D. and McDermott, M. F. (2006) A proposed classification of the immunological diseases. PLoS medicine 3, e297.
16. Shah, C., Hari-Dass, R., Raynes, J. G. (2006) Serum amyloid A is an innate immune opsonin for Gram-negative bacteria. Blood 108, 1751-7.
17. Christenson, K., Björkman, L., Ahlin, S., Olsson, M., Sjöholm, K., Karlsson, A., Bylund, J. (2013) Endogenous Acute Phase Serum Amyloid A Lacks Pro-Inflammatory Activity, Contrasting the Two Recombinant Variants That Activate Human Neutrophils through Different Receptors. Frontiers in immunology 4, 92.
18. Volanakis, J. E. (2001) Human C-reactive protein: expression, structure, and function. Mol. Immunol. 38, 189-97.
19. Ryan, G. B. and Majno, G. (1977) Acute inflammation. A review. Am. J. Pathol. 86, 183-276.
20. Li, S., Ballou, L. R., Morham, S. G., Blatteis, C. M. (2001) Cyclooxygenase-2 mediates the febrile response of mice to interleukin-1beta. Brain Res. 910, 163-73.
21. Dinarello, C. A., Cannon, J. G., Wolff, S. M., Bernheim, H. A., Beutler, B., Cerami, A., Figari, I. S., Palladino, M. A., Jr., O'Connor, J. V. (1986) Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. 163, 1433-50.
22. Dinarello, C. A., Cannon, J. G., Mancilla, J., Bishai, I., Lees, J., Coceani, F. (1991) Interleukin-6 as an endogenous pyrogen: induction of prostaglandin E2 in brain but not in peripheral blood mononuclear cells. Brain Res. 562, 199-206.
23. Dinarello, C. A. (2004) Infection, fever, and exogenous and endogenous pyrogens: some concepts have changed. Journal of endotoxin research 10, 201-22.
24. Schuetz, P., Albrich, W., Mueller, B. (2011) Procalcitonin for diagnosis of infection and guide to antibiotic decisions: past, present and future. BMC medicine 9, 107.
25. Brown, K. L., Wekell, P., Osla, V., Sundqvist, M., Sävman, K., Fasth, A., Karlsson, A., Berg, S. (2010) Profile of blood cells and inflammatory mediators in periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome. BMC pediatrics 10, 65.
26. Athens, J. W., Raab, S. O., Haab, O. P., Mauer, A. M., Ashenbrucker, H., Cartwright, G. E., Wintrobe, M. M. (1961) Leukokinetic studies. III. The distribution of granulocytes in

the blood of normal subjects. J. Clin. Invest. 40, 159-64.
27. Summers, C., Rankin, S. M., Condliffe, A. M., Singh, N., Peters, A. M., Chilvers, E. R. (2010) Neutrophil kinetics in health and disease. Trends in immunology 31, 318-24.
28. Dotta, L., Tassone, L., Badolato, R. (2011) Clinical and genetic features of Warts, Hypogammaglobulinemia, Infections and Myelokathexis (WHIM) syndrome. Current molecular medicine 11, 317-25.
29. Zeidler, C., Germeshausen, M., Klein, C., Welte, K. (2009) Clinical implications of ELA2-, HAX1-, and G-CSF-receptor (CSF3R) mutations in severe congenital neutropenia. Br. J. Haematol. 144, 459-67.
30. Liu, F., Wu, H. Y., Wesselschmidt, R., Kornaga, T., Link, D. C. (1996) Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice. Immunity 5, 491-501.
31. Lieschke, G. J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., Fowler, K. J., Basu, S., Zhan, Y. F., Dunn, A. R. (1994) Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood 84, 1737-46.
32. Bainton, D. F., Ullyot, J. L., Farquhar, M. G. (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J. Exp. Med. 134, 907-34.
33. Borregaard, N., Kjeldsen, L., Rygaard, K., Bastholm, L., Nielsen, M. H., Sengeløv, H., Bjerrum, O. W., Johnsen, A. H. (1992) Stimulus-dependent secretion of plasma proteins from human neutrophils. J. Clin. Invest. 90, 86-96.
34. Borregaard, N. and Cowland, J. B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 89, 3503-21.
35. Day, R. B. and Link, D. C. (2012) Regulation of neutrophil trafficking from the bone marrow. Cell. Mol. Life Sci. 69, 1415-23.
36. Le Cabec, V., Cowland, J. B., Calafat, J., Borregaard, N. (1996) Targeting of proteins to granule subsets is determined by timing and not by sorting: The specific granule protein NGAL is localized to azurophil granules when expressed in HL-60 cells. Proc. Natl. Acad. Sci. U. S. A. 93, 6454-7.
37. Damiano, V. V., Kucich, U., Murer, E., Laudenslager, N., Weinbaum, G. (1988) Ultrastructural quantitation of peroxidase- and elastase-containing granules in human neutrophils. Am. J. Pathol. 131, 235-45.
38. Faurschou, M. and Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. Microbes and infection / Institut Pasteur 5, 1317-27.
39. Rørvig, S., Østergaard, O., Heegaard, N. H., Borregaard, N. (2013) Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. J. Leukoc. Biol. 94, 711-21.
40. Sengeløv, H., Follin, P., Kjeldsen, L., Lollike, K., Dahlgren, C., Borregaard, N. (1995) Mobilization of granules and secretory vesicles during in vivo exudation of human neutrophils. J. Immunol. 154, 4157-65.
41. Nordenfelt, P. and Tapper, H. (2011) Phagosome dynamics during phagocytosis by neutrophils. J. Leukoc. Biol. 90, 271-84.
42. Nauseef, W. M. (2007) How human neutrophils kill and degrade microbes: an integrated view. Immunol. Rev. 219, 88-102.
43. Bylund, J., Brown, K. L., Movitz, C., Dahlgren, C., Karlsson, A. (2010) Intracellular generation of superoxide by the phagocyte NADPH oxidase: How, where, and what for? Free Radic. Biol. Med. 49, 1834-1845.
44. Dahlgren, C. and Karlsson, A. (1999) Respiratory burst in human neutrophils. J. Immunol. Methods 232, 3-14.
45. Sundqvist, M., Osla, V., Jacobsson, B., Rudin, A., Sävman, K., Karlsson, A. (2013) Cord blood neutrophils display a galectin-3 responsive phenotype accentuated by vaginal delivery. BMC pediatrics 13, 128.
46. Sundqvist, M., Wekell, P., Osla, V., Bylund, J., Christenson, K., Sävman, K., Foell, D., Cabral, D. A., Fasth, A., Berg, S., Brown, K. L., Karlsson, A. (2013) Increased intracellular oxygen radical production in neutrophils during febrile episodes of periodic Fever, aphthous stomatitis, pharyngitis, and cervical adenitis syndrome. Arthritis Rheum. 65, 2971-83.
47. Nauseef, W. M. (2008) Nox enzymes in immune cells. Seminars in immunopathology 30, 195-208.
48. Brown, D. I. and Griendling, K. K. (2009) Nox proteins in signal transduction. Free Radic. Biol. Med. 47, 1239-53.
49. Novo, E. and Parola, M. (2008) Redox mechanisms in hepatic chronic wound healing and fibrogenesis. Fibrogenesis Tissue Repair 1, 5.

50. Bonekamp, N. A., Volkl, A., Fahimi, H. D., Schrader, M. (2009) Reactive oxygen species and peroxisomes: struggling for balance. Biofactors 35, 346-55.
51. Fossati, G., Moulding, D. A., Spiller, D. G., Moots, R. J., White, M. R., Edwards, S. W. (2003) The mitochondrial network of human neutrophils: role in chemotaxis, phagocytosis, respiratory burst activation, and commitment to apoptosis. J. Immunol. 170, 1964-72.
52. Zhou, R., Yazdi, A. S., Menu, P., Tschopp, J. (2011) A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221-5.
53. Bauernfeind, F., Ablasser, A., Bartok, E., Kim, S., Schmid-Burgk, J., Cavlar, T., Hornung, V. (2011) Inflammasomes: current understanding and open questions. Cell. Mol. Life Sci. 68, 765-83.
54. Martinon, F. (2010) Signaling by ROS drives inflammasome activation. Eur. J. Immunol. 40, 616-9.
55. Gloire, G., Legrand-Poels, S., Piette, J. (2006) NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem. Pharmacol. 72, 1493-505.
56. Nordberg, J. and Arner, E. S. (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic. Biol. Med. 31, 1287-312.
57. Borregaard, N., Heiple, J. M., Simons, E. R., Clark, R. A. (1983) Subcellular localization of the b-cytochrome component of the human neutrophil microbicidal oxidase: translocation during activation. J. Cell Biol. 97, 52-61.
58. Sengeløv, H., Nielsen, M. H., Borregaard, N. (1992) Separation of human neutrophil plasma membrane from intracellular vesicles containing alkaline phosphatase and NADPH oxidase activity by free flow electrophoresis. J. Biol. Chem. 267, 14912-7.
59. Bokoch, G. M. and Zhao, T. (2006) Regulation of the phagocyte NADPH oxidase by Rac GTPase. Antioxidants & redox signaling 8, 1533-48.
60. Klebanoff, S. J. Oxygen metabolites from phagocytes. In Inflammation: Basic principles and clinical correlates, Eds. Gallin, J. I., Snyderman, R., 3rd ed, (1999) Lippincott, Williams & Wilkins, 721-768.
61. Bylund, J., Goldblatt, D., Speert, D. P. (2005) Chronic granulomatous disease: from genetic defect to clinical presentation. Adv. Exp. Med. Biol. 568, 67-87.
62. Holland, S. M. (2010) Chronic granulomatous disease. Clin. Rev. Allergy Immunol. 38, 3-10.
63. Segal, B. H., Leto, T. L., Gallin, J. I., Malech, H. L., Holland, S. M. (2000) Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine (Baltimore). 79, 170-200.
64. Kuhns, D. B., Alvord, W. G., Heller, T., Feld, J. J., Pike, K. M., Marciano, B. E., Uzel, G., DeRavin, S. S., Priel, D. A., Soule, B. P., Zarember, K. A., Malech, H. L., Holland, S. M., Gallin, J. I. (2010) Residual NADPH oxidase and survival in chronic granulomatous disease. N. Engl. J. Med. 363, 2600-10.
65. Winkelstein, J. A., Marino, M. C., Johnston, R. B., Jr., Boyle, J., Curnutte, J., Gallin, J. I., Malech, H. L., Holland, S. M., Ochs, H., Quie, P., Buckley, R. H., Foster, C. B., Chanock, S. J., Dickler, H. (2000) Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore). 79, 155-69.
66. Kuhns, D. B. and Gallin, J. I. (1995) Increased cell-associated IL-8 in human exudative and A23187-treated peripheral blood neutrophils. J. Immunol. 154, 6556-62.
67. Roos, D., de Boer, M., Kuribayashi, F., Meischl, C., Weening, R. S., Segal, A. W., Ahlin, A., Nemet, K., Hossle, J. P., Bernatowska-Matuszkiewicz, E., Middleton-Price, H. (1996) Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood 87, 1663-81.
68. Ahlin, A., Fugelang, J., de Boer, M., Ringden, O., Fasth, A., Winiarski, J. (2013) Chronic granulomatous disease - haematopoietic stem cell transplantation versus conventional treatment. Acta Paediatr. 102,1087-94.
69. Parkos, C. A., Dinauer, M. C., Jesaitis, A. J., Orkin, S. H., Curnutte, J. T. (1989) Absence of both the 91kD and 22kD subunits of human neutrophil cytochrome b in two genetic forms of chronic granulomatous disease. Blood 73, 1416-20.
70. Dahlgren, C., Karlsson, A., Bylund, J. (2007) Measurement of respiratory burst products generated by professional phagocytes. Methods Mol. Biol. 412, 349-63.
71. Matute, J. D., Arias, A. A., Wright, N. A., Wrobel, I., Waterhouse, C. C., Li, X. J., Marchal, C. C., Stull, N. D., Lewis, D. B., Steele, M., Kellner, J. D., Yu, W., Meroueh, S. O., Nauseef, W. M., Dinauer, M. C. (2009) A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in p40 phox and selective defects in neutrophil NADPH oxidase activity. Blood 114, 3309-15.

72. Diamond, R. D. and Clark, R. A. (1982) Damage to Aspergillus fumigatus and Rhizopus oryzae hyphae by oxidative and nonoxidative microbicidal products of human neutrophils in vitro. Infect. Immun. 38, 487-95.
73. Dupre-Crochet, S., Erard, M., Nubetae, O. (2013) ROS production in phagocytes: why, when, and where? J. Leukoc. Biol. 94, 657-670.
74. Betten, A., Bylund, J., Christophe, T., Boulay, F., Romero, A., Hellstrand, K., Dahlgren, C. (2001) A proinflammatory peptide from Helicobacter pylori activates monocytes to induce lymphocyte dysfunction and apoptosis. J. Clin. Invest. 108, 1221-8.
75. Aurelius, J., Martner, A., Brune, M., Palmqvist, L., Hansson, M., Hellstrand, K., Thoren, F. B. (2012) Remission maintenance in acute myeloid leukemia: impact of functional histamine H2 receptors expressed by leukemic cells. Haematologica 97, 1904-8.
76. Olofsson, P., Holmberg, J., Tordsson, J., Lu, S., Åkerström, B., Holmdahl, R. (2003) Positional identification of Ncf1 as a gene that regulates arthritis severity in rats. Nat. Genet. 33, 25-32.
77. Hultqvist, M., Olofsson, P., Gelderman, K. A., Holmberg, J., Holmdahl, R. (2006) A new arthritis therapy with oxidative burst inducers. PLoS medicine 3, e348.
78. Dahlgren, C. (1987) Difference in extracellular radical release after chemotactic factor and calcium ionophore activation of the oxygen radical-generating system in human neutrophils. Biochim. Biophys. Acta 930, 33-8.
79. Dahlgren, C., Johansson, A., Orselius, K. (1989) Difference in hydrogen peroxide release between human neutrophils and neutrophil cytoplasts following calcium ionophore activation. A role of the subcellular granule in activation of the NADPH-oxidase in human neutrophils? Biochim. Biophys. Acta 1010, 41-8.
80. Karlsson, A. and Dahlgren, C. (2002) Assembly and activation of the neutrophil NADPH oxidase in granule membranes. Antioxidants & redox signaling 4, 49-60.
81. Ferguson, P. J., Lokuta, M. A., El-Shanti, H. I., Muhle, L., Bing, X., Huttenlocher, A. (2008) Neutrophil dysfunction in a family with a SAPHO syndrome-like phenotype. Arthritis Rheum. 58, 3264-9.
82. Turrens, J. F. (2003) Mitochondrial formation of reactive oxygen species. J Physiol 552, 335-44.
83. Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Jr., Dionne, L., Lu, N., Huang, S., Matzuk, M. M. (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 93, 9782-7.
84. Figueira, T. R., Barros, M. H., Camargo, A. A., Castilho, R. F., Ferreira, J. C., Kowaltowski, A. J., Sluse, F. E., Souza-Pinto, N. C., Vercesi, A. E. (2013) Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health. Antioxidants & redox signaling 18, 2029-74.
85. Bulua, A. C., Simon, A., Maddipati, R., Pelletier, M., Park, H., Kim, K. Y., Sack, M. N., Kastner, D. L., Siegel, R. M. (2011) Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). J. Exp. Med. 208, 519-533.
86. Dahlgren, C. and Stendahl, O. (1983) Role of myeloperoxidase in luminol-dependent chemiluminescence of polymorphonuclear leukocytes. Infect. Immun. 39, 736-41.
87. Mukhopadhyay, P., Rajesh, M., Yoshihiro, K., Hasko, G., Pacher, P. (2007) Simple quantitative detection of mitochondrial superoxide production in live cells. Biochem. Biophys. Res. Commun. 358, 203-8.
88. Mukhopadhyay, P., Rajesh, M., Hasko, G., Hawkins, B. J., Madesh, M., Pacher, P. (2007) Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confocal microscopy. Nat Protoc 2, 2295-301.
89. Wardman, P. (2007) Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. Free Radic. Biol. Med. 43, 995-1022.
90. Nauseef, W. M. (2013) Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases. Biochim. Biophys. Acta. E-pub ahead of print.
91. Dikalov, S. I. and Harrison, D. G. (2012) Methods for Detection of Mitochondrial and Cellular Reactive Oxygen Species. Antioxidants & redox signaling. E-pub ahead of print.
92. Forkink, M., Smeitink, J. A., Brock, R., Willems, P. H., Koopman, W. J. (2010) Detection and

manipulation of mitochondrial reactive oxygen species in mammalian cells. Biochim. Biophys. Acta 1797, 1034-44.
93. Follin, P., Briheim, G., Dahlgren, C. (1991) Mechanisms in neutrophil priming: characterization of the oxidative response induced by formylmethionyl-leucyl-phenylalanine in human exudated cells. Scand. J. Immunol. 34, 317-22.
94. Karlsson, A., Follin, P., Leffler, H., Dahlgren, C. (1998) Galectin-3 activates the NADPH-oxidase in exudated but not peripheral blood neutrophils. Blood 91, 3430-8.
95. Kuhns, D. B., Long Priel, D. A., Gallin, J. I. (1995) Loss of L-selectin (CD62L) on human neutrophils following exudation in vivo. Cell. Immunol. 164, 306-10.
96. Ashkenazi, M. and Dennison, D. K. (1989) A new method for isolation of salivary neutrophils and determination of their functional activity. J. Dent. Res. 68, 1256-61.
97. Qureshi, S. S., Lewis, S. M., Gant, V. A., Treacher, D., Davis, B. H., Brown, K. A. (2001) Increased distribution and expression of CD64 on blood polymorphonuclear cells from patients with the systemic inflammatory response syndrome (SIRS). Clin. Exp. Immunol. 125, 258-65.
98. Rosenbloom, A. J., Pinsky, M. R., Bryant, J. L., Shin, A., Tran, T., Whiteside, T. (1995) Leukocyte activation in the peripheral blood of patients with cirrhosis of the liver and SIRS. Correlation with serum interleukin-6 levels and organ dysfunction. JAMA 274, 58-65.
99. Harper, L., Cockwell, P., Adu, D., Savage, C. O. (2001) Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis. Kidney Int. 59, 1729-38.
100. Naegele, M., Tillack, K., Reinhardt, S., Schippling, S., Martin, R., Sospedra, M. (2011) Neutrophils in multiple sclerosis are characterized by a primed phenotype. J. Neuroimmunol. 242, 60-71.
101. Molad, Y., Fridenberg, A., Bloch, K., Langevitz, P., Mukamel, M., Sulkes, J., Pras, M., Livneh, A. (2004) Neutrophil adhesion molecule expression in familial Mediterranean fever: discordance between the intravascular regulation of beta2 integrin and L-selectin expression in acute attack. J. Investig. Med. 52, 58-61.
102. Middleton, J., Patterson, A. M., Gardner, L., Schmutz, C., Ashton, B. A. (2002) Leukocyte extravasation: chemokine transport and presentation by the endothelium. Blood 100, 3853-60.
103. Sperandio, M. (2006) Selectins and glycosyltransferases in leukocyte rolling in vivo. The FEBS journal 273, 4377-89.
104. Li, Y., Brazzell, J., Herrera, A., Walcheck, B. (2006) ADAM17 deficiency by mature neutrophils has differential effects on L-selectin shedding. Blood 108, 2275-9.
105. Choi, E. Y., Santoso, S., Chavakis, T. (2009) Mechanisms of neutrophil transendothelial migration. Frontiers in bioscience (Landmark edition) 14, 1596-605.
106. Woodfin, A., Voisin, M. B., Nourshargh, S. (2010) Recent developments and complexities in neutrophil transmigration. Curr. Opin. Hematol. 17, 9-17.
107. Stossel, T. P. (1993) On the crawling of animal cells. Science 260, 1086-94.
108. Almkvist, J., Fäldt, J., Dahlgren, C., Leffler, H., Karlsson, A. (2001) Lipopolysaccharide-induced gelatinase granule mobilization primes neutrophils for activation by galectin-3 and formylmethionyl-Leu-Phe. Infect. Immun. 69, 832-7.
109. Follin, P. and Dahlgren, C. (2007) A skin chamber technique as a human model for studies of aseptic inflammatory reactions. Methods Mol. Biol. 412, 333-46.
110. Christenson, K., Björkman, L., Karlsson, J., Sundqvist, M., Movitz, C., Speert, D. P., Dahlgren, C., Bylund, J. (2011) In vivo-transmigrated human neutrophils are resistant to antiapoptotic stimulation. J. Leukoc. Biol. 90, 1055-1063.
111. Briheim, G., Coble, B., Stendahl, O., Dahlgren, C. (1988) Exudate polymorphonuclear leukocytes isolated from skin chambers are primed for enhanced response to subsequent stimulation with chemoattractant f-Met-Leu-Phe and C3-opsonized yeast particles. Inflammation 12, 141-52.
112. Berends, C., Dijkhuizen, B., de Monchy, J. G., Gerritsen, J., Kauffman, H. F. (1994) Induction of low density and up-regulation of CD11b expression of neutrophils and eosinophils by dextran sedimentation and centrifugation. J. Immunol. Methods 167, 183-93.
113. Watson, F., Robinson, J. J., Edwards, S. W. (1992) Neutrophil function in whole blood and after purification: changes in receptor expression, oxidase activity and responsiveness to cytokines. Biosci. Rep. 12, 123-33.
114. Yamaoka, A., Kuwabara, I., Frigeri, L. G., Liu, F. T. (1995) A human lectin, galectin-3 (epsilon

bp/Mac-2), stimulates superoxide production by neutrophils. J. Immunol. 154, 3479-87.
115. Almkvist, J., Dahlgren, C., Leffler, H., Karlsson, A. (2004) Newcastle disease virus neuraminidase primes neutrophils for stimulation by galectin-3 and formyl-Met-Leu-Phe. Exp. Cell Res. 298, 74-82.
116. Kolaczkowska, E. and Kubes, P. (2013) Neutrophil recruitment and function in health and inflammation. Nature reviews. Immunology 13, 159-75.
117. Tak, T., Tesselaar, K., Pillay, J., Borghans, J. A., Koenderman, L. (2013) What's your age again? Determination of human neutrophil half-lives revisited. J. Leukoc. Biol. 94, 595-601.
118. Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A., Tesselaar, K., Koenderman, L. (2010) In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. Blood 116, 625-7.
119. Tofts, P. S., Chevassut, T., Cutajar, M., Dowell, N. G., Peters, A. M. (2011) Doubts concerning the recently reported human neutrophil lifespan of 5.4 days. Blood 117, 6050-2; author reply 6053-4.
120. Li, K. W., Turner, S. M., Emson, C. L., Hellerstein, M. K., Dale, D. C. (2011) Deuterium and neutrophil kinetics. Blood 117, 6052-3; author reply 6053-4.
121. Hengartner, M. O. (2000) The biochemistry of apoptosis. Nature 407, 770-6.
122. Suratt, B. T., Young, S. K., Lieber, J., Nick, J. A., Henson, P. M., Worthen, G. S. (2001) Neutrophil maturation and activation determine anatomic site of clearance from circulation. American journal of physiology. Lung cellular and molecular physiology 281, L913-21.
123. Martin, C., Burdon, P. C., Bridger, G., Gutierrez-Ramos, J. C., Williams, T. J., Rankin, S. M. (2003) Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence. Immunity 19, 583-93.
124. Colotta, F., Re, F., Polentarutti, N., Sozzani, S., Mantovani, A. (1992) Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 80, 2012-20.
125. van Raam, B. J., Drewniak, A., Groenewold, V., van den Berg, T. K., Kuijpers, T. W. (2008) Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3. Blood 112, 2046-54.
126. Christenson, K., Björkman, L., Karlsson, A., Bylund, J. (2013) Regulation of neutrophil apoptosis differs after in vivo transmigration to skin chambers and synovial fluid: a role for inflammasome-dependent interleukin-1beta release. Journal of innate immunity 5, 377-88.
127. Lotz, S., Aga, E., Wilde, I., van Zandbergen, G., Hartung, T., Solbach, W., Laskay, T. (2004) Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2. J. Leukoc. Biol. 75, 467-77.
128. Silva, M. T., do Vale, A., dos Santos, N. M. (2008) Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. Apoptosis : an international journal on programmed cell death 13, 463-82.
129. Squier, M. K., Sehnert, A. J., Cohen, J. J. (1995) Apoptosis in leukocytes. J. Leukoc. Biol. 57, 2-10.
130. Martelli, A. M., Zweyer, M., Ochs, R. L., Tazzari, P. L., Tabellini, G., Narducci, P., Bortul, R. (2001) Nuclear apoptotic changes: an overview. J. Cell. Biochem. 82, 634-46.
131. Zimmermann, K. C., Bonzon, C., Green, D. R. (2001) The machinery of programmed cell death. Pharmacol. Ther. 92, 57-70.
132. Kayagaki, N., Kawasaki, A., Ebata, T., Ohmoto, H., Ikeda, S., Inoue, S., Yoshino, K., Okumura, K., Yagita, H. (1995) Metalloproteinase-mediated release of human Fas ligand. J. Exp. Med. 182, 1777-83.
133. Fadeel, B. (2004) Plasma membrane alterations during apoptosis: role in corpse clearance. Antioxidants & redox signaling 6, 269-75.
134. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. Science 303, 1532-5.
135. Christenson, K., Thoren, F. B., Bylund, J. (2012) Analyzing cell death events in cultured leukocytes. Methods Mol. Biol. 844, 65-86.
136. Badley, A. D., McElhinny, J. A., Leibson, P. J., Lynch, D. H., Alderson, M. R., Paya, C. V. (1996) Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes. J. Virol. 70, 199-206.
137. Splawski, J. B., Jelinek, D. F., Lipsky, P. E. (1991) Delineation of the functional capacity

of human neonatal lymphocytes. J. Clin. Invest. 87, 545-53.
138. Splawski, J. B. and Lipsky, P. E. (1991) Cytokine regulation of immunoglobulin secretion by neonatal lymphocytes. J. Clin. Invest. 88, 967-77.
139. Hassan, J. and Reen, D. J. (1996) Reduced primary antigen-specific T-cell precursor frequencies in neonates is associated with deficient interleukin-2 production. Immunology 87, 604-8.
140. Firth, M. A., Shewen, P. E., Hodgins, D. C. (2005) Passive and active components of neonatal innate immune defenses. Animal health research reviews / Conference of Research Workers in Animal Diseases 6, 143-58.
141. Carr, R. (2000) Neutrophil production and function in newborn infants. Br. J. Haematol. 110, 18-28.
142. Koenig, J. M. and Yoder, M. C. (2004) Neonatal neutrophils: the good, the bad, and the ugly. Clin. Perinatol. 31, 39-51.
143. Khalfan, L., Björksten, B., Dahlgren, C., Follin, P. (1992) Increased respiratory burst response and alkaline phosphatase activity in human cord blood neutrophils. Pediatr. Allergy Immunol. 3, 91-5.
144. Khalfan, L., Karlsson, A., Lundqvist, H., Björksten, B., Dahlgren, C. (1995) Lack of correlation between NADPH-oxidase priming and elevated alkaline phosphatase activity in cord blood neutrophils. Pediatr. Allergy Immunol. 6, 161-4.
145. Cook, G. M. (1986) Cell surface carbohydrates: molecules in search of a function? J. Cell Sci. Suppl. 4, 45-70.
146. Barondes, S. H., Castronovo, V., Cooper, D. N., Cummings, R. D., Drickamer, K., Feizi, T., Gitt, M. A., Hirabayashi, J., Hughes, C., Kasai, K., et al. (1994) Galectins: a family of animal beta-galactoside-binding lectins. Cell 76, 597-8.
147. Cooper, D. N. and Barondes, S. H. (1999) God must love galectins; he made so many of them. Glycobiology 9, 979-84.
148. Cooper, D. N. (2002) Galectinomics: finding themes in complexity. Biochim. Biophys. Acta 1572, 209-31.
149. Leffler, H., Carlsson, S., Hedlund, M., Qian, Y., Poirier, F. (2004) Introduction to galectins. Glycoconj. J. 19, 433-40.
150. Hughes, R. C. (1999) Secretion of the galectin family of mammalian carbohydrate-binding proteins. Biochim. Biophys. Acta 1473, 172-85.
151. Nickel, W. (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur. J. Biochem. 270, 2109-19.
152. Hirabayashi, J. and Kasai, K. (1993) The family of metazoan metal-independent beta-galactoside-binding lectins: structure, function and molecular evolution. Glycobiology 3, 297-304.
153. Herrmann, J., Turck, C. W., Atchison, R. E., Huflejt, M. E., Poulter, L., Gitt, M. A., Burlingame, A. L., Barondes, S. H., Leffler, H. (1993) Primary structure of the soluble lactose binding lectin L-29 from rat and dog and interaction of its non-collagenous proline-, glycine-, tyrosine-rich sequence with bacterial and tissue collagenase. J. Biol. Chem. 268, 26704-11.
154. Seetharaman, J., Kanigsberg, A., Slaaby, R., Leffler, H., Barondes, S. H., Rini, J. M. (1998) X-ray crystal structure of the human galectin-3 carbohydrate recognition domain at 2.1-A resolution. J. Biol. Chem. 273, 13047-52.
155. Nieminen, J., Kuno, A., Hirabayashi, J., Sato, S. (2007) Visualization of galectin-3 oligomerization on the surface of neutrophils and endothelial cells using fluorescence resonance energy transfer. J. Biol. Chem. 282, 1374-83.
156. Ho, M. K. and Springer, T. A. (1982) Mac-2, a novel 32,000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. J. Immunol. 128, 1221-8.
157. John, C. M., Jarvis, G. A., Swanson, K. V., Leffler, H., Cooper, M. D., Huflejt, M. E., Griffiss, J. M. (2002) Galectin-3 binds lactosaminylated lipooligosaccharides from Neisseria gonorrhoeae and is selectively expressed by mucosal epithelial cells that are infected. Cellular microbiology 4, 649-62.
158. Liu, F. T. (2005) Regulatory roles of galectins in the immune response. Int. Arch. Allergy Immunol. 136, 385-400.
159. Nieminen, J., St-Pierre, C., Sato, S. (2005) Galectin-3 interacts with naive and primed neutrophils, inducing innate immune responses. J. Leukoc. Biol. 78, 1127-35.
160. Ochieng, J., Fridman, R., Nangia-Makker, P., Kleiner, D. E., Liotta, L. A., Stetler-Stevenson, W. G., Raz, A. (1994) Galectin-3 is a novel substrate for human matrix metalloproteinases-2 and -9. Biochemistry (Mosc). 33, 14109-14.
161. Puthenedam, M., Wu, F., Shetye, A., Michaels, A., Rhee, K. J., Kwon, J. H. (2010) Matrilysin-1

(MMP7) cleaves galectin-3 and inhibits wound healing in intestinal epithelial cells. Inflamm. Bowel Dis. 17, 260-7.
162. Hsu, D. K., Zuberi, R. I., Liu, F. T. (1992) Biochemical and biophysical characterization of human recombinant IgE-binding protein, an S-type animal lectin. J. Biol. Chem. 267, 14167-74.
163. Massa, S. M., Cooper, D. N., Leffler, H., Barondes, S. H. (1993) L-29, an endogenous lectin, binds to glycoconjugate ligands with positive cooperativity. Biochemistry (Mosc). 32, 260-7.
164. Pelletier, I. and Sato, S. (2002) Specific recognition and cleavage of galectin-3 by Leishmania major through species-specific polygalactose epitope. J. Biol. Chem. 277, 17663-70.
165. Morris, S., Ahmad, N., Andre, S., Kaltner, H., Gabius, H. J., Brenowitz, M., Brewer, F. (2004) Quaternary solution structures of galectins-1, -3, and -7. Glycobiology 14, 293-300.
166. Sato, S. and Nieminen, J. (2004) Seeing strangers or announcing "danger": galectin-3 in two models of innate immunity. Glycoconj. J. 19, 583-91.
167. Yang, R. Y., Hill, P. N., Hsu, D. K., Liu, F. T. (1998) Role of the carboxyl-terminal lectin domain in self-association of galectin-3. Biochemistry (Mosc). 37, 4086-92.
168. Kuklinski, S. and Probstmeier, R. (1998) Homophilic binding properties of galectin-3: involvement of the carbohydrate recognition domain. J. Neurochem. 70, 814-23.
169. Lepur, A., Salomonsson, E., Nilsson, U. J., Leffler, H. (2012) Ligand induced galectin-3 self-association. J. Biol. Chem. 287, 21751-6.
170. Colnot, C., Fowlis, D., Ripoche, M. A., Bouchaert, I., Poirier, F. (1998) Embryonic implantation in galectin 1/galectin 3 double mutant mice. Dev. Dyn. 211, 306-13.
171. Hsu, D. K., Yang, R. Y., Pan, Z., Yu, L., Salomon, D. R., Fung-Leung, W. P., Liu, F. T. (2000) Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. Am. J. Pathol. 156, 1073-83.
172. Bhaumik, P., St-Pierre, G., Milot, V., St-Pierre, C., Sato, S. (2013) Galectin-3 facilitates neutrophil recruitment as an innate immune response to a parasitic protozoa cutaneous infection. J. Immunol. 190, 630-40.
173. Zuberi, R. I., Hsu, D. K., Kalayci, O., Chen, H. Y., Sheldon, H. K., Yu, L., Apgar, J. R., Kawakami, T., Lilly, C. M., Liu, F. T. (2004) Critical role for galectin-3 in airway inflammation and bronchial hyperresponsiveness in a murine model of asthma. Am. J. Pathol. 165, 2045-53.
174. Saegusa, J., Hsu, D. K., Chen, H. Y., Yu, L., Fermin, A., Fung, M. A., Liu, F. T. (2009) Galectin-3 is critical for the development of the allergic inflammatory response in a mouse model of atopic dermatitis. Am. J. Pathol. 174, 922-31.
175. MacKinnon, A. C., Liu, X., Hadoke, P. W., Miller, M. R., Newby, D. E., Sethi, T. (2013) Inhibition of galectin-3 reduces atherosclerosis in apolipoprotein E-deficient mice. Glycobiology 23, 654-63.
176. Forsman, H., Islander, U., Andreasson, E., Andersson, A., Önnheim, K., Karlström, A., Sävman, K., Magnusson, M., Brown, K. L., Karlsson, A. (2011) Galectin 3 aggravates joint inflammation and destruction in antigen-induced arthritis. Arthritis Rheum. 63, 445-54.
177. Farnworth, S. L., Henderson, N. C., Mackinnon, A. C., Atkinson, K. M., Wilkinson, T., Dhaliwal, K., Hayashi, K., Simpson, A. J., Rossi, A. G., Haslett, C., Sethi, T. (2008) Galectin-3 reduces the severity of pneumococcal pneumonia by augmenting neutrophil function. Am. J. Pathol. 172, 395-405.
178. Bernardes, E. S., Silva, N. M., Ruas, L. P., Mineo, J. R., Loyola, A. M., Hsu, D. K., Liu, F. T., Chammas, R., Roque-Barreira, M. C. (2006) Toxoplasma gondii infection reveals a novel regulatory role for galectin-3 in the interface of innate and adaptive immunity. Am. J. Pathol. 168, 1910-20.
179. Kuwabara, I. and Liu, F. T. (1996) Galectin-3 promotes adhesion of human neutrophils to laminin. J. Immunol. 156, 3939-44.
180. Danella Polli, C., Alves Toledo, K., Franco, L. H., Sammartino Mariano, V., de Oliveira, L. L., Soares Bernardes, E., Roque-Barreira, M. C., Pereira-da-Silva, G. (2013) Monocyte Migration Driven by Galectin-3 Occurs through Distinct Mechanisms Involving Selective Interactions with the Extracellular Matrix. ISRN inflammation 2013, 259256.
181. Sato, S., Ouellet, N., Pelletier, I., Simard, M., Rancourt, A., Bergeron, M. G. (2002) Role of galectin-3 as an adhesion molecule for neutrophil extravasation during streptococcal pneumonia. J. Immunol. 168, 1813-22.
182. Liu, F. T., Hsu, D. K., Zuberi, R. I., Kuwabara, I., Chi, E. Y., Henderson, W. R., Jr. (1995) Expression and function of galectin-3, a beta-galactoside-binding lectin, in human

monocytes and macrophages. Am. J. Pathol. 147, 1016-28.
183. Almkvist, J., Dahlgren, C., Leffler, H., Karlsson, A. (2002) Activation of the neutrophil nicotinamide adenine dinucleotide phosphate oxidase by galectin-1. J. Immunol. 168, 4034-41.
184. Carlsson, S., Öberg, C. T., Carlsson, M. C., Sundin, A., Nilsson, U. J., Smith, D., Cummings, R. D., Almkvist, J., Karlsson, A., Leffler, H. (2007) Affinity of galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. Glycobiology 17, 663-76.
185. Feuk-Lagerstedt, E., Jordan, E. T., Leffler, H., Dahlgren, C., Karlsson, A. (1999) Identification of CD66a and CD66b as the major galectin-3 receptor candidates in human neutrophils. J. Immunol. 163, 5592-8.
186. Linden, J. R., Kunkel, D., Laforce-Nesbitt, S. S., Bliss, J. M. (2013) The role of galectin-3 in phagocytosis of Candida albicans and Candida parapsilosis by human neutrophils. Cellular microbiology 15, 1127-42.
187. Sano, H., Hsu, D. K., Apgar, J. R., Yu, L., Sharma, B. B., Kuwabara, I., Izui, S., Liu, F. T. (2003) Critical role of galectin-3 in phagocytosis by macrophages. J. Clin. Invest. 112, 389-97.
188. Karlsson, A., Christenson, K., Matlak, M., Björstad, Å., Brown, K. L., Telemo, E., Salomonsson, E., Leffler, H., Bylund, J. (2009) Galectin-3 functions as an opsonin and enhances the macrophage clearance of apoptotic neutrophils. Glycobiology 19, 16-20.
189. Cortegano, I., del Pozo, V., Cardaba, B., de Andres, B., Gallardo, S., del Amo, A., Arrieta, I., Jurado, A., Palomino, P., Liu, F. T., Lahoz, C. (1998) Galectin-3 down-regulates IL-5 gene expression on different cell types. J. Immunol. 161, 385-9.
190. Jeng, K. C., Frigeri, L. G., Liu, F. T. (1994) An endogenous lectin, galectin-3 (epsilon BP/Mac-2), potentiates IL-1 production by human monocytes. Immunol. Lett. 42, 113-6.
191. Hsu, D. K., Hammes, S. R., Kuwabara, I., Greene, W. C., Liu, F. T. (1996) Human T lymphotropic virus-I infection of human T lymphocytes induces expression of the beta-galactoside-binding lectin, galectin-3. Am. J. Pathol. 148, 1661-70.
192. Christenson, R. H., Duh, S. H., Wu, A. H., Smith, A., Abel, G., deFilippi, C. R., Wang, S., Adourian, A., Adiletto, C., Gardiner, P. (2010) Multi-center determination of galectin-3 assay performance characteristics: Anatomy of a novel assay for use in heart failure. Clin. Biochem. 43, 683-90.
193. de Boer, R. A., van Veldhuisen, D. J., Gansevoort, R. T., Muller Kobold, A. C., van Gilst, W. H., Hillege, H. L., Bakker, S. J., van der Harst, P. (2012) The fibrosis marker galectin-3 and outcome in the general population. J. Intern. Med. 272, 55-64.
194. Gruson, D. and Ko, G. (2012) Galectins testing: new promises for the diagnosis and risk stratification of chronic diseases? Clin. Biochem. 45, 719-26.
195. Dhirapong, A., Lleo, A., Leung, P., Gershwin, M. E., Liu, F. T. (2009) The immunological potential of galectin-1 and -3. Autoimmunity reviews 8, 360-3.
196. Iurisci, I., Tinari, N., Natoli, C., Angelucci, D., Cianchetti, E., Iacobelli, S. (2000) Concentrations of galectin-3 in the sera of normal controls and cancer patients. Clin. Cancer Res. 6, 1389-93.
197. Takenaka, Y., Fukumori, T., Raz, A. (2004) Galectin-3 and metastasis. Glycoconj. J. 19, 543-9.
198. Yu, L. G. (2010) Circulating galectin-3 in the bloodstream: An emerging promoter of cancer metastasis. World journal of gastrointestinal oncology 2, 177-80.
199. de Boer, R. A., Voors, A. A., Muntendam, P., van Gilst, W. H., van Veldhuisen, D. J. (2009) Galectin-3: a novel mediator of heart failure development and progression. European journal of heart failure 11, 811-7.
200. de Boer, R. A., Lok, D. J., Jaarsma, T., van der Meer, P., Voors, A. A., Hillege, H. L., van Veldhuisen, D. J. (2011) Predictive value of plasma galectin-3 levels in heart failure with reduced and preserved ejection fraction. Ann. Med. 43, 60-8.
201. Kang, E. H., Moon, K. C., Lee, E. Y., Lee, Y. J., Lee, E. B., Ahn, C., Song, Y. W. (2009) Renal expression of galectin-3 in systemic lupus erythematosus patients with nephritis. Lupus 18, 22-8.
202. Lee, Y. J., Kang, S. W., Song, J. K., Park, J. J., Bae, Y. D., Lee, E. Y., Lee, E. B., Song, Y. W. (2007) Serum galectin-3 and galectin-3 binding protein levels in Behcet's disease and their association with disease activity. Clin. Exp. Rheumatol. 25, S41-5.
203. Ohshima, S., Kuchen, S., Seemayer, C. A., Kyburz, D., Hirt, A., Klinzing, S., Michel, B. A., Gay, R. E., Liu, F. T., Gay, S., Neidhart, M. (2003) Galectin 3 and its binding protein in

rheumatoid arthritis. Arthritis Rheum. 48, 2788-95.
204. Ezzat, M. H., El-Gammasy, T. M., Shaheen, K. Y., Osman, A. O. (2011) Elevated production of galectin-3 is correlated with juvenile idiopathic arthritis disease activity, severity, and progression. International journal of rheumatic diseases 14, 345-52.
205. Frol'ova, L., Smetana, K., Jr., Borovska, D., Kitanovicova, A., Klimesova, K., Janatkova, I., Malickova, K., Lukas, M., Drastich, P., Benes, Z., Tuckova, L., Manning, J. C., Andre, S., Gabius, H. J., Tlaskalova-Hogenova, H. (2009) Detection of galectin-3 in patients with inflammatory bowel diseases: new serum marker of active forms of IBD? Inflamm. Res. 58, 503-12.
206. Weigert, J., Neumeier, M., Wanninger, J., Bauer, S., Farkas, S., Scherer, M. N., Schnitzbauer, A., Schaffler, A., Aslanidis, C., Scholmerich, J., Buechler, C. (2010) Serum galectin-3 is elevated in obesity and negatively correlates with glycosylated hemoglobin in type 2 diabetes. J. Clin. Endocrinol. Metab. 95, 1404-11.
207. Ten Oever, J., Giamarellos-Bourboulis, E. J., van de Veerdonk, F. L., Stelma, F. F., Simon, A., Janssen, M., Johnson, M., Pachot, A., Kullberg, B. J., Joosten, L. A., Netea, M. G. (2013) Circulating galectin-3 in infections and non-infectious inflammatory diseases. Eur. J. Clin. Microbiol. Infect. Dis. E-pub ahead of print.
208. Stojanov, S., Lapidus, S., Chitkara, P., Feder, H., Salazar, J. C., Fleisher, T. A., Brown, M. R., Edwards, K. M., Ward, M. M., Colbert, R. A., Sun, H. W., Wood, G. M., Barham, B. K., Jones, A., Aksentijevich, I., Goldbach-Mansky, R., Athreya, B., Barron, K. S., Kastner, D. L. (2011) Periodic fever, aphthous stomatitis, pharyngitis, and adenitis (PFAPA) is a disorder of innate immunity and Th1 activation responsive to IL-1 blockade. Proc. Natl. Acad. Sci. U. S. A. 108, 7148-7153.
209. Kolly, L., Busso, N., von Scheven-Gete, A., Bagnoud, N., Moix, I., Holzinger, D., Simon, G., Ives, A., Guarda, G., So, A., Morris, M. A., Hofer, M. (2012) Periodic fever, aphthous stomatitis, pharyngitis, cervical adenitis syndrome is linked to dysregulated monocyte IL-1beta production. J. Allergy Clin. Immunol. 131, 1635-43
210. Førsvoll, J., Kristoffersen, E. K., Øymar, K. (2013) Elevated levels of CXCL10 in the Periodic Fever, Aphthous stomatitis, Pharyngitis and cervical Adenitis syndrome (PFAPA) during and between febrile episodes; an indication of a persistent activation of the innate immune system. Pediatric rheumatology online journal 11, 38.
211. Fleer, A., Gerards, L. J., Verhoef, J. (1988) Host defence to bacterial infection in the neonate. J. Hosp. Infect. 11 Suppl A, 320-7.
212. Doverhag, C., Hedtjärn, M., Poirier, F., Mallard, C., Hagberg, H., Karlsson, A., Sävman, K. (2010) Galectin-3 contributes to neonatal hypoxic-ischemic brain injury. Neurobiol. Dis. 38, 36-46.
213. Sävman, K., Heyes, M. P., Svedin, P., Karlsson, A. (2013) Microglia/Macrophage-Derived Inflammatory Mediators Galectin-3 and Quinolinic Acid are Elevated in Cerebrospinal Fluid from Newborn Infants After Birth Asphyxia. Translational stroke research 4, 228-235.
214. Mukaro, V. R., Bylund, J., Hodge, G., Holmes, M., Jersmann, H., Reynolds, P. N., Hodge, S. (2013) Lectins offer new perspectives in the development of macrophage-targeted therapies for COPD/emphysema. PloS one 8, e56147.
215. Björkman, L., Raynes, J. G., Shah, C., Karlsson, A., Dahlgren, C., Bylund, J. (2010) The proinflammatory activity of recombinant serum amyloid A is not shared by the endogenous protein in circulation. Arthritis Rheum. 62, 1660-1665.
216. McDermott, M. F., Aksentijevich, I., Galon, J., McDermott, E. M., Ogunkolade, B. W., Centola, M., Mansfield, E., Gadina, M., Karenko, L., Pettersson, T., McCarthy, J., Frucht, D. M., Aringer, M., Torosyan, Y., Teppo, A. M., Wilson, M., Karaarslan, H. M., Wan, Y., Todd, I., Wood, G., Schlimgen, R., Kumarajeewa, T. R., Cooper, S. M., Vella, J. P., Amos, C. I., Mulley, J., Quane, K. A., Molloy, M. G., Ranki, A., Powell, R. J., Hitman, G. A., O'Shea, J. J., Kastner, D. L. (1999) Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell 97, 133-44.
217. Consortium, T. I. F. (1997) Ancient missense mutations in a new member of the RoRet gene family are likely to cause familial Mediterranean fever. The International FMF Consortium. Cell 90, 797-807.
218. Consortium, F. F. (1997) A candidate gene for familial Mediterranean fever. Nat. Genet. 17, 25-31.

219. Kastner, D. L., Aksentijevich, I., Goldbach-Mansky, R. (2010) Autoinflammatory disease reloaded: a clinical perspective. Cell 140, 784-90.
220. Grateau, G., Hentgen, V., Stojanovic, K. S., Jeru, I., Amselem, S., Steichen, O. (2013) How should we approach classification of autoinflammatory diseases? Nature reviews. Rheumatology 9, 624-9.
221. Agostini, L., Martinon, F., Burns, K., McDermott, M. F., Hawkins, P. N., Tschopp, J. (2004) NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. Immunity 20, 319-25.
222. Dinarello, C. A. (2004) Unraveling the NALP-3/IL-1beta inflammasome: a big lesson from a small mutation. Immunity 20, 243-4.
223. Chow, A. and Robinson, J. L. (2011) Fever of unknown origin in children: a systematic review. World journal of pediatrics : WJP 7, 5-10.
224. Obici, L. and Merlini, G. (2012) AA amyloidosis: basic knowledge, unmet needs and future treatments. Swiss medical weekly 142, w13580.
225. Drenth, J. P., Cuisset, L., Grateau, G., Vasseur, C., van de Velde-Visser, S. D., de Jong, J. G., Beckmann, J. S., van der Meer, J. W., Delpech, M. (1999) Mutations in the gene encoding mevalonate kinase cause hyper-IgD and periodic fever syndrome. International Hyper-IgD Study Group. Nat. Genet. 22, 178-81.
226. Hoffman, H. M., Mueller, J. L., Broide, D. H., Wanderer, A. A., Kolodner, R. D. (2001) Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. Nat. Genet. 29, 301-5.
227. Aksentijevich, I., C, D. P., Remmers, E. F., Mueller, J. L., Le, J., Kolodner, R. D., Moak, Z., Chuang, M., Austin, F., Goldbach-Mansky, R., Hoffman, H. M., Kastner, D. L. (2007) The clinical continuum of cryopyrinopathies: novel CIAS1 mutations in North American patients and a new cryopyrin model. Arthritis Rheum. 56, 1273-85.
228. Simon, A., Park, H., Maddipati, R., Lobito, A. A., Bulua, A. C., Jackson, A. J., Chae, J. J., Ettinger, R., de Koning, H. D., Cruz, A. C., Kastner, D. L., Komarow, H., Siegel, R. M. (2010) Concerted action of wild-type and mutant TNF receptors enhances inflammation in TNF receptor 1-associated periodic fever syndrome. Proc. Natl. Acad. Sci. U. S. A. 107, 9801-6.
229. Ryan, J. G. and Kastner, D. L. (2008) Fevers, genes, and innate immunity. Curr. Top. Microbiol. Immunol. 321, 169-84.
230. Almeida de Jesus, A. and Goldbach-Mansky, R. (2013) Monogenic autoinflammatory diseases: concept and clinical manifestations. Clin. Immunol. 147, 155-74.
231. Onen, F. (2006) Familial Mediterranean fever. Rheumatol. Int. 26, 489-96.
232. Ben-Chetrit, E. and Touitou, I. (2009) Familial mediterranean Fever in the world. Arthritis Rheum. 61, 1447-53.
233. Wekell, P., Friman, V., Balci-Peynircioglu, B., Yilmaz, E., Fasth, A., Berg, S. (2013) Familial Mediterranean Fever -- an increasingly important childhood disease in Sweden. Acta Paediatr. 102, 193-8.
234. Berg, S. and Fasth, A. Autoinflammatory disorders. In Primary Immunodeficiency Disorders. Edited by: Rezaei N., Agahamohammadi A., Notarangelo, L. D. (2008) Springer. 215-233
235. Chae, J. J., Cho, Y. H., Lee, G. S., Cheng, J., Liu, P. P., Feigenbaum, L., Katz, S. I., Kastner, D. L. (2011) Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1beta activation and severe autoinflammation in mice. Immunity 34, 755-68.
236. Booty, M. G., Chae, J. J., Masters, S. L., Remmers, E. F., Barham, B., Le, J. M., Barron, K. S., Holland, S. M., Kastner, D. L., Aksentijevich, I. (2009) Familial Mediterranean fever with a single MEFV mutation: where is the second hit? Arthritis Rheum. 60, 1851-61.
237. Rebelo, S. L., Bainbridge, S. E., Amel-Kashipaz, M. R., Radford, P. M., Powell, R. J., Todd, I., Tighe, P. J. (2006) Modeling of tumor necrosis factor receptor superfamily 1A mutants associated with tumor necrosis factor receptor-associated periodic syndrome indicates misfolding consistent with abnormal function. Arthritis Rheum. 54, 2674-87.
238. Aksentijevich, I., Masters, S. L., Ferguson, P. J., Dancey, P., Frenkel, J., van Royen-Kerkhoff, A., Laxer, R., Tedgard, U., Cowen, E. W., Pham, T. H., Booty, M., Estes, J. D., Sandler, N. G., Plass, N., Stone, D. L., Turner, M. L., Hill, S., Butman, J. A., Schneider, R., Babyn, P., El-Shanti, H. I., Pope, E., Barron, K., Bing, X., Laurence, A., Lee, C. C., Chapelle, D., Clarke, G. I., Ohson, K., Nicholson, M., Gadina, M., Yang, B., Korman, B. D., Gregersen, P. K., van

Hagen, P. M., Hak, A. E., Huizing, M., Rahman, P., Douek, D. C., Remmers, E. F., Kastner, D. L., Goldbach-Mansky, R. (2009) An autoinflammatory disease with deficiency of the interleukin-1-receptor antagonist. N. Engl. J. Med. 360, 2426-37.
239. Reddy, S., Jia, S., Geoffrey, R., Lorier, R., Suchi, M., Broeckel, U., Hessner, M. J., Verbsky, J. (2009) An autoinflammatory disease due to homozygous deletion of the IL1RN locus. N. Engl. J. Med. 360, 2438-44.
240. Bellehumeur, C., Blanchet, J., Fontaine, J. Y., Bourcier, N., Akoum, A. (2009) Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Hum. Reprod. 24, 2193-204.
241. Cochard, M., Clet, J., Le, L., Pillet, P., Onrubia, X., Gueron, T., Faouzi, M., Hofer, M. (2010) PFAPA syndrome is not a sporadic disease. Rheumatology (Oxford). 49, 1984-7.
242. Førsvoll, J., Kristoffersen, E. K., Øymar, K. (2013) Incidence, clinical characteristics and outcome in Norwegian children with periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis syndrome; a population-based study. Acta Paediatr. 102, 187-92.
243. Schulman, S. (1999) Periodic fever: A not-so-rare pediatric syndrome. American Academy of Pediatrics Grand Rounds 2, 39-40.
244. Marshall, G. S., Edwards, K. M., Butler, J., Lawton, A. R. (1987) Syndrome of periodic fever, pharyngitis, and aphthous stomatitis. J. Pediatr. 110, 43-6.
245. Marshall, G. S., Edwards, K. M., Lawton, A. R. (1989) PFAPA syndrome. Pediatr. Infect. Dis. J. 8, 658-9.
246. Thomas, K. T., Feder, H. M., Jr., Lawton, A. R., Edwards, K. M. (1999) Periodic fever syndrome in children. J. Pediatr. 135, 15-21.
247. Gattorno, M., Caorsi, R., Meini, A., Cattalini, M., Federici, S., Zulian, F., Cortis, E., Calcagno, G., Tommasini, A., Consolini, R., Simonini, G., Pelagatti, M. A., Baldi, M., Ceccherini, I., Plebani, A., Frenkel, J., Sormani, M. P., Martini, A. (2009) Differentiating PFAPA syndrome from monogenic periodic fevers. Pediatrics 124, e721-8.
248. Feder, H. M. and Salazar, J. C. (2010) A clinical review of 105 patients with PFAPA (a periodic fever syndrome). Acta Paediatr. 99, 178-84.
249. Burton, M. J., Pollard, A. J., Ramsden, J. D. (2010) Tonsillectomy for periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis syndrome (PFAPA). Cochrane Database Syst Rev, CD008669.
250. Garavello, W., Romagnoli, M., Gaini, R. M. (2009) Effectiveness of adenotonsillectomy in PFAPA syndrome: a randomized study. J. Pediatr. 155, 250-3.
251. Renko, M., Salo, E., Putto-Laurila, A., Saxen, H., Mattila, P. S., Luotonen, J., Ruuskanen, O., Uhari, M. (2007) A randomized, controlled trial of tonsillectomy in periodic fever, aphthous stomatitis, pharyngitis, and adenitis syndrome. J. Pediatr. 151, 289-92.
252. Chiesa, S., Pelagatti, M., Finetti, M., Prigione, I., Caorsi, R., Lasiglie, D., D'Agostino, R., Sementa, A., Omenetti, A., Traggiai, E., Martini, A., Gattorno, M. (2011) Characterization of tonsil infiltration and gene expression profile of innate sensors in PFAPA patients. Pediatric rheumatology online journal 9, O30.
253. Long, S. S. (1999) Syndrome of Periodic Fever, Aphthous stomatitis, Pharyngitis, and Adenitis (PFAPA)--what it isn't. What is it? J. Pediatr. 135, 1-5.
254. Padeh, S., Brezniak, N., Zemer, D., Pras, E., Livneh, A., Langevitz, P., Migdal, A., Pras, M., Passwell, J. H. (1999) Periodic fever, aphthous stomatitis, pharyngitis, and adenopathy syndrome: clinical characteristics and outcome. J. Pediatr. 135, 98-101.
255. Wurster, V. M., Carlucci, J. G., Feder, H. M., Jr., Edwards, K. M. (2011) Long-term follow-up of children with periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis syndrome. J. Pediatr. 159, 958-64.
256. Førsvoll, J. A. and Øymar, K. (2007) C-reactive protein in the periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis (PFAPA) syndrome. Acta Paediatr. 96, 1670-3.
257. Dinarello, C. A. (2007) Mutations in cryopyrin: bypassing roadblocks in the caspase 1 inflammasome for interleukin-1beta secretion and disease activity. Arthritis Rheum. 56, 2817-22.
258. Farber, J. M. (1997) Mig and IP-10: CXC chemokines that target lymphocytes. J. Leukoc. Biol. 61, 246-57.
259. Kessel, C., Holzinger, D., Foell, D. (2012) Phagocyte-derived S100 proteins in autoinflammation: Putative role in pathogenesis and usefulness as biomarkers. Clin. Immunol. 147, 229-41.
260. Sunahori, K., Yamamura, M., Yamana, J., Takasugi, K., Kawashima, M., Yamamoto, H., Chazin, W. J., Nakatani, Y., Yui, S., Makino, H.

(2006) The S100A8/A9 heterodimer amplifies proinflammatory cytokine production by macrophages via activation of nuclear factor kappa B and p38 mitogen-activated protein kinase in rheumatoid arthritis. Arthritis research & therapy 8, R69.
261. Tamayo, E., Gomez, E., Bustamante, J., Gomez-Herreras, J. I., Fonteriz, R., Bobillo, F., Bermejo-Martin, J. F., Castrodeza, J., Heredia, M., Fierro, I., Alvarez, F. J. (2012) Evolution of neutrophil apoptosis in septic shock survivors and nonsurvivors. J. Crit. Care 27, 415e1-11.
262. Chen, A. and Seifert, H. S. (2011) Neisseria gonorrhoeae-mediated inhibition of apoptotic signalling in polymorphonuclear leukocytes. Infect. Immun. 79, 4447-58.
263. Blomgran, R., Patcha Brodin, V., Verma, D., Bergström, I., Söderkvist, P., Sjöwall, C., Eriksson, P., Lerm, M., Stendahl, O., Särndahl, E. (2012) Common genetic variations in the NALP3 inflammasome are associated with delayed apoptosis of human neutrophils. PloS one 7, e31326.
264. Christenson, K., Björkman, L., Karlsson, A., Bylund, J. (2013) Regulation of Neutrophil Apoptosis Differs after in vivo Transmigration to Skin Chambers and Synovial Fluid: A Role for Inflammasome-Dependent Interleukin-1beta Release. Journal of innate immunity. 5, 377-88.
265. Hirata, J., Kotani, J., Aoyama, M., Kashiwamura, S., Ueda, H., Kuroda, Y., Usami, M., Okamura, H., Marukawa, S. (2008) A role for IL-18 in human neutrophil apoptosis. Shock 30, 628-33.