Dual Role of Neutrophils in Inflammation

Janesh Pillay

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Dual Role of Neutrophils in Inflammation

Tweeledige Rol van Neutrofielen in Inflammatie
(met een samenvatting in het Nederlands)

Proefschrift

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Janesh Pillay

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Prof. dr. L. Koenderman
Prof. dr. L.P.H. Leenen

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To Dad, Mum, Darsin, Zoran,
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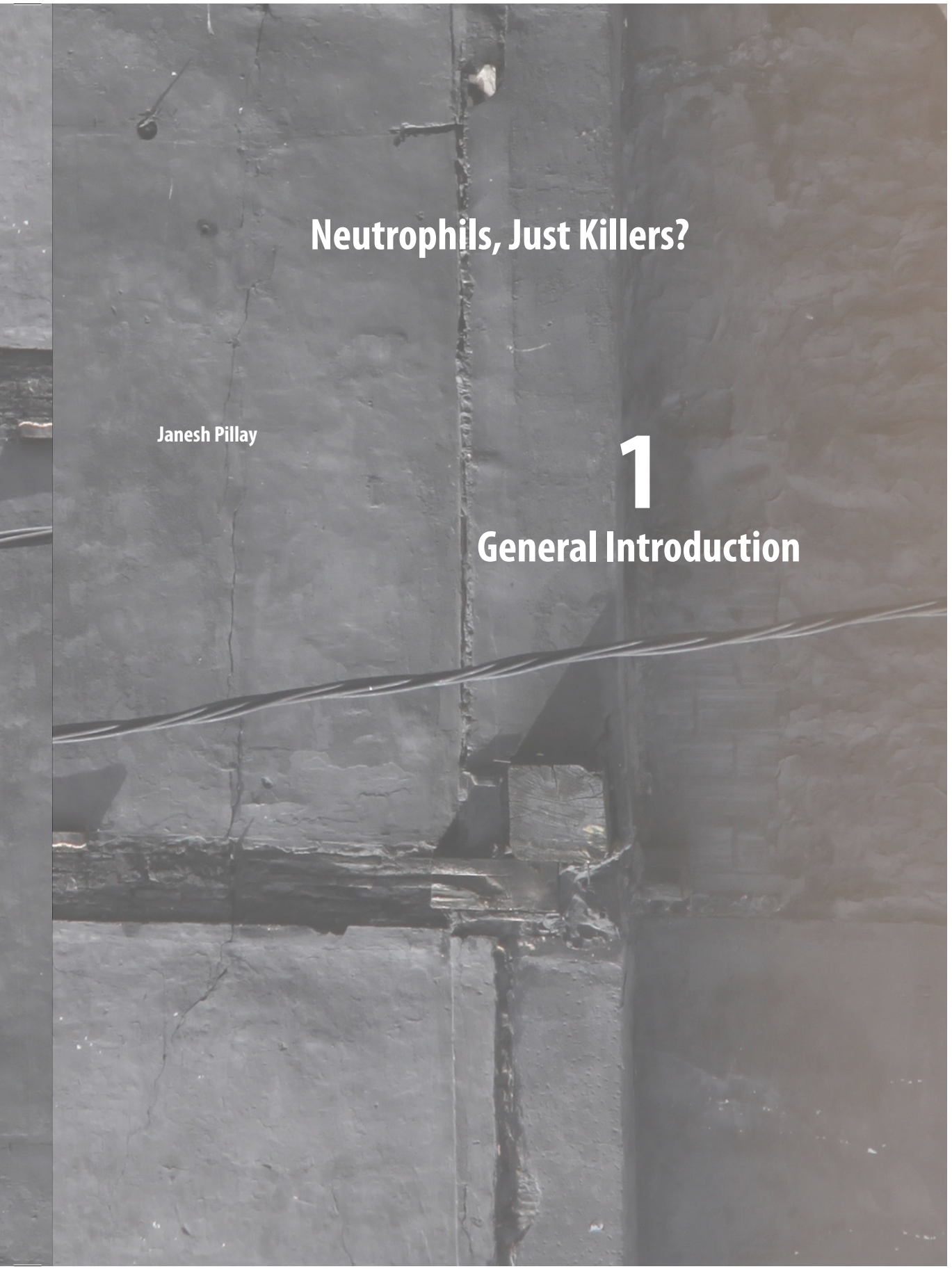
Contents



Neutrophils, Just Killers?

Janesh Pillay

1 General Introduction

Protecting the host: killing the invaders

Neutrophils are essential in mediating effective host defense against invading microorganisms. These cells are abundant in the circulation and comprise up to 70% of total circulating leukocytes under homeostatic conditions in humans. Upon invasion of the host by microorganisms these cells are rapidly recruited to the site of infection. Neutrophils posses a large repertoire of anti-microbial mechanisms such as phagocytosis of microbes, activation of NADPH-oxidase, and an array of anti-microbial peptides and proteins pre-stored in their granules. Recently a new mechanism has been identified that facilitates microbial killing: NET formation. This mechanism is mediated by expulsion of DNA, which forms an extracellular trap that facilitates the immobilization and binding of microorganisms. These major antimicrobial mechanisms will briefly be discussed.

Neutrophil priming and activation

Neutrophils possess powerful mechanisms for microbial killing. However many of these mechanisms described below can lead to tissue damage when insufficiently controlled. Neutrophil activation by soluble stimuli, such as tissue factors and cytokines, is involved in neutrophil hyperactivation. Neutrophils possess a control mechanism in which pre-activation (priming) is necessary before a stimulus can lead to full activation of cytotoxic function such as the respiratory burst[1]. For example, quiescent neutrophils do not produce a respiratory burst on stimulation with fMLF. 'Priming' of these neutrophils with platelet activating factor before stimulation with fMLF results in full activation of the respiratory burst[2]. Priming and activation are differentially regulated for various neutrophil functions. For instance, PAF is a potent primer for the respiratory burst, however in the same concentration activates neutrophil degranulation and chemotaxis[3]. Apart from the protection against aberrant neutrophil activation, priming can lead to enhancement of neutrophil antimicrobial activity[4].

Phagocytosis – engulfment of pathogens

Neutrophils are professional phagocytes, which use their immunoglobulin receptors FcgR (IgG receptors: CD16 and CD32) and FcaRI (IgA receptor: CD89) to bind and engulf IgG- and IgA - opsonized microorganisms respectively[5,6]. Microorganisms opsonized by complement are phagocytosed by neutrophils by engagement of CR1 (CD35) and αMβ2 integrin (Mac-1, CD11b/CD18). These are receptors for C3b and C3bi respectively. Other complement receptors on the surface of neutrophils, such as C5aR (CD88) do not directly participate in phagocytosis, but are involved in neutrophil activation[7].

Synergy between complement receptors and Ig-receptors has been reported as engagement of Mac-1 (CD11b/CD18) facilitates phagocytosis of IgG and IgA opsonized particles[8]. Mac-1 is rapidly upregulated on the surface of the neutrophil upon activation by cytokines and chemokines[9]. The αMβ2 integrin (Mac-1) is also the major receptor on human neutrophils for β-glucan, a polysaccharide mainly expressed on yeasts and

fungi[10]. Other receptors for opsonized particles includes the CR1 receptor (CD35), which binds C3b and mannose binding lectin[11].

Apart from opsonin receptors binding to opsonized microorganisms, neutrophils posses a large array of receptors directly recognizing microbial structures. These 'pattern-recognition' receptors include but are not limited to the Toll-like Receptors (TLR) and the fMLF-receptor[12,13]. Engagement of these receptors by these pathogen associated molecular patterns (PAMP's) enhances several neutrophil functions, which are important for pathogen clearance, such as chemotaxis towards the site of infection and phagocytosis of pathogens[14,15].

After phagocytosis of micro-organisms, neutrophils kill ingested pathogens by activating powerful antimicrobial mechanisms. Pathogens are engulfed into a specialized phagolysosome compartment. When this phagosome is formed it is not immediately antimicrobial. Sequential interactions are required to form an antimicrobial phagosome[16]. This results in large changes of the phagosomal contents and membrane. Translocation and fusion with preformed neutrophil granules is required for the formation of an antimicrobial phagolysosome. In short, neutrophil azurophilic granules containing a range of anti-microbial peptides such as myeloperoxidase (MPO), elastase and bactericidal/permeability increasing protein, fuse with and release their content into the the phagosome[17]. In addition, secondary granules, which are a major source of phagosomal NADPH oxidase, fuse with the phagosome.

Fusion of granules with the phagosome is essential for killing pathogens as is illustrated by the mechanisms by which pathoghens are able to live and replicate within the phagosome. The parasite Leishmania major, once phagocytosed by a neutrophil, specifically recruited azurophilic granules to the phagosome, however tertiary and specific granules, involved in vacuole acidification and superoxide anion generation did not fuse with the phagosome, allowing the parasite to survive inside the neutrophil[18]. Mycobacteria reduce the fusion of azurophilic granules with the phagosome, enabling their survival within neutrophils[19,20]. These strategies used by the pathogens demonstrate that inhibition of fusion of either azurophil granulus (containing MPO and elastase/cathepsin G) or specific and tertiary granules is sufficient to limit the phagosome's antimicrobial capacity. This shows that synergy between granule contents is needed for effective microbial killing. The NADPH-oxidase, one of these granule contents, best explains this synergy:

There are multiple mechanisms underlying the role of NADPH-oxidase in anti-microbial killing. Firstly, hydrogen peroxide allows MPO, which is present in the phagolysome, to convert chloride and iodide to hypochlorous acid and iodine, respectively. These are potent anti-microbial metabolites[21]. Secondly, the generation of superoxide anion, which is rapidly converted to hydrogen peroxide, lowers the intraphagosomal pH required for effective anti-microbial function of proteases such as elastase and cathepsin G[22].

The reactive oxygen species (ROS) produced by the NADPH oxidase system do not remain confined to the phagolysosome. This can be seen in figure 1. in which serum-

opsonized zymosan (green) is phagocytosed by neutrophils resulting in a localized intracellular generation of hydrogen peroxide (red-orange) as depicted by the arrows. Importantly, in time, the extracellular environment becomes red-orange as well, suggesting leakage of hydrogen peroxide into the microenvironment when neutrophils phagocytose and kill microorganisms. This extracellular production of ROS is thought to play a minor role in antimicrobial killing, as they readily diffuse and are rapidly converted to less harmful metabolites, thus high concentrations of ROS are required for extracellular microbial killing[23]. However, ROS may contribute to microbial killing and tissue damage together with neutrophil granule content as described above when extracellular ROS is accompanied by neutrophil degranulation. In contrast, extracellular ROS in high concentrations might contribute to neutrophil apoptosis, decreased neutrophil activation and suppression of adaptive immunity[24-26].

Degranulation – release of anti-microbial contents into the microenvironment

Not all microbes can be phagocytosed effectively, such as large parasites or bacteria which create biofilms[27]. Therefore, neutrophils possess additional anti-microbial mechanisms. Neutrophils can deploy a vast array of anti-microbial factors extracellularly, which can disrupt the microbial cell wall and membrane and/or inhibit microbial growth. These factors consist of highly charged peptides and proteins such as defensins, chathelicidins (LL-37), myeloperoxidase (MPO), bacterial permeability increasing protein, cationic serine proteases (serocidins) such as neutrophil elastase (NE), cathepsin G, proteinase 3, and azurocidin. These factors are stored in neutrophil granules, specialized compartments within the neutrophil cytoplasm[28]. These granules contain oxidase-dependent and oxidase-independent killing mechanisms. They can either fuse and degranulate within the phagolysosome, for intracellular killing of ingested pathogens as described above, or fuse with the extracellular membrane, thereby releasing anti-microbials in the direct vicinity of infections.

At least four different types of neutrophil granules exist, with different functions, which can be deployed separately from each other[29]. These types are: 1. Highly mobilizable secretory vesicles, 2. peroxidase negative tertiary and 3. secondary granules (gelatinase and specific granules respectively) and 4. peroxidase positive primairy granules (azurophilic granules).

Azurophil and specific granules contain the most anti-microbial peptides and proteins and are under a more stringent control of degranulation compared to secretory vesicles and secondary granules. Secretory vescicles and secondary granules contain neutrophil surface proteins such as CD11b/CD18 (Mac-1)[9]. These pre-stored proteins enable neutrophils to rapidly upregulate essential surface receptors upon activation, which are necessary for cellular adhesion and phagocytosis. The current hypothesis is that sequential release of these granules represents different stages of neutrophil activation; Fusion of secretory vesicles with the neutrophil membrane is implicated to facilitate

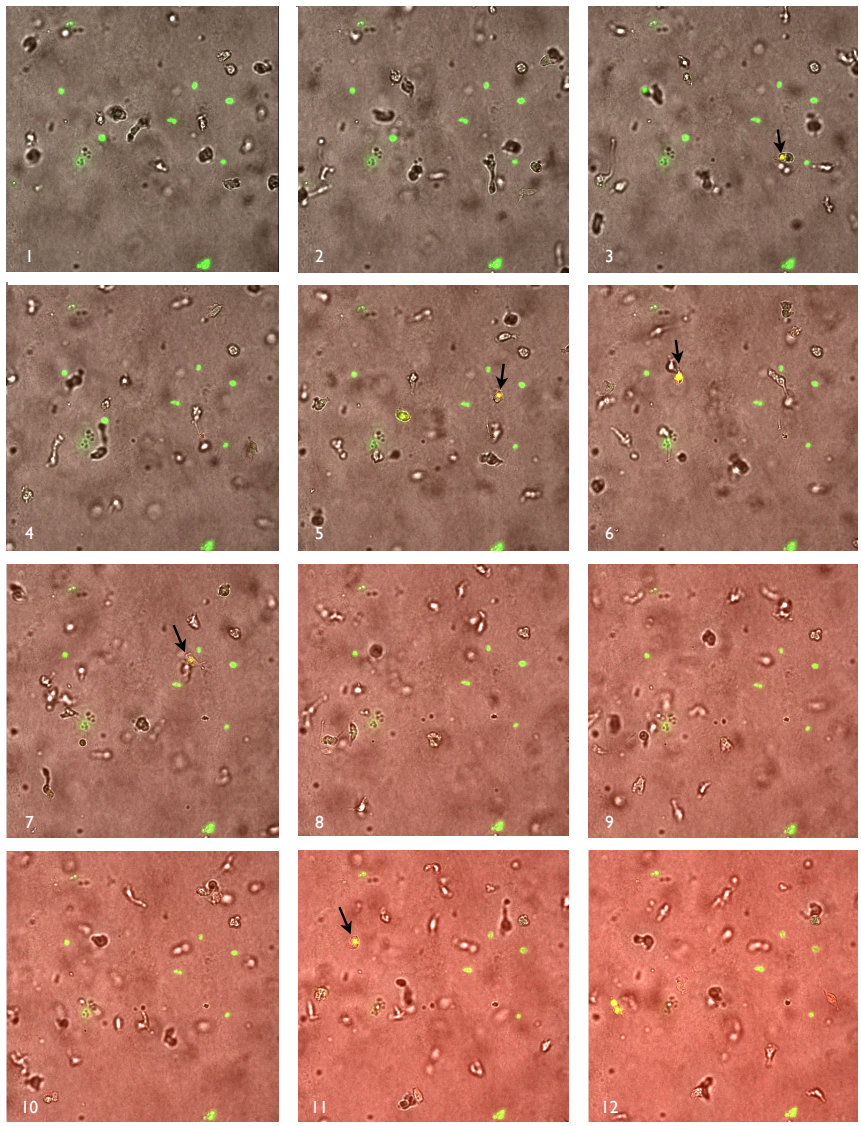


Figure 1. Phagocytosis of FITC (green) labeled zymosan by neutrophils in a fibrin matix. Neutrophils and serum opsonized FITC labeled zymosan were trapped in a fibrin gel in the presence of HEPES3+ containing Amplex Red probe for detection of hydrogenperoxide. Images 1 to 12 represent sequential timepoints of up to 15 minutes. Arrows represent phagocytosing neutrophils. After phagocytosis neutrophils generate an intracellular respiratory burst (orange colour) as depicted by the arrows indicating phagocytosis. Of note is the general oxidative environment these activated phagocytosing neutrophils create as shown by a gradually increasing background redness, which may cause further immune activation and collateral tissue damage.

neutrophil egression from the circulation by increasing the surface expression of integrins and integrin associated proteins such as CD47[30]. In addition, matrix metalloproteases

such as MMP-8 stored in specific granules facilitates neutrophil migration by degradation of collagen matrices[31].
Arrived at the site of infection, additional signals allow fusion of specific and azurophil granules with the plasma membrane, thus releasing potent anti-microbial proteins into the environment. Cytotoxic proteins, such as myeloperoxidase (MPO) are converted by hydrogen peroxide to hypochlorous acid, and contribute to killing of extracellular pathogens[32].
Unfortunately, release of granular MPO into the microenvironment, combined with extracellular release of hydrogenperoxide by other neutrophils, either during phagocytosis or by hyperactivation might greatly contribute to neutrophil induced tissue damage.

Neutrophil Extracellular Traps

A recent discovery has added a third anti-microbial strategy to the neutrophils arsenal[33]. Extensive *in vitro* research revealed that activated neutrophils enter a cell-death program, distinct from the classical apoptosis or cell necrosis, in which the nuclear and granular membranes dissolve and the nuclear contents condense into the cytoplasm[34]. Finally the plasma membrane ruptures and chromatin decorated with granular anti-microbial proteins is released into the extracellular space[35]. It is still unknown how this process is triggered or regulated, however the RAF-MERK-ERK pathway has recently been shown to be necessary for NADPH-oxidase dependent NET formation[36]. In addition, both elastase and myeloperoxidase are implicated to dissolve the granular membrane, allowing nuclear contents to condense in the cytoplasm[37,38].
NETs share anti-microbial features of the classical killing mechanisms. Firstly, the NADPH-oxidase is involved in the formation of NETs[35]. Secondly, the chromatin structures are covered with anti-microbial granule proteins, such as elastase[33].
These initial reports of NET formation were based on *in vitro* simulation with PMA, and were shown to be NADPH oxidase dependent[33]. This concept was strengthened by the observation that neutrophils acquired from CGD patients cannot form NETs after stimulation with PMA and Aspergillus nidulans[39]. In addition, genetic restoration of the NADPH – gene results in restoration of NET formation[39].
Recently alternative mechanisms have been shown for *in vitro* and *in vivo* NET generation, which are NADPH oxidase independent[40,41]. Hartl et al. showed that neutrophils in chronic inflammation require CXCR2 for their NADPH-oxidase independent *in vivo* NET generation[40]. However the exact mechanisms for NET release of neutrophils when activated by pathogens such as S. aureus, anti-neutrophil antibodies, activated platelets, have not been studied and thus remain elusive[41-43].
In vivo NET formation has been documented under several pathological condition[42,43]. In sepsis neutrophils can generate NETs within minutes under flow conditions, when exposed to platelets and TLR4 ligands[42]. Other reports have shown NET formation in pneumonia and aspergillus infections[44,45].

It should be noted that interpretation of data on *in vivo* detection of NETs is much more difficult. It was recently reported that fibrin deposits could not be distinguished from NETs by scanning electron microscopy (SEM) on the basis of morphological criteria[46]. In addition, both NETs and fibrin deposits are sensitive to DNAse-I cleavage, an experimental setup often used to confirm the presence of NETs[33]. It has recently been shown that fibrin strands can be formed around bacteria and contribute to bacterial containment *in vivo*[47,48]. Distinguishing NETs from fibrin strands in inflammation may further be complicated by free circulating neutrophil degranulation products such as elastase, circulating free DNA and histones released in systemic inflammation, binding to these fibrin strands[49]. However it is still unknown whether fibrin strands formed intravascular can bind free DNA and circulating neutrophil antimicrobial products thereby mimicking neutrophil NETs.

Whatever their origin, the added value of NETs in comparison to the previously mentioned anti-microbial strategies (phagocytosis and degranulation) may be to promote the physical containment of bacteria. This allows synergy between anti-microbial agents and their containment in the vicinity of the neutrophil.

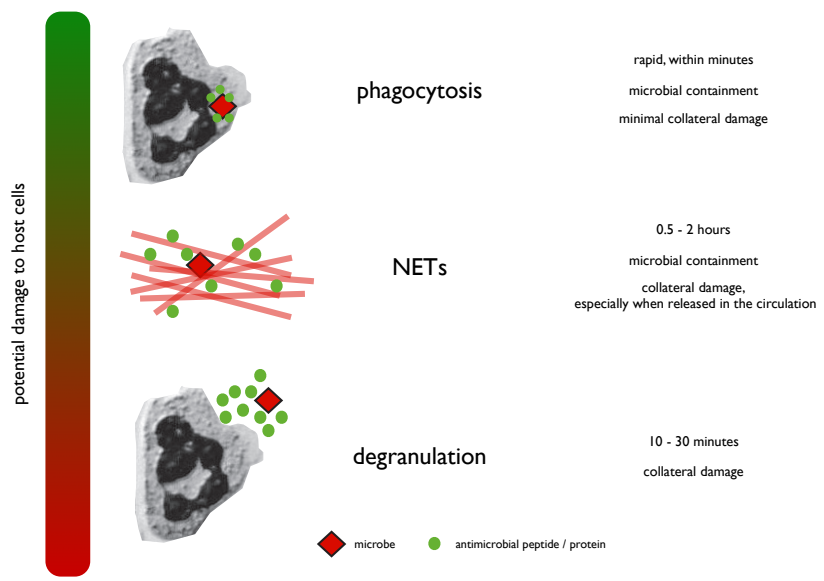


Figure 2. Choice of neutrophil antimicrobial strategy. Adapted from Papayannopoulos et al.[53] Neutrophil deployment of anti-microbial strategy may be determined by their activation state and the duration of activating stimuli. Phagocytosis occurs within minutes, degranulation within 30 minutes and the formation of NETs takes hours *in vitro*. Phagocytosis of microorganisms results in relatively little collateral damage, whereas neutrophil degranulation has the potential cause major damage to the surrounding tissue.

However, extracellular DNA covered by cytotoxic and antimicrobial proteins, such as histones, might lead to unwanted tissue damage as is suggested in conditions such as sepsis, SLE and vascular thrombosis[43,50-52].

Choice of anti-microbial strategy

As reviewed above, neutrophils posses at least three major antimicrobial strategies. The question arises how the choice for the different strategies is made by the neutrophils under different conditions (figure 2). An interesting hypothesis suggested by Papayannopoulos et al. is that the timing of initiation of antimicrobial strategies is important for the hierarchy in these events[53]. Neutrophils can engage microbes *ex vivo* by phagocytosis within seconds to minutes. The rate of neutrophil degranulation varies on the type of granules but the fastest secretory vesicles fuse with the membrane within seconds after stimulation[29,54]. The anti-microbial specific and azurophil granules are released within minutes of neutrophil stimulation[29]. NET formation occurring in minutes to hours seems to be an endstage process *ex vivo*, which is coupled to neutrophil death.

Killing the host

Genetic mutations, which result in disorders of neutrophil functions and numbers, are very rare, but illustrate the importance of neutrophils to immunity.

1. Severe congenital neutropenia (SCN); neutrophil numbers are severely decreased in SCN, due to an arrest in neutrophil development at the pro-myelocytic stage. This arrest is due to a variety of genetic mutations reviewed by Bouma et al.[55]. These patients present within their first year of life with life-threatening infections[56].
2. Chronic granulomatous disease (CGD); as discussed above the NADPH-oxidase is important for microbial killing by neutrophils. Patients lacking functional NADPH-oxidase through genetic mutations leading to defect in any one of its components, fail to produce a respiratory burst. This leads to susceptibility to infections by bacteria, particularly Aspergillus species, S. aureus and Salmonella species[57].
3. Chediak-Higashi and specific granule deficiency (SGD); both disorders affect neutrophil granules. Chediak-Higashi syndrome is cause by a mutation in the LYST-gene, which encodes a lysosomal trafficking regulator. Lack of this protein results in neutrophils displaying giant granules with impaired degranulation and phagolysosome generation[58]. SGD neutrophils lack essential granule proteins such as lactoferrin caused by a mutation in the CEBPE gene[59,60]. Patients suffering from Chediak-Higashi or SGD are extremely susceptible to pyogenic infections.
4. Leukocyte adhesion deficiency (LAD); essential for neutrophil antimicrobial functions is effective exit from the bloodstream, migration to the site of infections and phagocytosis of pathogens. β2-integrins play an important role in all of these functions[61-64]. Patients suffering from leukocyte adhesion deficiency type I have neutrophils with very low expression or lacking β2-integrins. Unless treated by haematopoetic stem cell

transplantation LAD results in lethal non-pussing infections[65].

The above-discussed genetic mutations are only few deficiencies in neutrophil function to illustrate the importance of neutrophil antimicrobial defense.
Apart from lack of function causing suppressed immunity, hyperactivation of neutrophils can cause tissue damage. As neutrophils possess powerful mechanisms to eradicate pathogens, use of these potentially damaging tools must be specific and controlled to ensure minimal damage to the host tissue. Proper recognition of pathogens by neutrophils is essential to ensure effective clearance and minimal tissue damage. Neutrophils posses several pattern recognition receptors such as the fMLF –receptor, TLRs and receptors for the recognition of bacterial polysaccharides[12,13]. In addition, recognition and phagocytosis of pathogens is facilitated by their opsonization. Opsonization with complement or immunoglobulins allows neutrophils to specifically recognize pathogens, through their complement receptors and FcR, respectively[66]. However, neutrophil activation even during phagocytosis is accompanied by extracellular ROS production or their leakage. This is shown in figure 1 as phagocytosis of serum opsonized zymosan by neutrophils is accompanied by leakage of ROS into the environment causing oxidation of the Amplex Red, which is a specific probe for H_2O_2.
Deposition of opsonins such as complement or immuneglobulins to ischemic cells is thought to result in neutrophil activation and tissue damage in ischemia reperfusion injury[67,68]. In a similar way, neutrophil FcRs can cause detrimental immune reactions in patients receiving blood products as can occur in transfusion related lung injury (TRALI)[69,70].
Neutrophil degranulation can occur when these cells are activated by soluble stimuli or a combination of soluble stimuli and neutrophil adhesion[71]. This results in the release of cytotoxic proteins and peptides into the environment. Combined with extracellular ROS production these proteins and peptides (such as MPO and elastase) contribute to tissue damage and organ failure in critically ill patients[72,73].
Neutrophil priming and activation has been causally linked to the acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) in various animals, although other mechanisms resulting in lung damage may exist[74,75]. In addition, products secreted from neutrophils increase vascular endothelial permeability increasing fluid leakage and inflammation and may be important in the development of ARDS and ALI[76]. Finally the most recent discovered neutrophil anti-microbial function (NETs) might not be deployed without collateral damage. Kubes et al. suggest that *in vitro* and *in vivo* generation of NETs in sepsis is accompanied with host tissue damage[42]. Neutrophils and platelets activated with LPS produce NETs. This activation is accompanied by liver damage *in vivo*, which can be prevented by platelet and neutrophil depletion. However this does not causally link NETs to tissue damage as neutrophil degranulation also occurs. In contrast, it is tempting to speculate that binding of histones and antimicrobial proteins to DNA strands

might prevent them from freely circulating, thus protecting the host.

Endogenous inhibitors of neutrophil activation

As aberrant neutrophil activation *in vivo* can lead to detrimental consequences as described above, tight control of their functions is necessary. Apart from intrinsic control such as neutrophil priming and their presumed short lifespan, endogenous inhibitors of neutrophil functions are scarce. Glucocorticoids are powerful inhibitors of the adaptive immune system[77]. However they have not proven to be useful to limit inflammation induced by the innate immune system and may even lead to increased neutrophil survival and activation[78,79].

Endogenous inhibitors of neutrophil function can be found within the inflammatory environment. Proteases cleave neutrophil receptors such as CD62L and CXCR1, leading to down regulation of neutrophil function[80,81]. Factors isolated from exudates of resoving inflammation have revealed promising anti-inflammatory agents. 'Resolvins' have been shown to limit neutrophil chemotaxis to inflammatory foci and prevented β2-integrin upregulation[82]. Neutrophils have been shown to posses an anti-inflammatory feedback mechanism. Upon activation and adhesion to endothelial cells annexin-1 is mobilized and externalized. This limited subsequent neutrophil transmigration[83].

It is tempting to speculate that inhibitors of neutrophil function can be found in the circulation, as excessive circulating neutrophil activation is highly unwanted. Serum albumin has been shown to inhibit neutrophil functions by blocking the shedding of CD43[84]. In addition endothelial DEL-1 is upregulated in inflammation on vascular endothelial cells and limits neutrophil recruitment to the tissues[85]. Acute phase proteins have the potential to be powerful regulations of neutrophil functions as they are upregulated inflammation when they are most needed. Two such proteins which are upregulated in acute inflammation will be evaluated in this thesis: soluble fibrinogen and C1-esterase inhibitor.

Neutrophil lifespan

Perfectly in line with neutrophil classical antimicrobial function is their short lifespan. Neutrophils have been reported to leave the circulation within hours after exit from the bone marrow, and their tissue half-life is unknown but presumed to be short (7 hours)[86]. The classical hypothesis is that neutrophils home to tissues and are cleared by tissue macrophages when they become apoptotic[87,88]. This has resulted in the general acceptance of a classical model in which neutrophils are generated in the bone marrow, circulate for a 8-12 hours and enter the tissues were they go into apoptosis (figure 3).

However, studies addressing neutrophil clearance from the circulation under homeostatic conditions have not been performed. Reduced *in vitro* neutrophil apoptosis in mice deficient in β2 integrins have been non-causally linked to neutrophilia observed in these animals[88]. In non-inflammatory conditions (pre-) apoptotic neutrophils are not observed

in the circulation.

Injection of low doses of endotoxin results in neutrophil sequestration in liver, lungs and spleen[87]. In this model P-selectin dependent neutrophil clearance by liver Kupffer cells has been observed. The pattern of this neutrophil sequestration is similar as seen during the injection of isolated (labeled) neutrophil in various murine and human studies[89,90]. Recently an additional mechanism has been proposed in mice in which neutrophil clearance from the circulation under homeostatic conditions has been attributed to the CXCR4-SDF1α axis[91]. Isolated neutrophils, express CXCR4 after 6-24 hours of *ex vivo* culture and home to the bone marrow in a CXCR4 dependent way when re-infused[92].

In vitro neutrophils enter their apoptotic program within 24 hours in the absence of growth factors and very few studies have reported substantially longer *in vitro* lifespans using cytokines and growth factors[93]. This short lifespan is thought to be an important control mechanism to limit deleterious neutrophilic inflammation as their cytotoxic potential is contained by programmed cell death[94].

The *in vivo* lifespan of circulating neutrophils has been studied using techniques requiring neutrophil isolation and manipulation[86]. Neutrophils were labeled using incorporation of ^{3}H-thymidine *in vivo*, whereafter they were isolated and reinfused into another individual[86]. As an alternative, *in vivo* labeling with ^{32}P-DFP has been used which specifically labels neutrophil *in vivo*[95]. However, protease inhibition by DFP has been shown to influence neutrophil activation[86,96]. Neutrophil priming and activation by *ex vivo* manipulation leads to margination and non-homeostatic homing of cells to lung, liver and spleen[89,90]. A study using *in vivo* labeling with ^{3}H-thymidine without subsequent neutrophil isolation and reinfusion yielded a much longer neutrophil lifespan of 17 hours[97]. Although in this study chronic lymphatic leukemia (CLL) patients and patients with gliolastoma multiforma were used which might have influenced neutrophil homing through cytokine production[98,99]. This might have underestimated true homeostatic neutrophil half-life.

Their presumed short lifespan has limited research efforts directed at putative non-classical immune modulatory functions. This has supported the claim that neutrophils are mainly killers.

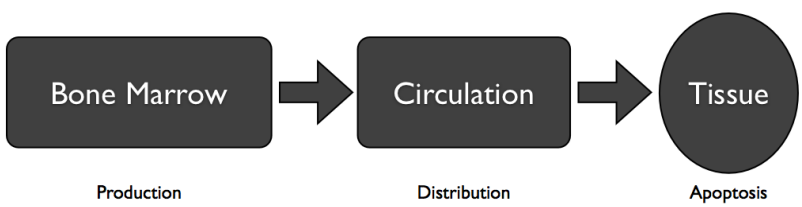


Figure 3. Classical neutrophil lifecycle.

Human endotoxemia as a tool to study neutrophil activation *in vivo*

The circulating neutrophil pool changes rapidly in response to inflammation. Primed or activated neutrophils rapidly sequester in the microvasculature of the lung, liver and spleen[89]. This sequestration is characterized by increased neutrophil transit time through the vasculature[90]. Sequestration can occur either through 'active' interactions with the vascular wall through selectin mediated rolling or through activation leading to increased rigidity of neutrophils, which consequently become trapped in the microvasculature[100]. In addition to rapid sequestration of activated neutrophils, it is known that within hours of inflammation large amounts of neutrophils are rapidly released from the post-mitotic pool in the bone marrow[101-103].

These rapid kinetics of circulating neutrophils complicate our understanding of neutrophil activation in systemic inflammation. Blood sampling of neutrophils might not assess the sequestered pool and freshly released non-activated neutrophils might 'dilute' signs of neutrophil activation. In most studies, which include patients with major burns, injuries or systemic infections (sepsis), sampling protocols usually stated that neutrophils were acquired within 12 hours of hospital admission. This has led to heterogeneity in sampling time. Moreover the exact onset of systemic inflammation was not known. As a result of the rapid kinetics of circulating neutrophil pool in inflammation, these sampling protocols have revealed no consensus on the *in vivo* neutrophil activation in humans.

An alternate way to study neutrophil activation in humans is the controlled induction of systemic inflammation, allowing accurate analysis of cellular activation and kinetics. Controlled systemic inflammation can be induced in humans in several ways. Injection of cytokines such as TNF has been performed. This resulted in a transient systemic inflammatory response with leukocytosis, neutrophil activation, changes in the coagulation cascade and metabolic changes similar to septic individuals[104-106].

A more physiological way is to systemically administer lipopolysaccharides of E.Coli (LPS). This Toll-like receptor 4 (TLR4) agonist induces a rapid and profound systemic immune response. This results in clinical symptoms corresponding with early systemic inflammatory response (increased heart rate, increased body temperature and vasodilatation), a transient cytokine storm, and leukocytosis[107]. These effects are transient, clinical symptoms subside after 2-3 hours whereas circulating cytokines and leukocytes remain elevated until 6-12 hours after LPS administration. This human inflammatory model was initially developed to simulate a state of sepsis and systemic infection.

During infection, the innate immune response is activated via microbial components (pathogen associated molecular patterns or PAMPs), while after injury the innate immune response is activated via damage associated molecular pattern (DAMPs). These consist of endogeneous cytosolic and mitochondrial components such as high mobility group box 1 (HMGB1), heat shock proteins, interleukin-1a (IL-1a), defensins and annexins[108,109].

Central to the recognition of both PAMPs and DAMPs are the pattern-recognition

receptors (PRRs)
The innate immune system recognizes micro-organisms via PRRs of which Toll-like receptors (TLR) are most well known[12]. In the human endotoxemia model LPS activates the immune system via TLR4 on antigen presenting cells, neutrophils and endothelial cells[110-112]. In addition, tissue injury induces release endogenous ligands such as mitochondrial peptides and DNA and activates the immune system via TLRs [74,109,113].
As PRRs recognize ligands from both microbial and endogenous origin, we hypothesize that inflammation induced by LPS administration and inflammation induced by tissue damage (severe injuries) is initiated through similar mechanisms. In this thesis we have studied both inflammation induced by LPS injection and induced by trauma.

Neutrophils - Not just killers
As reviewed above the last decades have firmly established the essential role for neutrophils in anti-microbial defense. Interestingly, neutrophil functions are emerging which suggest an additional more regulatory role for these cells in immune processes. In humans the discovery of these functions has been delayed because of the difficulties to culture viable neutrophils for longer than 24 hours. This short *in vitro* lifespan would not allow for novel immune regulatory functions, which might depend on protein synthesis in response to immune mediators, to fully emerge. Neutrophils stimulated *in vitro* can acquire MHCII and costimularory molecules CD80 and CD86[114]. In addition, these MCHII expressing neutrophils can present superantigens to T-cells[115].
As neutrophils respond rapidly to an inflammatory environment they come in close contact with a vast number of various immune cells, such as lymphocytes, macrophages, monocytes and dendritic cells. Neutrophils can interact with these immune cells and shape subsequent immune responses. For instance dying neutrophils, which are phagocytosed by macrophages shift immune responses of these cells towards a more anti-inflammatory cytokine profile, thus providing a negative regulation of inflammation[116,117]. In addition, dendritic cell interactions with neutrophil Mac-1 (CD11b/CD18) through dendritic cell DC-sign enables dendritic cells to more potently stimulate T-cell proliferation and results in a TH1 skewing profile[118]. In mice neutrophils are necessary for the induction of a TH1 response in response to various pathogens such as candida albicans and legionella pneumophila, as depletion of neutrophils results in TH2 skewing[119,120]. In these models neutrophil IL-12 is required for the TH1 inflammatory profile. Apart from producing cytokines that stimulate T-cell responses, murine neutrophils can suppress immune responses in mycobacterial infections by producing IL-10[121,122]. Neutrophils have been shown to suppress immunity through other mechanims apart from IL-10. Immature neutrophils are part of a heterogeneous group of cells referred to as myeloid-derived suppressor cells (MDSCs). These cells have been shown to directly suppress T-cell functions in murine models of cancer, infectious diseases, bone marrow transplantation and autoimmune diseases[123]. In humans, MDSCs have been identified in patients with

metastatic cancer, and consist of immature myeloid cells and granulocytes. However, mechanisms involved in induction of neutrophil immune suppressive or stimulative phenopytes are still unknown.

Scope of this thesis

The scope of this thesis is the response of the circulating neutrophil pool to human systemic inflammation, with a focus on neutrophil heterogeneity and immune modulation by neutrophils.

Systemic inflammation can cause complications due to immune suppression and immune hyperactivation, such as susceptibility to infections and organ failure respectively. To date these complications cannot adequately be predicted. In chapter 2 we will review the possibilities for immune monitoring with a focus on neutrophil surface expression. This leads to the realization that studies on neutrophil expression profiles in systemic inflammation (trauma and sepsis) yield conflicting results. We hypothesized that this was due to the unknown start of inflammation and the rapid kinetics of the circulating neutrophil pool. We, therefore, apply a model of human inflammation in which the exact timing of inflammation was known and serial blood sampling was possible (chapter 4, 5 and 6). Administration of LPS to humans induces systemic inflammation as described above.

The first question that is addressed is, '*Is the circulating neutrophil pool similarly activated in the human endotoxemia model and in patients with tissue injury?*' (chapter 4 and 6).

We use the human endotoxemia model to answer the following research questions.

Does functional neutrophil heterogeneity exist in systemic inflammation? (chapter 5 and 6)

Do any of these neutrophil subsets have immunemodulating functions? (chapter 6)

What is the mechanism of neutrophil induced T-cell suppression? (chapter 6)

In addition, we hypothesize that neutrophil heterogeneity would be reflected in their circulating lifespan. Therefore we firstly study the circulating lifespan of human neutrophils in homeostatic conditions. In chapter 3 we hypothesize that '*The circulating lifespan of in vivo labeled human neutrophils in much longer than previously reported*'.

Finally we investigate the possibility to limit systemic inflammation in order to reduce inflammatory complications. Two proteins upregulated within the acute phase of inflammation are studied, fibrinogen and C1-esterase inhibitor (C1INH).

We hypothesize that as soluble fibrinogen is a ligand of leukocyte integrin CD11b/CD18 and binds to ICAM-1, *Acute phase concentrations of fibrinogen limit neutrophil adherence to vascular endothelium* (chapter 7).

Finally we hypothesize that *C1INH can limit inflammation in vivo*, and use the human endotoxemia model to study this (chapter 8).

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Measurements of circulating neutrophil phenotypes to predict complications caused by dysregulated inflammation

Janesh Pillay
Leo Koenderman
Luke Leenen

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adapted from

The systemic inflammatory response induced by trauma is reflected by multiple phenotypes of blood neutrophils.

Pillay J, Hietbrink F, Koenderman L, Leenen LP.

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Inflammatory complications and systemic inflammation

Severe systemic inflammation as a consequence of injuries, major surgery and infections carries a risk for detrimental outcome. This is caused by immunological complications mediated by a dysfunctional immune system. This dysfunctionality can lead to impaired clearance of micro-organisms and overactivation of damaging immune mechanisms or both (figure 1).

The initial systemic inflammatory response is characterized by a robust activation of the innate immune system. The innate immune response consists of both a humoral and a cellular response[1]. The cellular response is typically mediated by neutrophils, monocytes and dendritic cells. The initial humoral response consists of bioactive proteins (cytokines, complement and acute phase proteins) and lipids (leukotrienes and platelet-activating factor)[2]. The initial cellular response is for an important part mediated by neutrophils. These cells are essential in the first line of defense to microbial threats. The amount of neutrophils can be rapidly increased by release from the bone marrow[3]. Apart from immune surveillance, innate immune cells play an important role in tissue repair mechanisms after tissue injury[4]. Aberrant activation of these cells, however contributes to the pathogenesis of many acute and chronic inflammatory diseases[5-7].

Following tissue injury, damaged local tissue releases humoral danger signals, which attract and activate neutrophils[8-10]. Excessive local activation of neutrophils in the tissues leads to a vicious circle of inappropriate homing, adhesion and activation of these cells and may result in subsequent tissue damage. The role of neutrophils has clearly been shown in various models of tissue injury, such as caused by reperfusion after peripheral ischemia. In these studies neutrophil influx into ischemic tissue is the main cause of further tissue damage[11-13].

As neutrophils are potentially damaging to the host, these cells require multiple signals to reach their full cytotoxic potential. Classically a first 'priming' stimulus is needed whereafter a second 'activating' stimulus is required for maximal responsiveness (figure 2). Primed circulating neutrophils are prone to home to and become activated in the tissues or vasculature when they encounter additional local inflammatory stimuli[14]. In particular, the lung is a preferred site for homing and activation of primed neutrophils due to the large microvascular bed and long cellular transit time[15,16]. As a consequence systemic neutrophil priming can lead to excessive local neutrophil entrapment and activation in the lung. This local activation occurs when lung vascular endothelium becomes damaged and/or activated[17]. Excessive neutrophil activation in the lung can lead to complications when increased permeability or destruction of the alveolar- capillary barrier occurs, resulting in influx of protein rich oedema fluid and impairment of arterial oxygenation. This is seen in clinical conditions such as the acute respiratory distress syndrome (ARDS) and acute lung injury (ALI).

Surgery and inflammation

Every type of tissue injury, including surgical intervention, leads to an inflammatory response[18]. Therefore, in patients with multiple injuries, additional tissue injury induced by surgical intervention can increase the overall inflammatory reaction, and thereby increase the risk of inflammatory complications. Surgical-induced tissue injury can be seen as an important risk factor for systemic inflammatory complications. This is particularly relevant in those patients who are already characterised by a borderline inflammatory response (i.e. a situation with a high risk to develop inflammatory complications) to the initial trauma or disease. Minimising this surgically induced inflammatory response through limiting surgical procedures is the basis of damage control procedures[19,20]. In order to adjust surgical strategy it is essential to identify the multiple-injured patient at risk by accurately assessing and quantifying the extent and type of the inflammatory state.

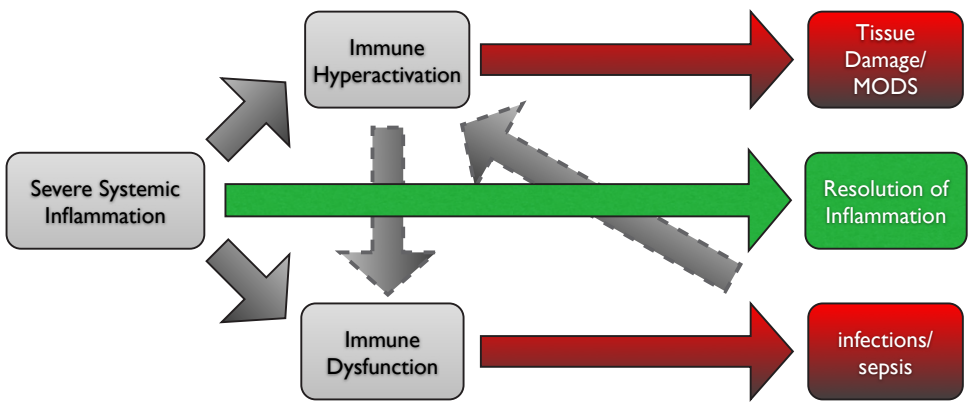

Figure 1. Systemic inflammation can lead to organ failure trough hyperactivation. Initial hyperactivication can lead to tissue damage. Immune dysfunction can lead to an increased susceptibility to sepsis. Sepsis leads to immune hyperactivation and tissue damage.

Prediction of inflammatory complications – the role of systemic inflammation

In marked contrast to primary mortality, available scoring tools based purely on anatomical and physiological injury severity are not adequate to predict secondary morbidity and mortality due to inflammatory complications. The initial inflammatory response to major surgery, injuries and burns may predict the development of subsequent sepsis, Acute respiratory distress syndrome (ARDS) or Multiple organ disfunction syndrome (MODS)[21].

As shown in figure 1. an initial stimulus will lead to a complicated chain of events, which in the best case results in immune homeostasis, sufficient to clear micro-organisms or contribute to tissue repair. Hyperactivation of the immune system leads to accumulation of neutrophils, which can damage tissues. On the other hand, immune dysfunction, either mediated by direct negative - regulatory mechanims or depletion of functionally mature leukocytes may lead to infections, sepsis and eventually hyperactivation of immune

responses and tissue damage[22].
It is tempting to speculate that determination of the systemic inflammatory response will aid in risk assessment for these inflammatory complications[23]. In the following section we will discuss current literature for measuring the inflammatory response.

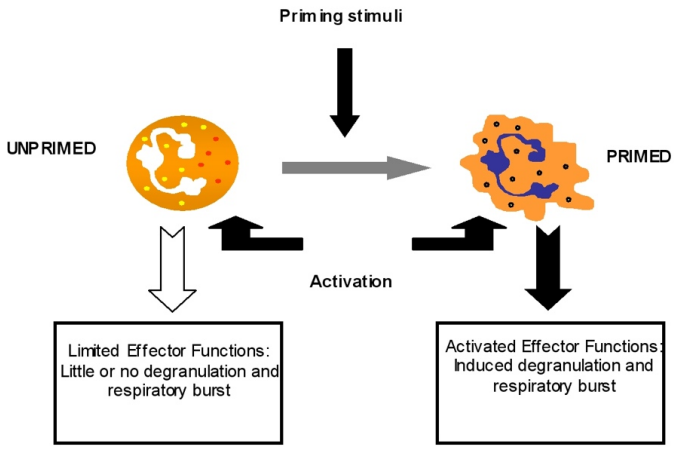

Figure 2. Neutrophil priming and function after injury: Activated unprimed neutrophils have limited effector function, activated primed neutrophils are capable of an enhanced functional response.

Conventional serum markers

The most commonly used inflammatory markers are acute phase proteins, including C-reactive protein (CRP), procalcitonine (PCT) and phospholypase A_2 (PLA_2). Acute phase proteins are defined as proteins whose plasma concentrations increase or decrease during inflammation. CRP as well as PLA_2, are significantly elevated at and during admission in patients developing MODS compared to patients who do not[24]. In addition, PLA_2 and CRP levels remain significantly higher (respectively from day 2 and 4 post injury) in multiple injured patients with lethal MODS compared to those who survive MODS[25]. The positive prognostic value for lethality is 74% for PLA_2 on day 2 whereas that of CRP reaches 86% on day 4 (table 1). Yet, most studies show that both these markers are non-specific and therefore have a low predictive value[2,24,26-29].
PCT is proposed to be a better and more specific marker for inflammation than CRP as the kinetics of PCT more closely resemble the kinetics of inflammation[30,31]. Two studies, investigating inflammation-induced complications after trauma, describe significantly increased PCT concentrations with peak levels on day 0 to 3 after trauma[32,33]. The extent of the elevated PCT concentrations showed a correlation to ISS and development of severe sepsis and septic shock. One study found a correlation between the occurrence

of MODS[33], whereas the other one did not[32]. Both studies mentioned that the predictive value strongly depends on the cut off value of PCT plasma levels. When the cut off point of plasma PCT levels increases the sensitivity decreases while the specificity increases, changing thereby the positive and negative predictive values.

Table 1. Positive predictive value of different markers for organ failure, septic complications and mortaility in prospective series with multitrauma patients

Marker	Result	PPV	Group size	Ref.
CRP	Increased until 10 days PI in patients developing MOF.	?	75	24
	Increased from day 3 days PI in patients developing MOF	73%	67	25
	Increased preoperative in patients developing postoperative MOF	75%	104	47
	Decreased from admission in patients developing MOF	?	57	27
PCT	Increased from day 1 to 21 PI in patients developing sepsis	38-67%	405	33
	Increased at admission in patients deveolping sepsis	?	78	32
PLA_2	Increased from day 0 - 10 PI in patients developing MOF	?	75	24
	Increased from day 2 PI in patients developing MOF	74%	67	10
Platelet count	Decreased preoperative in patients with postoperative MOF	71%	104	47
IL-6	Increased from day 0 - 4 PI in patients with lethal MOF	?	66	21
	Increased from day 0 - 10 PI in patients developing MOF	?	75	24
	Increased at day 0-1 PI for early MOF, increased at 5-10 for late MOF	70%	352	40
IL-8	Increased from day 0-1 PI in patients developing MOF	?	66	21
	Increased at day 0-1 PI for early MOF, increased at 5-10 for late MOF	69%	352	40
IL-10	Increased from day 0-10 PI in patients developing MOF	?	75	24
	Increased ad admission and declining in the following days in patienst developing MOF	60%	352	40

PI (Post-injury); PPV (Positive predictive value) is the portion of patients with a positve test who are correctly diagnosed; CRP (C-reactive protein); MOF (Multiple organ failure); PCT (Procalcitonin); PLA_2 (Phospholipase A_2)

Cytokines

The innate immune response is modulated by cytokines, which are rapidly released directly after injury. The release of several cytokines depends on the severity of injury. Levels of several important cytokines can easily be measured in the peripheral circulation and, therefore, cytokines have been proposed as potential markers. Plasma levels of tumor necrosis factor-α (TNF-α) and IL-1β rise within 1 to 2 hours after initial trauma followed

by, among others, IL-6, IL-8, IL-12 and the anti-inflammatory cytokine IL-10[2,34,35]. The levels of the latter cytokines are maximal between 6 to 24 hours post-injury and gradually decline during the following days.
Enhanced IL-6 levels evidently correlate with the severity of injury as well as the incidence on MODS, ARDS, sepsis and mortality[21,26,36,37].
Elevated IL-8 plasma levels also correlates with injury severity and outcome[21,36]. Yet, contrasting results concerning IL-8 have been reported, probably due to the short half-life time of this cytokine in peripheral blood[26,38]. For that same reason, IL-1β and TNF-α have turned out to be poor markers[2,26]. In addition, the wide variety in value of the cytokine levels among individuals makes it difficult to predict outcome[21]. Yet, positive predictive values of 80 to 90% have been described in studies combining cytokine levels with biochemical markers and clinical signs[24,39]. Remarkably, few studies took in account the difference in the pathogenesis between early or late-onset MODS when investigating correlation between cytokine release and MODS[21,40]. These studies showed that cytokine patterns clearly differ in patients with early-onset and late-onset MODS (see table 1). Maier et al. studied correlation between cytokine levels and the onset of MODS and showed that IL-6 and IL-8 had a positive predictive value of 70 and 69% respectively[40]. Unfortunately, this enfolds that still 30% is false positive.

Cellular response

Immune cells play a key role in the inflammatory response and differences in functionality and/or immune phenotype of these cells have been reported to correlate with complications and outcome of trauma-patients. Within hours after injury the number of leukocytes markedly increases, while the number of lymphocytes and monocytes decreases[41]. Post-injury leukocytosis is mainly the result of an increased number of neutrophils (chapter 5). Immediately after trauma circulating neutrophils are primed, resulting in not only in an increased migratory capacity but also in enhanced cytotoxic functions[42-44]. The levels of neutrophil elastase as indicator of systemic neutrophil activation, rise shortly after injury[21]. In addition, significantly elevated neutrophil elastase levels are seen during the first hours and days in patients with organ failure[21,45]. In surgical patients, neutrophil elastase levels are even suggested to have good positive prognostic value for complications with a sensitivity of 88% and a specificity of 83%[46].
Studies that have focussed on systemic (pre)activation of neutrophils in chronic inflammatory diseases mainly used the expression of L-selectin (CD62L) and Mac-1 (CD11b/CD18) (Table 2). Despite the fact that differential expression of these epitopes have been found in patients with different trauma severities, no correlation has been demonstrated between the extent of expression of these markers on immune cells and trauma severity scores[14,27,47,48]. Botha *et al.* used the base excess as a more precise measure for severity of injury and found a correlation between the expression of αM (CD11b) on neutrophils and injury severity[49]. In marked contrast, others have even found decreased

CD11b expression after trauma[50,51].
Similar inconclusive data were obtained with an other broadly used activation epitope L-Selectin (CD62L), which is shed upon activation[52-56]. In an interesting review on this topic by Stengel et al. it was concluded from a meta-analysis that soluble L-Selectin shows some correlation with the ISS, although inadequate for individual assessment[56].
A possible explanation for the differences in studies, describing modulation of αM (CD11b) expression on neutrophils after trauma, is that activated cells home for the tissues. Subtle differences in design of study (e.g. time of blood sampling) can introduce a large variation of αM (CD11b) expression[57]. Recent data from our laboratory supports the hypothesis that activated neutrophils disappear from the peripheral blood during severe inflammatory conditions. This hypothesis is corroborated by our finding that neutrophils harvested from the lung fluid by lung aspiration were characterised by a fully activated phenotype in the context of expression of αM (CD11b) and L-selectin (CD62L)[48]. In addition, our antibodies (A17 and A27), recognising primed innate immune cells *in vitro* and *in vivo*, allowed us to follow the kinetics of systemic neutrophil activation[58]. We provided suggestive evidence that neutrophils rapidly disappear from the circulation upon trauma, leaving behind cells that were refractory for activation of the innate immune stimulus N-formyl-Met-Leu-Phe (fMLF)[48]. In this study maximal neutrophil CD11b expression after fMLF stimulation decreased when injury severity increased in the moderate range of trauma.
In addition to the above mentioned modulation of expression of adhesion molecules, activation of neutrophils is associated with differential expression of chemoattractant receptors for IL-8 (CXCR1 [CD181] & CXCR2 [CD182]) and C5a (C5aR [CD88])[59-61]. The expression of these G-protein coupled receptors is down-regulated upon interaction with their ligand in a process generally referred to as homologous desensitisation[62]. In addition, chemokines can also induce heterologous desensitisation by which ligand binding to certain chemokine receptors leads to down-regulation of other non-ligand bound chemokine receptors[63,64]. Therefore, the modulated expression of these receptors can be used as markers of neutrophil activation. Few studies have tested whether this could be applied for evaluation of neutrophil activation in the context of acute inflammation[65].

Table 2. Description of dicussed neutrophil receptors and their role in inflammation.

Description	Ligand	Cellular Function	Modulation in inflammation
CD181 & CD182 (CXCR 1 & 2) chemokine receptors	IL-8, GROα, ENA-78	chemotaxis	downregulation after interaction with ligand
CD11b αM subunit of the β2 integrin Mac-1	ICAM, iC3b, fibrinogen, heparin	adhesion, phagocytosis, respiratory burst	upregulation, functional activation
CD88 Complement 5a receptor	C5a	activation, chemotaxis	downregulation after interaction with C5a
CD62L L-selectin	MAdCam-1	rolling, secondary tethering	shed from cell surface upon activation
A17 & A27 Phage antibodies recognizing an activated complex of FcγRII		priming, phagocytosis	Increased responsiveness in mild inflammation and downregulation in severe inflammation

Neutrophil responsiveness

In addition to measuring *in vivo* differential expression of neutrophil surface proteins evaluation of neutrophil activation *in vivo* can be achieved by the determination of the responsiveness of the cells for the innate immune stimulus fMLF. This responsiveness can be measured by the modulated expression of activation epitopes induced by activation of whole blood with fMLF *in vitro*[66]. Application of this method on whole blood of normal donors shows that neutrophils are very sensitive for fMLF[67]. Interestingly, neutrophils in blood of patients with acute inflammatory disease are characterised by a marked refractory response towards fMLF[48,68].

Clinical studies performed to date have not shown a correlation between single neutrophil receptors (homeostatic and after activation) and injury severity in the individual patient. However, all of these studies have focused on single neutrophil markers and have not taken into account the rapid kinetics of neutrophil release and homing to the tissues (see also chapter 9).

Complex kinetics of neutrophil phenotypes in peripheral blood after injury

Our recent studies have clearly shown that neutrophils rapidly change their expression

repertoire of relevant receptors. It is not clear whether this occurs in distinct populations of neutrophils or whether one phenotype evolves from the other. In addition it does not take in account the contribution of the rapid release of neutrophils from the bone marrow into the circulation in inflammatory conditions. As multiple factors evidently contribute to the phenotype of the total circulating neutrophil pool, description of neutrophil phenotypes can only be reliable when multiple comparisons are made for expression of different receptors. Future studies should, therefore, focus on subtle differences in expression of an array of activation markers.

Markers	Min → Inflammatory state of the host → Max									
CD181	Bright	Bright	Bright	Dim	Low	Low	Low	Absent	Absent	Absent
CD182	Bright	Dim	Low	Low	Low	Absent	Absent	Absent	Absent	Absent
CD88	Bright	Bright	Dim	Dim	Dim	Dim	Dim	Dim	Low	Low
A17	Low	Dim	Bright	Dim	Low	Low	Low	Low	Low	Low
A27	Low	Dim	Bright	Dim	Low	Low	Low	Low	Low	Low
CD62L	Dim	Bright	Dim	Low	Low	Low	Low	Low	Low	Low
CD11b	Low	Dim	Dim	Bright	Bright	Bright	Bright	Bright	Dim	Dim
CD16	Bright	Bright	Bright	Bright	Bright	Bright	Dim	Dim	Dim	Low
A17 fMLF	High	High	Bright	Dim	Dim	Dim	Low	Low	Low	Low
A27 fMLF	High	High	Bright	Dim	Dim	Low	Low	Low	Low	Low
CD11b fMLF	High	High	High	High	High	Bright	Dim	Dim	Dim	Low

Figure 3. Example of post-injury neutrophil phenotypes. Neutrophil phenotypes are proposed on the basis of existing data and preliminary results. The table reviews expression of the markers on neutrophils *ex vivo* (top part of the table). For three markers CD11b, A17 and A27 expression is also given after *in vitro* activation of whole blood with fMLF (1 mM, 15 min at 37 C). Factors that can influence the expression of the different markes are: 1) dose of stimuli, 2) phase of neutrophil maturation 3) age and physical condition of the host 4) time of measurement. Neutrophil profiles were determined using existing literature and preliminary results. Factors of influence on expression profile: 1) dose of stimuli, 2) phase of neutrophil maturation 3) age and physical condition of the host 4) time of measurement

Little is known regarding the mechanisms underlying the differential expression of neutrophil receptors *in vivo* during injury. However, it is known that differences in expression of neutrophil receptors is determined by many factors. These multiple mechanisms (action of cytokines, adhesion, chemokines etc.) combined with the preferential homing of certain phenotypes of cells, results in a dynamic situation that cannot be studied using single markers.

We propose using multiple markers to assess the inflammatory state. This hypothesis

is illustrated by figure 3. This figure shows the existence of complex profiles of receptor expression on blood neutrophils found during post-injury systemic inflammation. These data are based on current literature and our results. Similar complex approaches have proven useful tools in various other medical fields such as genomics (transcriptome analysis), proteomics (protein profiles) and multiplex analysis of presence of multiple cytokines in peripheral blood[69,70]. In complex clinical situations, such as the inflammatory response following trauma, studying specific neutrophil profiles seems promising. Taking into account the rapid increase of circulating neutrophils and the homing of primed neutrophils to site of inflammation, we consider the circulating neutrophil pool capable of integrating multiple inflammatory signals and it may therefore function as a readout or the overall inflammatory response.

More insight into the fundamental neutrophil biology following acute inflammation will aid us in recognizing specific neutrophil receptor profiles, which reveal the inflammatory status of the patient and thus can guide in the surgical management of a multiple injured patient.

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In vivo labeling with ^2H_2O reveals a human neutrophil lifespan of 5.4 days

Janesh Pillay
Ineke den Braber Nienke
Vrisekoop Lydia Kwast
Rob de Boer
José Borghans
Kiki Tesselaar*
Leo Koenderman*

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** Authors contributed equally*

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Introduction

Neutrophils are indispensible for host defense[1]. In addition, these cells play a detrimental role in the pathogenesis of many acute and chronic inflammatory diseases. They can cause tissue damage through aspecific activation of their repertoire of anti-microbial mechanisms. Neutrophils also inform and shape subsequent immunity[2] and can prolong inflammation by release of cytokines[3] and chemokines[4]. There is an emerging concept that neutrophils directly influence adaptive immune responses through pathogen shuttling to draining lymph nodes[5,6], antigen presentation[7], and modulation of Th1/Th2 responses[8]. Along this line, neutrophils have recently been reported to be an important component of myeloid derived suppressor cells (MDSCs) mediating lymphocyte suppression in various experimental models of acute[9] and chronic inflammation[10].

Targeting neutrophils in disease has mainly been focused on limiting their damaging capacity or directing their cytotoxic machinery to tumors[11]. Their immune modulatory functions have received little attention as potential targets in inflammatory diseases. This may at least in part be due to the current paradigm that these functions are of limited importance because of the generally accepted short circulatory half-life of neutrophils. Neutrophil lifespans have mainly been assessed by determination of *ex-vivo* lifespans in culture (<24 hours) and by transfer studies of *ex vivo* manipulated neutrophils. The latter studies revealed an estimated circulating half-life of approximately 8 hours in humans[12]. *Ex vivo* manipulation has been shown to have dramatic effects on neutrophil redistribution *in vivo*[13]. In mice half-lives of 8-10 hours were reported when neutrophils were labeled *in vivo*[14]. In contrast, *ex vivo* labeling in mice revealed that after transfer 90% of labeled neutrophils were cleared from the circulation within 4 hours, resulting in a half-life of less than 1.5 hours[15]. These differences between *in vivo* and *ex vivo* labeling strengthens our hypothesis that neutrophil transfer experiments may lead to underestimation of neutrophil lifespan. The activation during *ex vivo* manipulation has probably lead to retention in the lungs[16], liver, spleen and bone marrow[15], which may drastically reduce their circulatory half-life. To circumvent the complications introduced by *ex vivo* manipulation, we labeled the neutrophil pool *in vivo* in healthy mice and humans by administration of $^{2}H_{2}O$ in drinking water. Acquisition of label and appearance of labeled neutrophils in the circulation is characterized by i) the rate of division in the mitotic pool (MP) in the BM, ii) the transit-time of newly-formed neutrophils through the post-mitotic pool (PMP) in the BM, and iii) the delay in mobilization of neutrophils from the PMP to the blood. Using a combination of gas chromatography and mass-spectrometry (GC/MS) the fraction of ^{2}H-labeled adenosine in the DNA of the proliferating neutrophil pool was measured and the kinetics of the neutrophil pool was determined.

Study design

Human volunteers

Five healthy male volunteers (characteristics described previously[17]) were included in

the study after giving written informed consent. (see supplement for labeling protocol and sample collection). This study was approved by the medical ethical committee of the Academic Medical Center Amsterdam.

Mice
C57Bl/6 mice were maintained under specific pathogen-free conditions in accordance with institutional and national guidelines. 12-week old mice obtained a boost injection (i.p.) of 16.5 ml/kg of 90% ^2H_2O in PBS (Cambridge Isotopes, Cambridge, MA), followed by feeding with 4% ^2H_2O in drinking water for one week.

Neutrophil isolation
Neutrophils were isolated as previously described. (see supplemental methods)

Mathematical modeling
Neutrophil life spans were estimated by mathematical modeling taking into account the availability of ^2H_2O in urine or plasma for men and mice, respectively (see supplement). Based on the assumption that in homeostasis neutrophils are a kinetically homogeneous population and that - as a consequence - the rate at which neutrophils are produced and enter the blood equals the rate at which labelled neutrophils are lost in a random manor from the circulation, we fitted the level of deuterium-enrichment of the DNA of neutrophils to the solution of:

$$dL(t)/dt = dcU(t-\Delta) - dL(t)$$

in which $L(t)$ is the fraction of labelled DNA in neutrophils at time t (in days), c is an amplification factor that is required because of the multiple hydrogen atoms in a single adenosine deoxyribose moiety that can be replaced by deuterium, d is the average turnover rate of neutrophils and $U(t-\Delta)$ is the ^2H_2O enrichment of body water as measured from the urine or serum of men and mice, respectively[17]. We allowed for a time delay of Δ days between production of neutrophils in the MP of the BM and measurement of labelled DNA in the PMP or the blood.

Results and Discussion
We first compared the dynamics of neutrophils in BM and blood of mice. We administered ^2H_2O to mice for 7 days and measured the accrual and loss of label in plasma, BM-derived and blood-derived murine neutrophils. Isolation of the BM PMP revealed a transit time of 1.6 days in the PMP, in addition to 0.7 days residence time in the mitotic pool (figure 1A). The resulting combined delay of 2.3 days in the BM was confirmed by mathematical modeling of the blood data, which revealed an estimated delay of 2.3 days (figure 1B). The average half-life of circulating neutrophils in mice was estimated to be 12.5 hours,

corresponding to an expected lifespan of 0.75 days (figure 1B).
These estimates are in perfect agreement with results from previous *in vivo* studies on neutrophil life spans in the blood and BM of mice[14]
We next evaluated the dynamics of neutrophils in the peripheral blood of healthy human volunteers. We applied our mathematical model for $^{2}H_{2}O$ labeling to data of five healthy individuals and estimated a median half-life of 3.8 days for circulating neutrophils, corresponding to an expected lifespan of 5.4 days in peripheral blood, and a delayed exit from the BM of 5.8 days (Table 1 and figure 1C). This delay is in line with the transit time through the PMP of 6 to 7 days that was previously reported based on *in vivo* ^{3}H-thymidine or ^{2}H-glucose labeling of BM in humans[12,18]. In summary, our estimated half-lives of murine circulating neutrophils and human and murine BM neutrophils are in good agreement with previously reported half-lives based on *in vivo* labeling[12,14,18]. Our estimated human circulating neutrophil half-life of approximately 90 hours, however, is at least 10-fold longer than previous estimates based on *ex vivo* labelled neutrophils[12].

Table 1. Best fitting parameters for the neutrophil labeling data of 5 healthy volunteers.

Individual	d	Half-life	Lifespan	Δ
A	0.186 [0.156– 0.241]*	3.73	5.38	5.80 [4.92 – 6.91]*
B	0.197 [0.160– 0.250]	3.52	5.08	6.01 [5.13 – 6.84]
C	0.185 [0.145– 0.218]	3.75	5.41	6.14 [5.22 – 6.54]
D	0.119 [0.077– 0.192]	5.83	8.40	3.94 [0.19 – 6.61]
E	0.135 [0.106– 0.211]	5.13	7.41	2.33 [0.23 – 4.70]
Median	0.185	3.75	5.41	5.80

d represents the average turnover rate of neutrophils (which represents their production and loss rate in steady state conditions). *Δ* represents the delay with which labelled cells from the BM reach the blood. Average lifespans were calculated as 1/*d*, or converted to half-lives as ln2/*d*.
* 95%-confidence intervals as determined by bootstrapping.

We investigated whether the discrepancies between the current and previous estimates for human neutrophil lifespans could be due to our assumption that neutrophils are kinetically homogeneous. To exclude the possibility that the longer estimated half-life could be due to a small contaminating population of relatively long-lived eosinophils, we fitted our data to a model including a second kinetic population (see supplemental methods) with a circulating lifespan of 12 days, which represented 5% of the isolated cell population. This hardly affected the estimated half-lives of the remaining 95% of

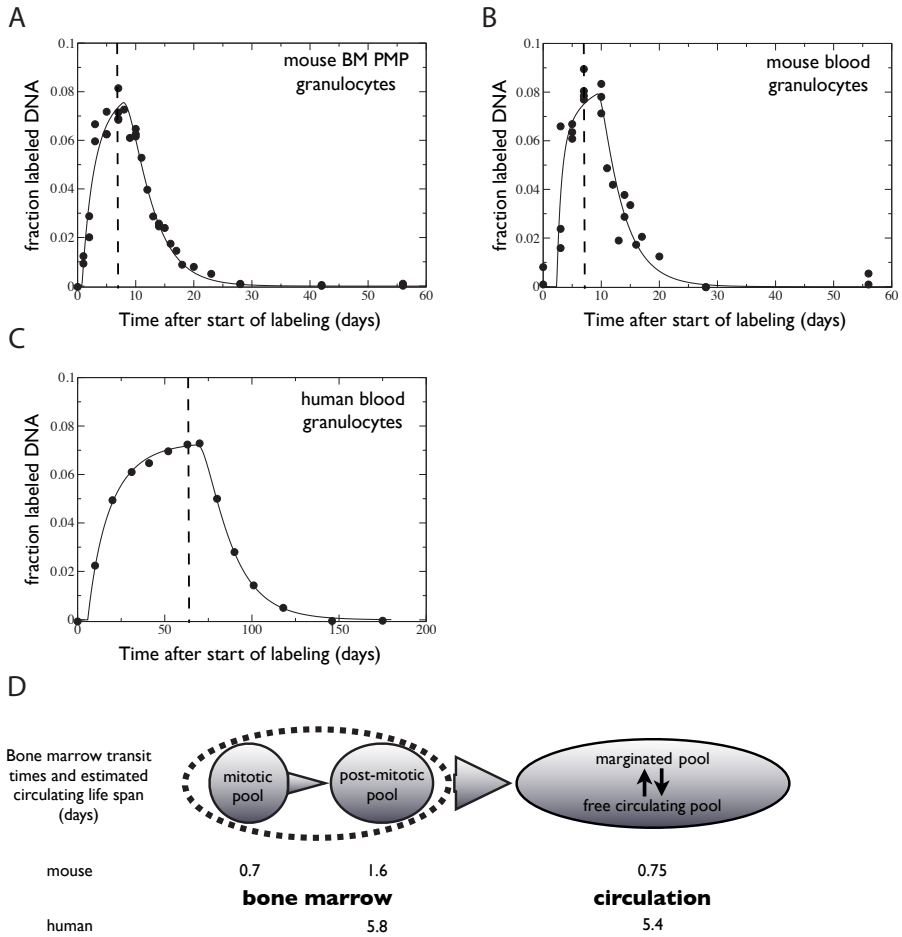

Figure 1. Analysis of neutrophil turnover by in vivo ^2H_2O labeling. Cross-sectional up- and down-labeling of murine **(A)** BM PMP neutrophils and **(B)** blood neutrophils. **(C)** Representative example of longitudinal up- and down-labeling of human blood neutrophils; parameter values of 5 individuals are given in Table 1. Dashed vertical lines indicate the time of label cessation on day 7 **(A&B)** or day 63 **(C)**. Curves were fitted as described in methods taking into account the actual level of label enrichment of plasma in mice or urine in humans (supplemental figure 1). **(D)** Estimated median neutrophil lifespans and transit times (in days) of mice and humans. Murine estimates for transit times in the BM could be calculated directly; from the BM labeling data we calculated that labeled cells entered the PMP with a delay of $\Delta = 0.7$ days, and that they had an expected lifespan in the PMP of $1/d = 1.6$ days. The total transit time in the BM of 0.7 + 1.6 days matches the estimated $\Delta = 2.3$ days that resulted from analysis of the murine blood labeling data. BM transit times in humans were estimated from the delay Δ with which labeled neutrophils were observed in the blood, while the circulating lifespan was calculated from 1/d.

neutrophils (not shown). Reversely, when forcing one sub-population of neutrophils to have an estimated lifespan as short as 12 hours, we found that in order to be compatible

with our labeling data, either i) this fast population had to be very small while the other neutrophils still had an expected lifespan of ~5 days, or ii) there had to be considerable heterogeneity between neutrophils, such that a second – slower – population of neutrophils would form a significant part of the cell population with estimated life spans of up to ~10-20 days (see supplemental table 1). Fits in which all neutrophils had an expected life span of 12 hours and BM neutrophils entered the blood with a delay of 7 days were incompatible with the labeling data.

Our data therefore suggest that the expected lifespan of non-activated neutrophils under homeostatic conditions is much longer than previously thought, and that previous studies using *ex vivo* manipulation have underestimated the circulating half-life of neutrophils because of activation and homing of these cells. Also clinical data, such as neutrophil kinetics after myeloablation and G-CSF administration, are likely to have underestimated normal neutrophil circulatory half-lives, because of neutrophil activation and differentential homing under these clinical conditions.[15]

These results have important implications for neutrophils during immune homeostasis. Firstly, the general paradigm that murine and human neutrophil half-lives are similar is apparently incorrect. Secondly, combined with the novel view that human neutrophils can perform various immune modulatory functions, their relatively long estimated circulatory half-life may provide the incentive to target neutrophils to modulate immunity in cancer, autoimmune disorders and vaccine development.

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Supplemental material and data

Human subjects (labeling protocol and sample collection)

Human volunteers received an initial dose of 10 ml of ^2H_2O per kilogram of body water in small portions throughout the first day. Body water was estimated to be 60% of body weight. As a maintenance dose, the subjects drank 1/8 of this initial dose daily at home for 9 weeks. Blood and urine were collected before labeling, after the boost of label at the end of day 0, and six times during the labeling protocol. In addition, during the downlabel phase of 16 weeks, blood and urine were collected seven times.

As it was important to avoid inflammatory stimuli in our volunteers, only healthy individuals were included, who were non-allergic and did not have any other chronic inflammatory disease. To evaluate short-term immunological challenges such as caused by for example common colds, the individuals were asked to fill out a questionnaire before the start of the experiment and at each blood donation. In addition, as we reported previously, the lymphocyte labeling data of these same volunteers showed no signs whatsoever of viral infections or other immunological disturbances throughout the experiment[1]. Finally, blood cell counts of all participants were routinely determined on a hemocytometer and no neutrophil counts or percentages above or below the normal values were reported.

Neutrophil isolation

Human neutrophils were isolated by centrifugation over a Ficoll gradient followed by hypertonic lysis of erythrocytes using ice-cold NH_4CL_2. This yielded a neutrophil purity of >97%. This purity is routinely checked by cytospin preparations. Isolation of the murine PMP-BM pool was performed by centrifugation over various Percoll-gradients[3]. Murine blood neutrophils were sorted based on their distinct forward and sideward scatter (figure S3).

Mathematical modeling

To correct for the fraction of heavy water in the body water, we fitted the measured label enrichment in urine (for humans) and plasma (for mice) during up- and downlabeling, as described previously,[1] by the following equations:

$U(t) = f\,(1 - e{-}\delta t) + \beta e{-}\delta t$ during label intake ($t \le \tau$), and
$U(t) = [f(1 - e{-}\delta\tau) + \beta e{-}\delta\tau]e{-}\delta(t{-}\tau)$ after label intake ($t > \tau$).

where U(t) is the fraction of ^2H_2O in the urine or plasma at day t, f represents the fraction of ^2H_2O in the drinking water, labeling was stopped at $t = \tau = 63$ days in humans and at $\tau = 7$ days in mice, δ represents the turnover rate of body water per day, and $U(0) = \beta$ is the baseline body water enrichment that is attained after the boost of label by the end of day 0. The derivation

of these equations has been published previously[1]. Best fits for the label enrichment in plasma and urine are shown in figure S1. Parameters resulting from these body water fits were used when fitting the label enrichment of the DNA of neutrophils as previously described[1].
The model described in the Methods section is identical to the one that we derived previously[1], with the exception that 1) neutrophils are assumed to be kinetically homogeneous, implying that the average rate at which new neutrophils are produced (p) equals the rate at which labeled neutrophils are lost (d), and 2) that newly produced neutrophils enter the blood with a delay of Δ days. Kinetic homogeneity of the neutrophil population in homeostatic conditions is a generally accepted phenomenon. Scarce evidence is available that neutrophils recirculate under homeostatic conditions, and the percentages found (0.23%) do not disturb our interpretations[4].

When we relaxed the assumption that the analysed neutrophil population was kinetically homogeneous, we fitted the data to a two-compartment model, in which each sub-population i (containing a fraction αi of the analysed cells) was described by

$$dLi(t)/dt = dicU(t-\Delta) - diLi(t)$$

such that the level of label enrichment in the total cell population followed:

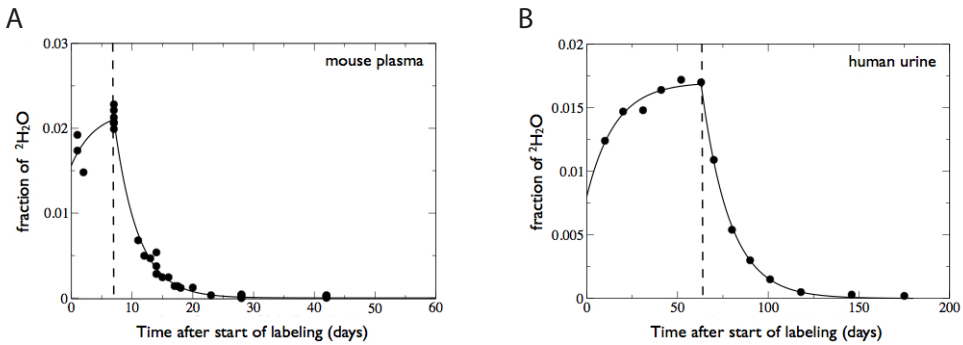
$$dL(t)/dt = \sum \alpha iLi(t)$$

To avoid overfitting of the data, when using the 2-compartment model the delay with which cells from the BM enter the blood was fixed to Δ=7 days, as previously estimated by *in vivo* labeling[5,6].

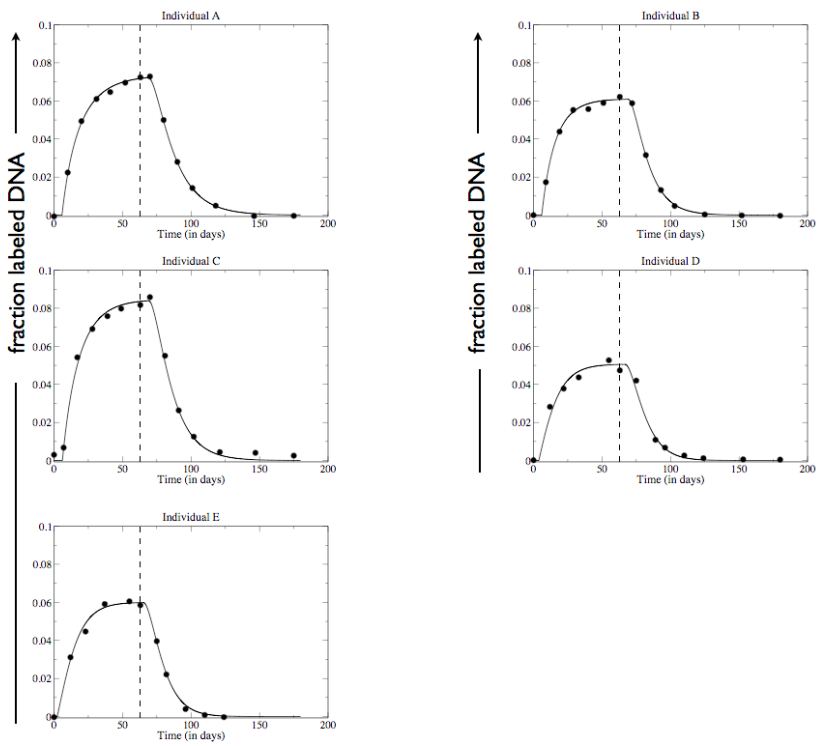
Supplemental Table 1: Results of a model with kinetic heterogeneity

Individual	h	d_2
A	0.27 (0.09 – 0.35)	0.18 (0.15 – 0.22)
B	0.24 (0.07 – 0.33)	0.19 (0.16 – 0.24)
C	0 (0.00 – 0.70)	0.21 (0.05 – 0.25)
D	0,51 (0.30 – 0.67)	0.08 (0.04 – 0.14)
E	0,68 (0.27 – 0.79)	0.11 (0.06 – 0.29)

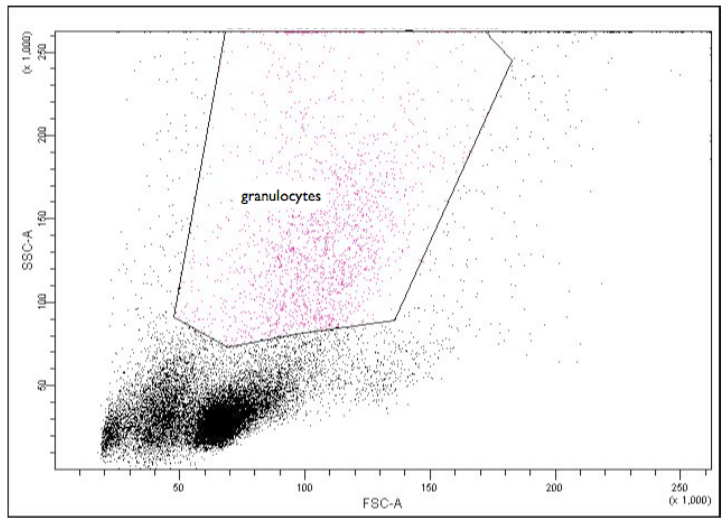
When the turnover rate of one sub-population of neutrophils was fixed to d_1 = 2 per day, the size of this fast sub-population (*h*) either had to be very small, or a considerable part (1-*h*) of the neutrophil population had to have a very slow rate of turnover (d_2) in order to be compatible with the labeling data. 95% confidence intervals are given in parentheses and were calculated using a bootstrap method. Note that high values of *h* in these confidence intervals always corresponded to low values of d_2, i.e. the larger the size of the fast population (with d_1 = 2 per day), the slower the rate of turnover of the slower population.



Supplemental figure 1. Fitted curves of ^2H_2O enrichment of (A) murine plasma and (B) human urine. Cessation of labeling is depicted by the dashed lines at day 7 **(A)** and day 63 **(B)**. Murine plasma data **(A)** are cross sectional data of individual mice. Human urine data **(B)** are a representative example of longitudinal data of the healthy controls.



Supplemental figure 2. Longitudinal up- and down-labeling of human blood neutrophils. Fitted curves of 5 human individuals taking into account the actual level of enrichment of plasma. Cessation of labeling is depicted by the dashed lines at day 63.



Supplemental figure 3. Example of FACS sort of murine blood granulocytes. The gated cells were kept on ice and sorted based on their forward and sideward scatter using a FACSaria.

Supplemental References

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Homology in systemic neutrophil response induced by human experimental endotoxemia and by trauma

Tjaakje Visser
Janesh Pillay
Peter Pickkers
Luke Leenen
Leo Koenderman

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adapted from submitted manuscript

Introduction

Tissue injury results in activation of the innate immune system by danger-associated molecular patterns (DAMPs)[1]. DAMPs exist of endogeneous cytosolic components such as high mobility group box 1 (HMGB-1), heat shock proteins, defensins and annexins[2]. After severe injury, activation of the immune system can lead to a systemic inflammatory response syndrome (SIRS) with an increased risk of inflammatory complications such as acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS). A comparable systemic innate immune response is seen during severe infectious diseases such as sepsis and septic shock[3]. Yet, during infection the innate immune response is activated by microbial components in general referred to as pathogen-associated molecular patterns (PAMPs) instead of by DAMPs[4,5]. PAMPs are recognized by a limited number of germline-encoded pattern-recognition receptors (PRRs), of which Toll-like receptors are most well known[4].

Recently, there has been much discussion about the distinction between PAMPs and DAMPs. It has been proposed that many micro-organism components and endogenous alarm signals belong to an ancient subfamily of universal DAMPs[6,7]. In addition, several studies have shown that DAMPs can also trigger the innate system via toll-like receptors[8-10]. We hypothesized that activation by PAMPs and DAMPs results in a similar early neutrophil response as part of the final common pathway of the innate immune response.

Human experimental endotoxemia can be used to investigate cellular innate immune response to PAMP in a standardized manner. Human experimental endotoxemia was developed as a model for the host response to infectious diseases and sepsis[11]. The model consists of a intravenous challenge of a human volunteer with endotoxin (lipopolysaccharide (LPS)) at low doses (1-4ng/kg)[12,13]. Earlier studies have already shown that the administration of LPS leads to a cytokine release, with increases of, e.g., tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and IL-10, comparable to that seen after trauma, albeit in a shorter timeframe[11-13].

It is unknown whether the early inflammation induced by DAMPs results in a similar cellular innate immune response compared to PAMP-induced inflammation. The inflammatory response to endotoxemia and to tissue injury have not been compared before. In this study we compared the neutrophil response after LPS exposure to that of trauma patients, in order to see if the experimental endotoxemia could be used to investigate the acute systemic cellular response after trauma. Since the inflammatory stimulus is short lived after a LPS challenge, the endotoxemia model is in particular useful for investigating the kinetics of the early innate immune response (during the first hours after onset of systemic inflammation). We compared activation phenotype of circulating neutrophils during this initial phase of the innate immune response, 3 hours after the insult. At this time point we were able to obtain blood samples from healthy volunteers undergoing endotoxemia as well as from trauma patients. In addition, at this time point

a prominent neutrophil response is seen both groups[13,14]. A second blood sample was drawn at a later time point at 24 hours after trauma and LPS challenge.
The endotoxemia model may facilitate the study of the detailed kinetics of the cellular innate immune reaction as it circumvents the heterogeneity seen in trauma patients. In addition, the endotoxemia model is ideal for testing the effect of immune modulating therapy on the innate immune response in a controlled experimental design.

Materials and Methods

Materials

U.S. Reference E.coli endotoxin (lot Ec-5, Centre for Biologic Evaluation and Research, Food and Drug Administration, Bethesda, MD); saline 0.9% (Baxter, The Netherlands); 2.5% glucose/0.45% saline (Baxter, The Netherlands); FITC-labelled mouse-antihuman monoclonal antibodies against: L-selectin (CD62L; clone Dreg56, BD Pharmingen, USA), CXCR1 (CD181; clone 42705, R&D Systems Europe, UK), C5aR (CD88; clone P12/1, Serotec, Germany); PE-labelled mouse-antihuman monoclonal antibodies against: αM (CD11b; clone 2LPM19c, DAKO, Denmark), CXCR2 (CD182; clone 48311, R&D Systems Europe, UK), FCγRII (CD32; clone FLI8.26, BD Pharmingen, USA); Alexa 647-labelled monoclonal antibodies against: FCγRIII (CD16; clone 3G8, BD Pharmingen, USA); FITC-labelled IgG1 negative control (clone MOPC-21, BD Biosciences, Belgium), and IgG2a negative control (clone MRC OX-34, Serotec, Germany); PE-labelled and IgG1 negative control (clone DD7, Chemicon, USA); Alexa 647-labelled IgG1 negative control (clone MOPC-21, BD Biosciences, Belgium); FITC-labelled monoclonal phage antibody A27 against active FCγRII (generated and characterized as described previously)[15]; N-formyl-methionylleucyl-phenylalanine (fMLF) (Sigma-Aldrich, USA); FACScalibur Flow cytometer (BD Biosciences, USA); SPSS version 15.0 software (The Apache Software Production 2008, USA)

Trauma patients

Trauma patients enrolled were part of an observational study performed at University Medical Centre Utrecht, investigating neutrophil activation in patients after chest injury[14]. Written informed consent was obtained from all patients or their legal representatives in accordance with the Declaration of Helsinki and the Good Clinical Practice guidelines.
12 patients suffering from chest injury with an abbreviated injury score (AIS) of 2 or more admitted to the Trauma department of the University Medical Center Utrecht were enrolled[16]. Exclusion criteria were age < 18 or > 70 years, death within 24 hours after admission and patients with an altered immunological status (e.g. chronic diseases, corticosteroid use or chemotherapy). Blood samples ware taken at 2-4 hours and 22-26 hours after the accident. On average, patients arrived between 1 to 1.5 hours after injury at the ER. In all trauma patients, the first blood sample was withdrawn and analyzed within 1 to 2 hours after arrival at the ER at t=2-4 hours after the insult.

Human experimental endotoxemia.

Healthy volunteers undergoing endotoxemia were part of three endotoxin trials (NCT00783068, NCT00916448 and NCT01091571 at www.clinicaltrials.gov) performed at the Radboud Medical Centre Nijmegen. Both study protocols were approved by the local Ethical Committees. Written informed consent was obtained from all healthy volunteers in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Human experimental endotoxemia was evoked exactly as described before[12]. In short, 9 male subjects were enrolled after screening and prehydrated with 1500 ml 2.5% glucose/0.45% saline infusion. E.coli endotoxin, was used in this study. Endotoxin was reconstituted in 5ml saline 0.9% and injected as single intravenous bolus (2ng/kg) during 1 minute at t=0. Blood samples were taken from the arterial catheter at 3 hours and 24 hours after administration of endotoxin.

FACS analysis

All blood samples were collected in a vacutainer® with sodium heparin as anticoagulant and cooled immediately on melting ice. Blood samples of 9 healthy lab co-workers served as controls. Red cells were lysed with icecold isotonic NH_4Cl. After lysis, white blood cells were washed and resuspended in phosphate buffered saline supplemented with sodium citrate (0.4% wt/vol) and pasteurised plasma protein solution (10% vol/vol) (PBS2+), as previously described[14]. Resuspended cells were incubated on ice with commercial obtained directly labelled mouse-antihuman monoclonal antibodies against L-selectin (CD62L), αM (CD11b), CXCR1 (CD181), CXCR2 (CD182), C5aR (CD88), FCγRII (CD32) and FCγRIII (CD16).

After incubation and final wash, labeling was measured on FACS calibur Flow cytometer. The neutrophils were identified according to their specific side-scatter and forward-scatter signal.

For measurement of active FCγRII expression, whole blood was incubated a FITC-labelled monoclonal phage antibody A27 for 45 min on ice[14]. Active up regulation of active FCγRII expression was measured after 5 min of stimulation of whole blood at 37°C with fMLF 10^{-6}M to evaluate the responsiveness of the cells for bacterial derived protein products/peptides. After stimulation, the samples were put on ice again and stained with phage antibody A27. After staining, red cells were lysed and expression was measured on FACS calibur as previously described[14].

Data from individual experiments are depicted as fluorescence intensity as the median fluorescence intesity (MFI) of at least 5000 neutrophils.

Leukocyte count and differentiation

Leukocyte counts were determined by routine laboratory test of the participating hospitals. Percentages of neutrophils and monocytes were calculated out of total amount

of white blood cells based on their specific forward-sideward scatter on the FACS plots.

Statistical analysis

Data were analyzed using SPSS version 15.0 software. Results are expressed by mean ± SE. Normality of variance was confirmed by the Lavene's test. Subsequently, a one-way ANOVA followed by a Bonferroni post hoc was used as appropriate to test differences between the study groups and control at the two different time point. Student's t-test was used to analyze difference in leukocyte count between the two experimental groups. Statistical significance was defined as $p<0.05$.

Results

Trauma patient demographics

From April 2008 until April 2009 twelve trauma patients were enrolled, of whom 9 were male and 3 female. The mean age was 53 years (range 25 – 69) and the mean injury severity score (ISS) 19 (range 9 – 56) (Tabel 1)[16]. None of the patients received blood products during the study period.

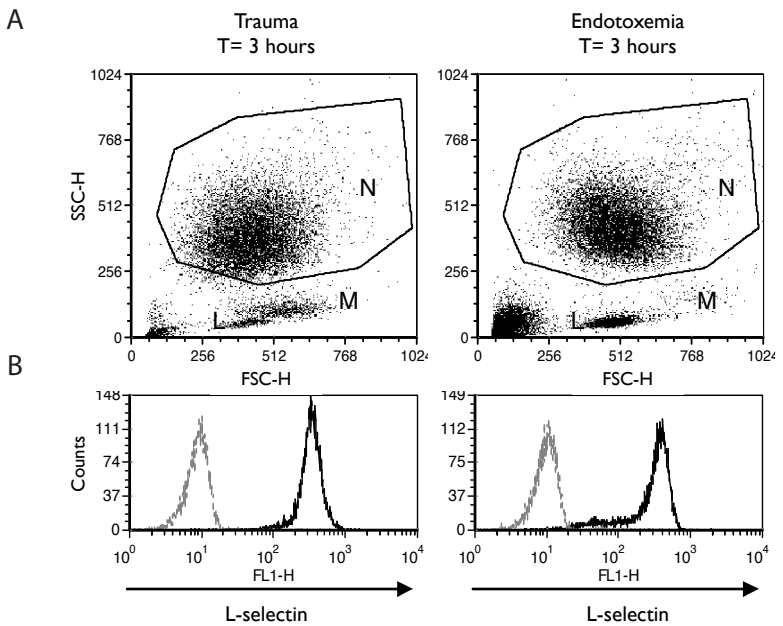

Figure 1. Representative example of FACS analysis of a trauma patient and a healthy volunteer undergoing endotoxemia at t=3 hours. (A) Neutrophils (N) are gated based on forward-sideward scatter; (M) = monocytes; (L) =Leukocytes. **(B)** Histogram showing L-selectin (black line) expression and expression of FITC-labelled IgG1 negative control (dotted line) on gated neutrophils.

Leukocyte count and differentiation

At 3 hours after the insult (trauma or LPS administration) leukocyte counts were evidently higher in the trauma group 12.8±1.5x 10^9 cells/l than the endotoxemia group 6.6± 1.8x 10^9 cells/l (p=0.002, students t-test). Earlier studies have shown that leukocyte counts increase during experimental endotoxemia, but not until 8 hours after infusion of endotoxin[12]. The percentage of neutrophils, however, was equally increased in the endotoxemia and trauma group (87±2% vs 81± 3%; p=0.229) compared to control values 57±2% (p<0.001 both groups) at 3 hours. The percentage of neutrophils remained high in the trauma group during the first 24 hours (76±2% p<0.001), whereas it restored to normal levels in the endotoxemia group at time point 24 hours (64±3%; p=0.138).

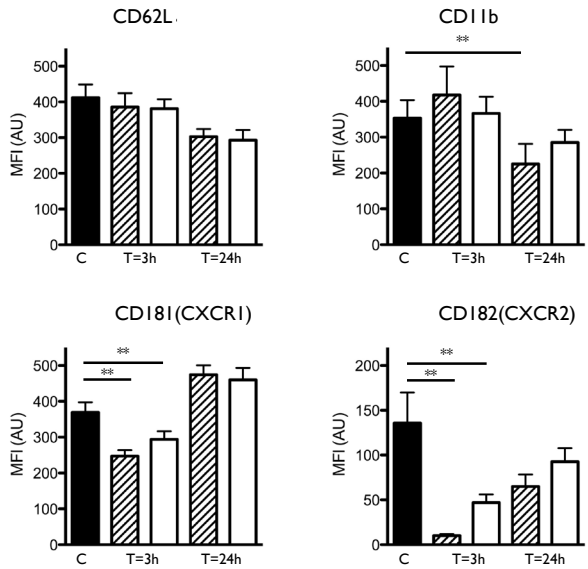

Figure 2. Expression of L-selectin, αM/CD11b, CXCR1 and CXCR 2 on circulating neutrophils measured by flowcytometry. Black bars (C) represents baseline values from healthy controls (n=9). Striped bars represent healthy volunteers undergoing endotoxemia (n=9) and open bars represent trauma patients (n=12) at 3 hours and 24 hours after onset of inflammation. Data are presented as mean±SEM. MFI (AU) = mean fluorescence intensity (arbitrary units); *p<0.05; ** p<0.01

A striking difference was seen in the percentage of monocytes between the trauma and the endotoxemia group at 3 hours. Monocytes almost completely disappeared from the circulation during endotoxemia. The percentage of monocytes was significantly lower in the endotoxemia group (0.7± 0.1%) at 3 hours compared to the trauma group (5.2±0.7% p<0.001) and control values (6.7±0.8% p<0.001). The percentage of circulating monocytes restored to normal at 24 hours (6.4±0.6% endotoxemia group vs. 7.6±0.7% trauma group; p=1.00 both groups compared to control values).

Table 1. Characteristics of included trauma patients. M = male, F = female, MVA = motor vehicle accident, ISS = injury severity score, NISS = new injury severity score[16].

Pt	Gender	Age	Mechanism of injury	Diagnosis	ISS	NISS	Apache II
1	M	25	MVA	8 rib fractures unilateral Pneumothorax bilateral	16	25	3
2	M	62	MVA	3 rib fractures unilateral Clavicula fracture Orbita roof fracture	17	17	15
3	M	51	Fall from height	2 rib fractures unilateral Pneumothorax unilateral	9	9	6
4	F	47	Fall from horse	10 rib fractures unilateral Flail thorax	17	17	3
5	M	60	MVA	5 rib fractures unilateral Pneumothorax unilateral Lungcontusion unilateral Pancreas contusion	21	29	9
6	M	59	Fall from height	6 rib fractures unilateral Pneumothorax unilateral Lungcontusion uniilateral	16	25	18
7	M	37	Fall from height	> 20 rib fractures bilateral Hematothorax bilateral Lungcontusion bilateral Pelvic fracture Cerebral hematoma Skull fracture	56	56	24
8	M	69	MVA	3 rib fractures unilateral	5	5	5
9	F	62	Bicycle accident	6 rib fractures unilateral Minor laceration kidney Facial hematoma	14	14	7
10	F	53	Fall from height	4 ribfractrures unilateral 1 rib fracture contra lateral Scapula fracture Fracture cervical vertebral body	17	17	2
11	M	62	MVA	3 ribfractures Lungcontusion bilateral Minor liver laceration	20	29	15
12	M	59	Attacked by cow	Multiple rib fractures bilateral Flail thorax bilateral Pneumothorax bilateral Lungcontusion bilateral Sternum fracutre Minor liver laceration	29	38	6

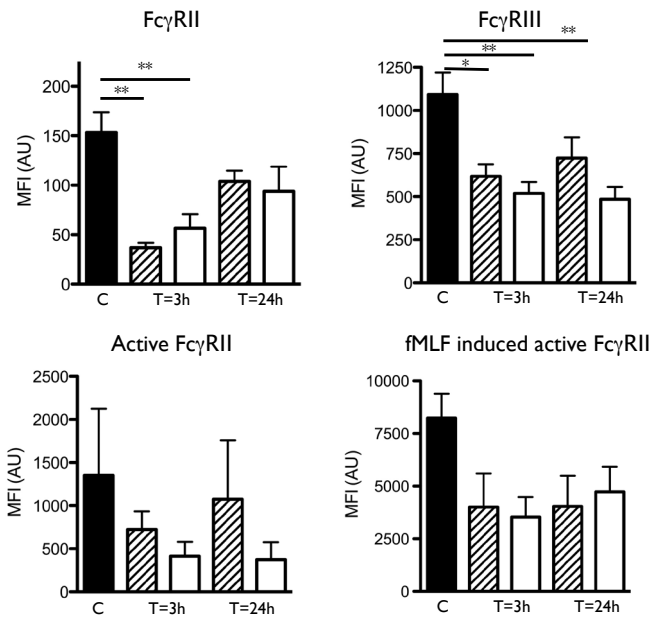

Figure 3. Expression of FcγRII, FcγRIII, active FcγRII and fMLF induced active FcγRII on circulating neutrophils measured by flowcytometry. Black bars (C) represent baseline values from healthy controls (n = 9). Striped bars represent healthy volunteers undergoing endotoxemia (n=9) and open bars represent trauma patients (n=12) at 3 hours and 24 hours after onset of inflammation. Data are presented as mean±SEM. MFI (AU)= mean fluorescence intensity (arbitrary units); *p<0.05; ** p<0.01

Receptor expression on the neutrophil surface

L-selectin (CD62L) and αM(CD11b)

It is well known that upon activation neutrophils shed L-selectin and at the same time increase the surface expression of αM(CD11b)[17]. Endotoxin as well as injury-induced inflammation resulted in a tendency towards lower L-selectin expression levels *in vivo*, but this decline did not reach statistical significance (endotoxemia p=0.140, trauma p=0.066 at t=24 hours; figure 2). In contrast, αM(CD11b) expression did not increase as seen during activation *in vitro*, but rather decreased during inflammation. At 24 hours after onset of inflammation αM(CD11b) expression was significantly lower in the endotoxemia group compared to control values ((p=0.003; figure 2). Although αM(CD11b) expression tended to decline in the trauma group at t=24 hours, expression was not significantly lower compared to control values (p=0.072). Between the trauma and endotoxemia group, expression of L-selectin and αM(CD11b) did not differ at any time point.

CXCR1(CD181) and CXCR29CD182)

Earlier studies have shown that CXCR1 and 2, surface receptors of the chemokine IL-8, are down-regulated upon activation of neutrophils both *in vitro* and *in vivo*[18]. In this study, systemic inflammation resulted in a reduced surface expression of CXCR1 and CXCR2 on circulating neutrophils at t=3 hours in both study groups in comparison to control values ($p<0.01$; figure 2), indicative for neutrophil activation. However, after 24 hours no significant difference in CXCR1 and CXCR2 expression between the endotoxemia group, trauma group and control values were found.

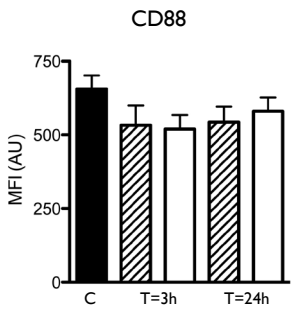

Figure 4. Expression of C5aR on circulating neutrophils measured by flowcytometry.
Black bars (C) represent baseline values from healthy controls (n = 9). Striped bars represent healthy volunteers undergoing endotoxemia (n=9) and open bars represent trauma patients (n=12) at 3 hours and 24 hours after onset of inflammation. Data are presented as mean±SEM. MFI (AU)= mean fluorescence intensity (arbitrary units); *$p<0.05$; ** $p<0.01$

FcγRII(CD32) and FcγRIII(CD16)

Fcγ receptors play in important role in activation of neutrophils. Fcγ receptors bind to immunoglobulins (IgG) either in aggregates or attached to pathogens[19]. Binding of IgG's to Fcγ receptors promotes the oxidative burst and induces phagocytosis[19]. Expression of Fcγ receptors on circulating neutrophils have been shown to decrease during systemic inflammation both in trauma patients as in healthy volunteers during endotoxemia[13,14]. In this study, expression of FcγRII and FcγRIII was significantly lower in both study groups in comparison to control values at 3 hours after onset of inflammation (FcγRII $p=0.001$ both groups; FcγRIII endotoxemia $p=0.028$, trauma group $p=0.001$; figure 3). FcγRIII expression remained low in the trauma group up until 24 hours ($p=0.001$), whereas FcγRII expression restored ($p=0.310$). Both intrinsic active FcγRII as well as fMLF induced active FcγRII expression showed a tendency to decline during inflammation (figure 3). Yet, this decline did not reach statistical difference in any of the groups at any time point. A decreased active FcγRII expression on circulating neutrophils, however, has been described in other studies during systemic inflammation after trauma as well as during experimental endotoxemia[13,14,20].

C5aR(CD88)

C5aR surface expression did not significantly change during inflammation (figure 4). C5a is a strong chemotaxin for neutrophils and facilitates the oxidative burst and phagocytosis of neutrophils. Decreased C5aR expression has been described in severely injured patients as well as in septic patients, but was not seen in this study[21,22].

Discussion

Understanding the circulating neutrophil response to injury in severely injured patients has proven to be problematic and has resulted in contradicting data[13,23]. The extent and duration of the innate immune response in trauma patients is influenced by several inevitable confounders such as differences in age, sex, medical history, received (blood) products and importantly heterogeneity of injuries and surgical interventions. In addition, lack of baseline values and an estimated time of the insult and onset of inflammation make the interpretation of data difficult. To circumvent these confounders, we propose the use of the well-established human endotoxemia model to accurately study the kinetics of a homogenous early cellular innate immune response *in vivo* for PAMP- as well as DAMP-associated diseases. The main finding of the present study is that neutrophils, as part of the final common pathway, are similarly activated by PAMPs and DAMPs. Both trauma patients as well as endotoxemia subjects show a transient activation of neutrophils, which was characterized most prominently by transient down regulation of chemokine receptors CXCR1 and 2.

Both endotoxin and injury resulted in an increased leukocyte count as well as an increased percentage of circulating neutrophils mounting up to approximately 85% of all leukocytes. These data indicate a prominent role for neutrophils in the early immune response in PAMP and DAMP associated diseases. Yet, a remarkable difference in percentage of circulating monocytes was seen between the endotoxemia and trauma group at 3 hours after the onset of inflammation. During endotoxemia, monocytes seem to almost totally disappear from the circulation. Interestingly, this phenomenon seems not to occur, or at least to a lesser extent, during trauma. The rapid decline in amount of circulating monocytes after LPS challenge is most probably due to homing of monocytes to the tissue. Several studies have reported increased apoptosis of monocytes and lymphocytes of septic patients after incubation *in vitro*[24]. However, the implication of this increased apoptosis during sepsis is not clear yet. To our knowledge, no one -including ourselves- has ever identified apoptotic circulating immune cells during inflammation. Therefore, we believe that drop of monocyte count in this study is rather the result of redistribution of monocytes than that of apoptosis.

Although neutrophil activation *in vitro* is typically characterized by shedding of L-selectin and up-regulation of αM(CD11b) expression[17], these expected changes in surface expression were not seen on circulating neutrophils *in vivo.* In both study groups L-selectin expression did not significantly decrease. Surprisingly, αM(CD11b)

expression *in vivo* showed a reversed activation phenotype compared to *in vitro*. *In vivo* αM(CD11b) surface expression decreased during inflammation whereas expression is known to increase after activation *in vitro*. We can only speculate why surface receptor expression on circulating neutrophils 3 to 24 hours after onset of inflammation differs form activation phenotype *in vitro*, but we assume that surface receptor expression *in vivo* is influenced by altered distribution of neutrophils. Activated neutrophils are prone to leave the circulation and home to tissue, whereas young non-activated neutrophils are released from the bone marrow and enter the circulation[3,5]. Changes in circulating neutrophil population can thus explain a different neutrophil receptor phenotype during inflammatory responses *in vivo* compared to neutrophil phenotypes seen after activation *in vitro*.

The overall decrease in αM(CD11b) expression 24 hours after inflammation *in vivo*, is most likely caused by an increased amount of young neutrophils, expressing αM(CD11b) at lower levels[25,26]. A recruitment of young neutrophils is suggested by an overall decreased expression of FcγRIII, as FcγRIII is known to be expressed at lower levels on banded neutrophils[13,27]. In this study, the appearance of young neutrophil after PAMP and DAMP induced inflammation was not only indicated by low overall FcγRIII expression, but was also confirmed by examination of cytospins showing high numbers of banded neutrophils. Recently, it has been show that neutrophil lifespan is approximately 5 days[28]. This can explain the presence of young neutrophils in the circulation at more than 20 hours after cytokine levels return to normal.

Redistribution of neutrophils (including homing of circulating neutrophils to tissue and release of young neutrophils from the bone marrow) plays a pivotal role during the initial phase of the innate immune response. Little is known about the kinetics and signalling pathways triggering the early neutrophil redistribution. In this study we show that DAMP- and PAMP-induced inflammation results in a similar composition of circulating neutrophil populations at 3 hours after the insult. An earlier study from our group emphasizes the importance of identifying neutrophil populations[13]. We showed that functionality of circulating neutrophils during inflammation varies between young and segmented neutrophils, The diversity in functionality clarifies why priming *in vivo* not necessarily results in a overall increased function, as it depends on the constitution of the circulating pool. This result indicates the essence of investigating each different neutrophil population separately; in particular when it involves functionality or activation of intracellular signalling pathways for it could very well differ among populations.

The complexity of the inflammatory reaction and the rapid kinetics of circulating neutrophils complicates the identification of the underlying mechanisms by which DAMPs and PAMPs trigger the early neutrophil response *in vivo*. Isolation of different neutrophil populations by cell sorting at different time points during the initial phase is essential. The controlled neutrophil reaction evoked by LPS challenge may well serve for this purpose and the endotoxemia model may provide better comprehension of

the kinetics and the induction of signalling pathways in the future. This study suggests that DAMP and PAMP induced inflammation results in a similar neutrophil activation phenotype, but it remains to be elucidated if same signalling pathways are involved.

At 24 hours after LPS challenge, the αM(CD11b) expression on circulating neutrophils was significantly lower compared to after injury. This is most likely explained by the fact that endotoxin administration leads to a short cytokine release that peaks after 2 to 3 hours and then gradually declines to undetectable levels after 24 hours[12,13], whereas trauma results in elevated cytokine levels during days[29]. Hence, the decline of αM(CD11b) expression is probably more pronounced after experimental endotoxemia due to less circulating inflammatory mediators after 24 -hours, resulting in less priming and less up-regulation of αM(CD11b) expression in circulating cells.

A relatively short lived inflammatory response after LPS challenge limits the extrapolation of the endotoxemia model to more persistent inflammation. Therefore, comparison between the LPS challenge and inflammatory diseases, in our opinion, can best be made during the first hours after onset of inflammation. The endotoxemia model is a reliable model for examining the kinetics of this early innate immune response. During this initial phase it is difficult to obtain blood samples from trauma patients at defined time points. In the future, the endotoxemia model may give us more insight on how early neutrophil priming *in vivo* relates to migration of neutrophils to tissue, a phenomenon that underlies the pathogenesis of organ failure (a complication often seen during severe inflammatory diseases).

In conclusion, trauma and experimental endotoxemia result in comparable activation of circulating neutrophils illustrating the similarities in DAMP- and PAMP-induced activation of the systemic innate immune response. The inevitable differences between trauma and the human endotoxemia model, such as diverse duration of inflammatory stimulus and the variety of tissue injuries lacking in the standardized endotoxemia model, does not seem to influence the early (<12 hours) neutrophil response. Therefore, the endotoxemia model might be a helpful tool not only for investigating the early cellular immune response, but also for testing potential immunomodulating drugs in conditions in which therapy can start shortly after onset of inflammation such as trauma, burn injury and major surgery.

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Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia

Janesh Pillay
Bart Ramakers
Vera Kamp
Adele Lo Tam Loi
Siu Lam
Falco Hietbrink
Luke Leenen
Anton Tool
Peter Pickkers
Leo Koenderman

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Introduction

Systemic inflammation following e.g. trauma, burn injury, major surgery and during sepsis induces a profound systemic neutrophil response. These cells rapidly respond to tissue damage and invading pathogens. Neutrophils form a first line of defense against invading microorganisms[1]. However, during uncontrolled inflammation their potent anti-microbial systems can cause tissue damage of the host[2,3]. This latter process contributes to the development of pro-inflammatory complications such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS). Paradoxically, systemic inflammation can lead to immune suppression associated with impaired bacterial clearance and increased risk of infectious complications such as sepsis and septic shock, despite large numbers of circulating neutrophils[4].

The mechanisms underlying the immune dysfunction induced by systemic inflammation are poorly understood. However, several studies have pointed at the importance of anti-inflammatory circulating cytokines (e.g. IL-10), dysfunction of monocytes and deregulated adaptive immune responses[5,6]. The role of neutrophils in this process has mainly been attributed to down regulation of their effector functions such as phagocytosis and chemotaxis[7,8].

The paradoxical role of neutrophils in mediating both excessive tissue damage and immune dysfunction has important implications for the prediction of complications in critically ill patient groups[9,10].

Understanding the role of neutrophils in both inflammatory and infectious complications has been hampered by a large variability of results[11]. For example, in systemic inflammation, some studies showed increased neutrophil reactive oxygen species (ROS) release[12,13] whereas others showed reduced ROS release[14]. Similar contradictory results were reported regarding the surface expression of the αM (CD11b) integrin chain[8,15,16].

Many studies are difficult to compare because of differences in study population, therapeutic interventions, unknown start of systemic inflammation and poorly defined blood-sampling schemes. In addition, differences found in neutrophil functionality and activation might be due to the assumption that in diseased conditions the neutrophil pool is homogeneous, as few studies addressed the putative existence of neutrophil functional phenotypes. Seligmann et al. have shown that the epitope recognized by the monoclonal antibody 31D8 (later identified as FcγRIII) can be used as a marker for neutrophil heterogeneity and showed a decrease in function of neutrophils rich in $31D8^{dim}$ cells[17,18]. However, these functional assays were only performed in healthy donors. In inflammation $31D8^{dim}$ cells are mobilized to the circulation, but their functional response under these conditions was not addressed[19].

Our study was designed to circumvent the above-mentioned issues by using a human model for induction of a controlled and acute systemic inflammation by intravenous LPS challenge. This was followed by the isolation of different neutrophil phenotype by cell sorting in order to directly functionally characterize these cells. Intravenous LPS

challenge is characterized by an acute inflammatory response, which resolves after 6-8 hours by induction of anti-inflammatory cytokines such as IL-1RA and IL-10[20].
We report the down regulation of neutrophil receptors necessary for chemotaxis, microbial recognition, and killing. Functionally, neutrophils showed a moderately decreased interaction with opsonized S.epidermis and impaired responsiveness towards the innate immune stimulus fMLF in the context of inside-out control of FcγRII (CD32). Paradoxically, this suppressed neutrophil phenotype was associated with a marked up-regulation of the capacity to produce ROS. An explanation for this paradoxical phenotype was found in simultaneous priming and activation of circulating neutrophils and release of refractory banded neutrophils with a low functionality from the bone marrow.

Methods

Subjects and study design

The study protocol was approved by the Ethics Committee of the St. Radboud University Nijmegen Medical Centre and complies with the Declaration of Helsinki and the Good Clinical Practice guidelines. Seven male volunteers gave written informed consent to participate in experiments, which were part of a larger endotoxin trial (NCT00513110 at www.clinicaltrials.gov). Subjects were enrolled after screening[21] and prehydrated with 1500 ml glucose/saline infusion[22]. U.S. Reference E.coli endotoxin (lot Ec-5, Centre for Biologic Evaluation and Research, Food and Drug Administration, Bethesda, MD) was used in this study. Ec-5 endotoxin was reconstituted in 5ml saline 0.9% and injected as single intravenous bolus (2ng/kg) during 1 minute at t=0. Blood samples anti-coagulated with sodium heparin were taken from the arterial and venous catheter at baseline and serially after administration of LPS. Blood taken from each control before the administration of LPS served as a control and is referred to as t=0 throughout the manuscript.

Reagents and antibodies

FITC-labeled phage-antibodies directed against active FcγRII were generated and characterized as previously described[23]. Monoclonal antibodies CD89 (clone A3/Santa Cruz Biotechnology CA, USA), CD11b (clone 2LPM19c/DAKO, Heverlee, Belgium), CD11b (clone cbrm1/5 /Biolegend, San Diego, CA, USA), TLR4 (clone HTA-125/ Imgenex, San Diego, CA, USA) were used. (1.) isotype controls IgG1 FITC (clone MOPC-21), IgG2a PE (clone G155-178), IgG2b FITC (clone 27-35) and IgG2b PE (clone MPC-11), CD16 Alexa Fluor 647 (clone 3G8); (2.) CD45 (clone 2D1), CD181 (clone 42705) and CD182 (clone 48311); (3.) CD88 (clone W17/1), CD29 (clone 4B7R) and isotype control IgG2a FITC (clone MRC OX-34); (4.) N-formyl-methionyl-leucyl-phenylalanine (fMLF), platelet-activating factor (PAF), phorbol myristate acetate (PMA) and Horseradish Peroxidase (HRP) and (5.) Amplex Red were purchased from (1)

Pharmingen (Erembodegem, Belgium), (2) R&D Systems (Minneapolis, MN, USA), (3) Serotec (Düsseldorf, Germany), (4) Sigma (St. Louis, MO, USA) and (5) Molecular Probes (Leiden, The Netherlands) respectively.
The following antibodies and reagents were used for western blotting, P67 rabbit antiserum (Upstate, Lake Placid, NY), P47 mouse monoclonal (clone D-10 Santa Cruz Biotechnology, Santa Cruz, CA), P22 mouse monoclonal (clone 48, Sanquin Reagents, Amsterdam, The Netherlands), GAPDH mouse monoclonal, (clone 374, Chemicon, Temecula, CA), nitrocellulose (Whatman/Schleicher & Schuell, Dassel, Germany) and diisopropyl fluorophosphates (DFP, Sigma (St. Louis, MO, USA).

Flow cytometry and cell counts
Blood was collected in sterile collection tubes containing sodium heparin at timepoints t=0 (before LPS) and 5, 30, 60, 90, 180 and 360 minutes after LPS administration. Blood was kept on ice and staining procedures were started within 1 hour. Blood samples were stained with A17 and A27 recognizing the active form of FcγRII as described previously[23]. In short, directly labeled antibody was added 1:20 to whole blood and incubated for 60 minutes on ice. After incubation erythrocytes were lysed with ice cold NH_4Cl. After a final wash, cells were resuspended in PBS containing trisodium citrate (0.4% w/v pH 7.4) and human pasteurized plasma solution (4 g/L) (PBS2+) and analyzed by flow cytometry. Commercially available antibodies were stained using a different protocol, whereby firstly erythrocytes were lysed using ice-cold NH_4Cl. After the lysing step, cells were resuspended in PBS 2+ and directly labeled antibodies were added according to the manufacturers' protocol.
In addition, the expression of active FcγRII, Mac-1 (CD11b) and CD45 were studied after activation of whole blood with fMLF. In short, whole blood was stimulated at 37°C for 5 minutes with N-formyl-methionyl-leucyl-phenylalanine (fMLF 10^{-6}M). Hereafter, red blood cells were lysed with NH_4Cl and the remaining total leukocytes were stained with the directly labeled antibodies to evaluate the responsiveness of the cells to a bacterial derived agonist.
Cells were analyzed in a FACScalibur flow-cytometer (Becton & Dickinson, Mountain View, California). The neutrophils were identified according to their specific forward- and side-scatter signals. Data from individual experiments are depicted as fluorescence intensity in arbitrary units (MFI) of at least 5000 events.
Total leukocyte counts were counted in whole blood kept on ice using Cell-Dyn 1800 cellcounter (Abbot Diagnostics, Abbot Park, IL). Using distinct forward and sideward scatter profiles on flow cytometry, percentages monocytes, lymphocytes and neutrophils were determined. Percentage eosinophils were calculated by identifying $CD16^{negative}$ cells in the granulocyte population. Using the percentages and the absolute leukocyte count, absolute numbers of circulating leukocytes could be calculated. In addition, cytospins stained with May Grunwald Giemsa revealed no circulating myeloid progenitors during

the course of the LPS model (results not shown).

Cytokines

Plasma was obtained from blood anticoagulated with sodium heparin after immediate centrifugation (2000G at 4°C for 15 minutes) and was stored at -80°C until analysis. Concentrations of cytokines were determined in one sample using a multiplex assay[24].

Interaction of neutrophils with opsonized S.epidermis

Neutrophils were isolated as described before[25]. In short, mononuclear cells were removed after ficoll density centrifugation (2000 rpm for 20 min), which was followed by isotonic lysis of remaining erythrocytes with NH_4Cl. Isolated neutrophils were incubated with live FITC-labeled S.epidermis in a 10:1 bacterium:neutrophil ratio, which were opsonized in the presence of 10% heat-inactivated (56°C, 30 minutes) human pooled serum for 2 hours[26].

Neutrophil interaction with opsonized S.epidermis was measured by flow cytometry[27]. Percentage neutrophils interacting with S.epidermis was identified by determination of the percentage FL-1 positive cells in the neutrophil gate in comparison to the FL-1 negative cells. The overall capacity of individual neutrophils to interact with S.epidermis was measured by the mean fluorescence intensity (MFI) of the FL-1 positive neutrophils

Reactive Oxygen Species

Total leukocytes, after erythrocyte lysis, were counted and the number of cells was adjusted to $0.5*10^6$ neutrophils/ml. Samples were incubated in a 96 wells plate in the presence of Amplex Red (10mM) and HRP (100U/ml). Stimuli or incubation buffer alone (as control) were added and H_2O_2 release was measured for 30 minutes at 37°C. H_2O_2 was determined in a fluoro-luminometer (FluostarOptima/BMGLABTECH)[28] by determination of fluorescent resorufin, which was formed from Amplex Red in the presence of H_2O_2 and horseradish peroxidase.

In addition, production of ROS of was studied by double staining shocked whole blood with an antibody against CD16 and loading with dihidrorhodamine (DHR) for 15 minutes at 37°C. After stimulation with fMLF or PAF/fMLF the relative increase in ROS production could be determined in $CD16^{dim}$ and $CD16^{bright}$ neutrophils.

Statistical analysis

Data were analyzed using Graphpad Prism 4.0. Repeated measure ANOVA was used to compare different time points. Data are plotted as mean ± SEM. Comparisons made between 0 and 6 hours were analyzed by a Mann Whitney U test unless stated otherwise. A p-value <0.05 was considered significant.

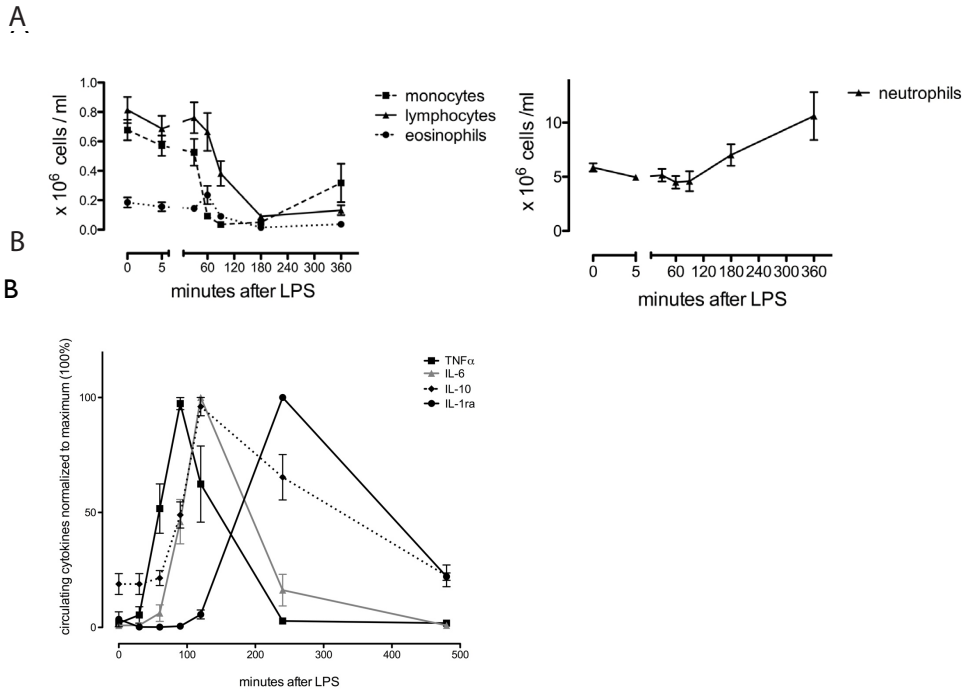

Figure 1. Kinetics of leukocytes and cytokines during LPS induced inflammation. Circulating leukocytes after LPS challenge are depicted in panel A and B showing a sharp decline of monocytes, lymphocytes and eosinophils 60-90 minutes after LPS administration **(A)** and an increase in neutrophil counts **(B)**. Mean ± SEM of 7 donors plotted. **(B)** Cytokines after LPS. The initial phase is characterized by TNFα and IL-6. The late phase is characterized by IL-10 and IL-ra. Cytokines are depicted as percentage of maximum concentration. All peaks were significantly different compared to timepoint 0 as tested by one-way ANOVA ($p<0.0001$) (n=7).

Results

Marked increase in neutrophils and decrease of monocyte, eosinophil and lymphocyte numbers in peripheral blood 3-6 hours after LPS challenge

As described previously, human endotoxemia led to a decrease in neutrophil number in the peripheral blood 1 hour after administration of LPS. This was mirrored by a decrease in monocytes, eosinophils and lymphocytes (figure 1A). The kinetics of circulating immune cell types in the peripheral blood differed greatly. After an initial drop, the monocyte counts recovered after 6 hours. In contrast, lymphocyte and eosinophil numbers remained decreased 6 hours after challenge. Neutrophil counts on the other hand dramatically increased from 1.5 hours onwards following the LPS challenge.

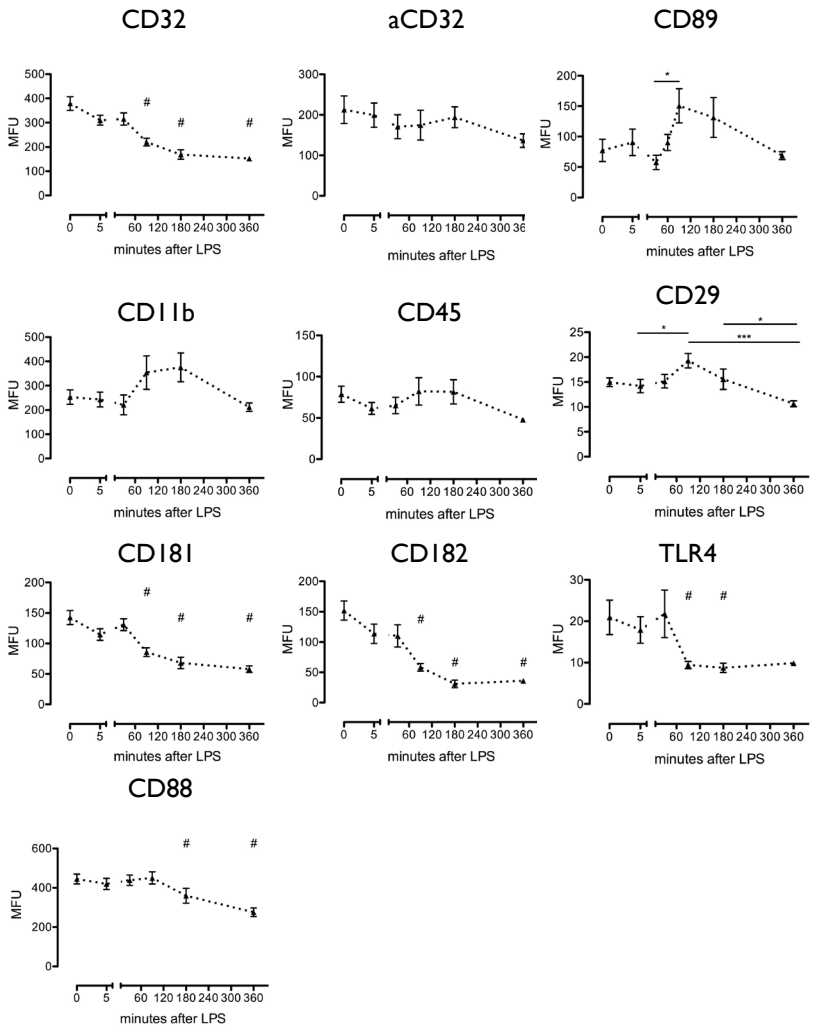

Figure 2. Receptor expression on circulating neutrophils. Blood was collected before and various times after LPS challenge. Erythrocytes were immediately lysed in NH_4Cl (see Materials and Methods). Hereafter, total leukocytes were stained with directly labeled antibodies and neutrophils were gated on their specific forward and sideward scatter characteristics. . # indicates a difference compared to t=0 P<0.05 (n=7). Data are shown as means ± SEM (n=7). * P<0.05 ** p< 0.005 *** p<0.001

Pro- and anti-inflammatory cytokine profile after LPS challenge

TNFα levels in the peripheral blood sharply increased after LPS challenge reaching a maximum value of 860±180 pg/ml 1.5 hours after LPS (figure 1B). The rise of TNFα levels was accompanied by flu-like symptoms as also previously reported[29]. This increase in TNFα was transient and was followed by normalization to basal levels within 4 hours after the LPS challenge. The TNFα peak was followed by a rise in IL-6 levels reaching a

maximum (1170±250 pg/ml) at 2 hours. IL-1β remained below the detection limit of our assays (results not shown). In addition to the release of the above mentioned pro-inflammatory cytokines, clear increases were found for the anti-inflammatory cytokines IL-10 and IL-1Ra with maximal values of 53±14 pg/ml and 27±2 ng/ml respectively. These values were reached after 2 (IL-6) and 4 (IL-1Ra) hours following LPS administration (figure 1B). 8 hours after LPS a marked decrease was seen in anti-inflammatory cytokines IL-10 and IL-1Ra demonstrating the transient nature of the LPS model.

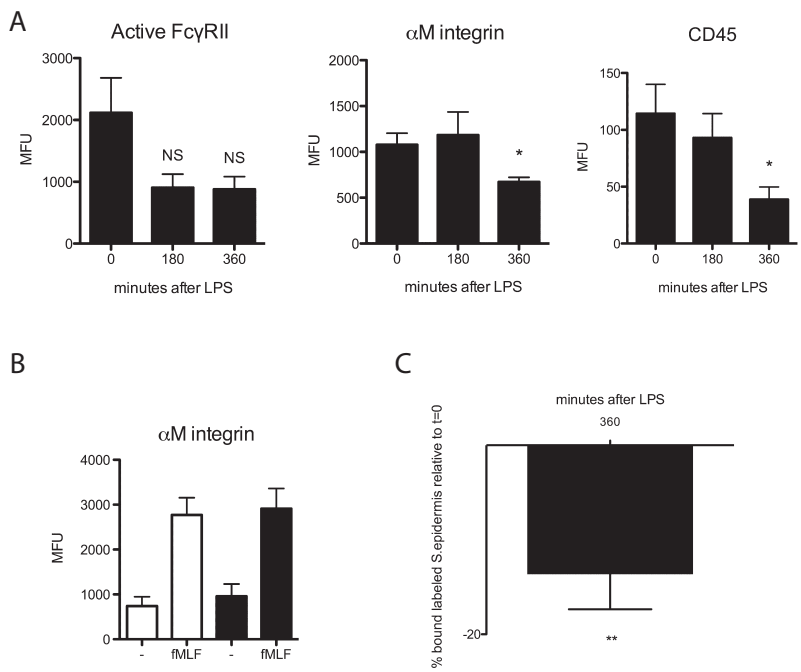

Figure 3. Responsiveness of circulating neutrophils to activation with fMLF and capacity to interact with S.epidermis. **(A)** Responsiveness of neutrophils towards *ex vivo* stimulation with fMLF (10^{-6} M, 5 minutes 37^{O}C) was measured by determination of the upregulation of activation markers αM integrin subunit, active FcγRII and CD45, before and 3 and 6 hours after LPS challenge. **(B)** Responsiveness of neutrophils from healthy controls to stimulation with fMLF (10^{-6} M, 5 minutes 37^{O}C) after pre-incubation of whole blood with plasma obtained before and 6 hours after LPS (15 minutes 37^{O}C added in a 1:1 ratio). **(C)** Neutrophil binding to opsonized FITC-labeled S.epidermis, neutrophils was determined by incubation of neutrophils with opsonized bacteria for 2 hours at 37^{O}C. Data are plotted as a percentage of t=0. Data represent means ± SEM of 7 donors. * $p<0.05$ and ** $p<0.01$ as analyzed by wilcoxon matched pairs test.

Expression of innate immune receptors on circulating neutrophils during LPS challenge
1-1.5 hours after the administration of LPS the circulating neutrophil pool showed a significant up-regulation of the Fcα-receptor (FcαR) and β1 integrin (figure 2). Classical activation markers such as the αM (CD11b) integrin chain and CD45 showed no statistically significant increases in expression.
A decrease of innate immune receptors was most apparent 3-6 hours after LPS challenge. For some receptors such as FcγRII (figure 2), the IL-8 receptors (CD181/182) and TLR4 (figure 2) this decrease was extensive, resulting in less than half of the expression 6 hours after LPS compared to the expression prior to administration of LPS. Expression of FcγRI (clone 10.1) was not detected on the surface of neutrophils in this model (results not shown).

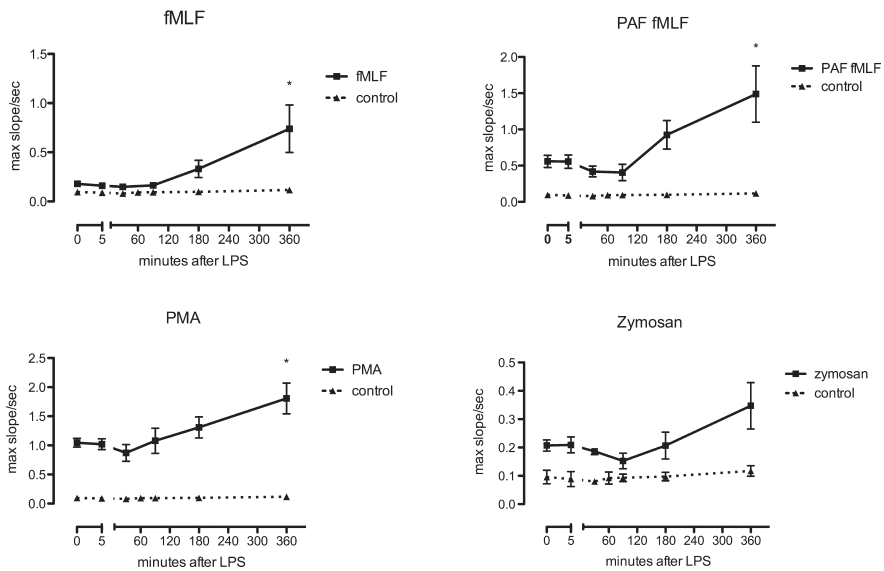

Figure 4. Production of reactive oxygen species before and after challenge with LPS. ROS production induced by PAF/fMLF (10^{-6}M/10^{-6}M), zymosan (1 mg/ml). PMA (100 ng/ml) and fMLF (10^{-6}M) was determined several times after LPS challenge. H_2O_2 release was measured by the oxidation of Amplex Red. The data are expressed as the maximal slope of the H_2O_2 production during total assay time (30 minutes). Incubation buffer served as a control for the addition of stimuli. Data are presented as means ± SEM. * $p<0.05$ compared to t=0 (n=7).

In addition to the decrease in surface receptors, circulating neutrophils showed a decreased capacity to respond to an inflammatory stimulus in the context of upregulation of the activation markers CD45, integrin αM and active FcγRII. *Ex vivo* stimulation with the bacterial peptide fMLF showed impaired responsiveness as visualized by the lowered expression of these innate receptors (figure 3A). Plasma obtained 360 minutes after administration of LPS could not induce impaired responsiveness for FMLF in cells from healthy controls (figure 3B).
In addition, arterial blood was compared to venous blood for all surface markers and no differences were observed (results not shown).

Interaction of neutrophils with opsonized S.epidermis
The observed decrease in Fcγ-receptors and impaired capacity to up-regulate the αM (CD11b) integrin chain and active FcγRII led us to examine the capacity of the neutrophils to interact with opsonized S.epidermis. No differences were observed in the percentage neutrophils that interacted with opsonized S.epidermis acquired before (45 ± 6 %; mean +/- SEM) versus six hours after LPS (46 ± 9%; mean +/- SEM). However a small but significant decrease of 14 ± 4% SEM ($p< 0.05$, n=7) was seen in the overall number of bacteria associated with neutrophils 6 hours after LPS (figure 3C).

Priming of respiratory burst in circulating neutrophils
Optimal activation of the NADPH-oxidase in neutrophils requires priming by inflammatory mediators such as PAF, TNF or LPS. Indeed, the ROS production of t=0 neutrophils was low in fMLF stimulated cells (maximal slope/sec of 0.13±0.01 arbitrary units) whereas it was higher in PAF/fMLF stimulated cells (0.42±0.06)). Interestingly, an increase in fMLF-induced respiratory burst was observed 3-6 hours after LPS challenge compared (figure 4) suggesting that *in vivo* priming occurred. This is in marked contrast to the poor fMLF-induced increase in expression of the αM integrin at 6 hours (figure 3A), which is considered a classical marker for neutrophil activation. An increased ROS release was also seen after stimulation with the soluble mediators PAF/fMLF and PMA and with zymosan at 3-6 hours after challenge. This suggests a primed NADPH-oxidase complex in circulating neutrophils 3 hours after administration of LPS.

Release of banded CD16^{dim} neutrophils 3 hours after infusion of LPS
Administration of LPS showed an increase in circulating neutrophils. This increase can partially be attributed to the release of CD16^{dim} neutrophils 3 hours after administration of LPS (figure 5A&B). To exclude that the decrease of CD16 was attributable to shedding as some have reported, we FACS sorted neutrophils at 3 hours after LPS on ice into a CD16^{dim} and a CD16^{bright} population. CD16^{dim} neutrophils displayed a "young" banded morphology whereas the CD16^{bright} population exhibited a normal segmented morphology.

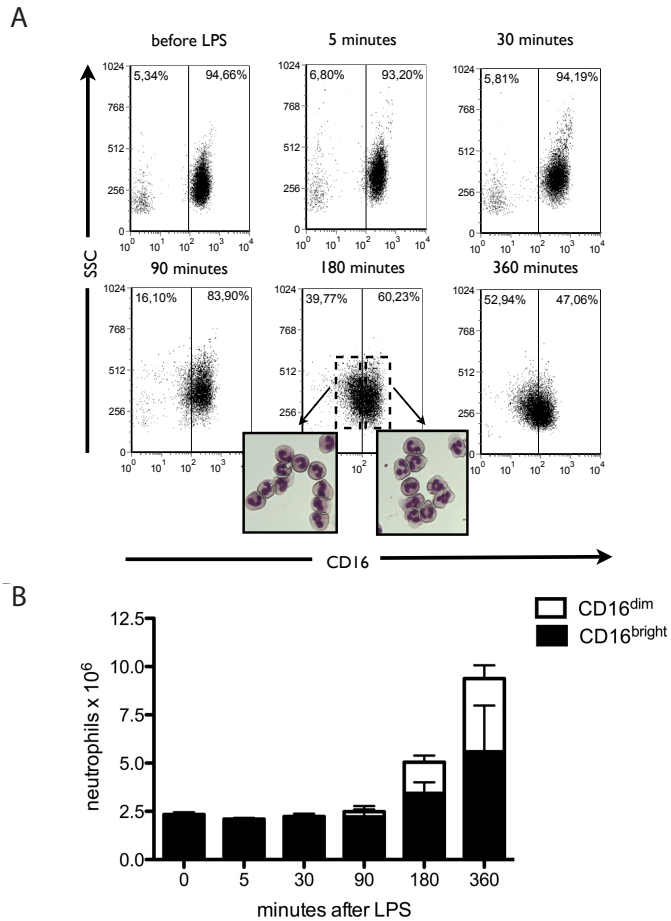

Figure 5. Release of CD16^{dim} neutrophils 3 hours after LPS challenge. (A)FACS scatter plot of neutrophils stained with an antibody against CD16. A release of CD16^{dim} with a banded morphology neutrophils was seen 3 hours after LPS challenge. **(B)** Absolute counts of circulating neutrophil populations as determined by flow cytometry. Mean ± SEM of 7 donors plotted.

Expression of innate immune receptors on CD16^{bright} and CD16^{dim} neutrophils

The largest changes in receptor expression on the entire circulating neutrophil pool were seen 3 hours after LPS. This coincided with the release of 30-40% CD16^{dim} neutrophils. We, therefore, examined the expression of innate immune receptors on the two circulating neutrophil populations separately by double staining various samples with CD16. This revealed that for most innate immune receptors studied expression markedly differed between the CD16^{bright} and CD16^{dim} neutrophils. Importantly expression of activation markers (αM integrin and active FcγRII), which were not found to be elevated when studying the entire neutrophil pool as a homogenous population were found to be upregulated in CD16^{bright} neutrophils, whereas the expression was lower or similar

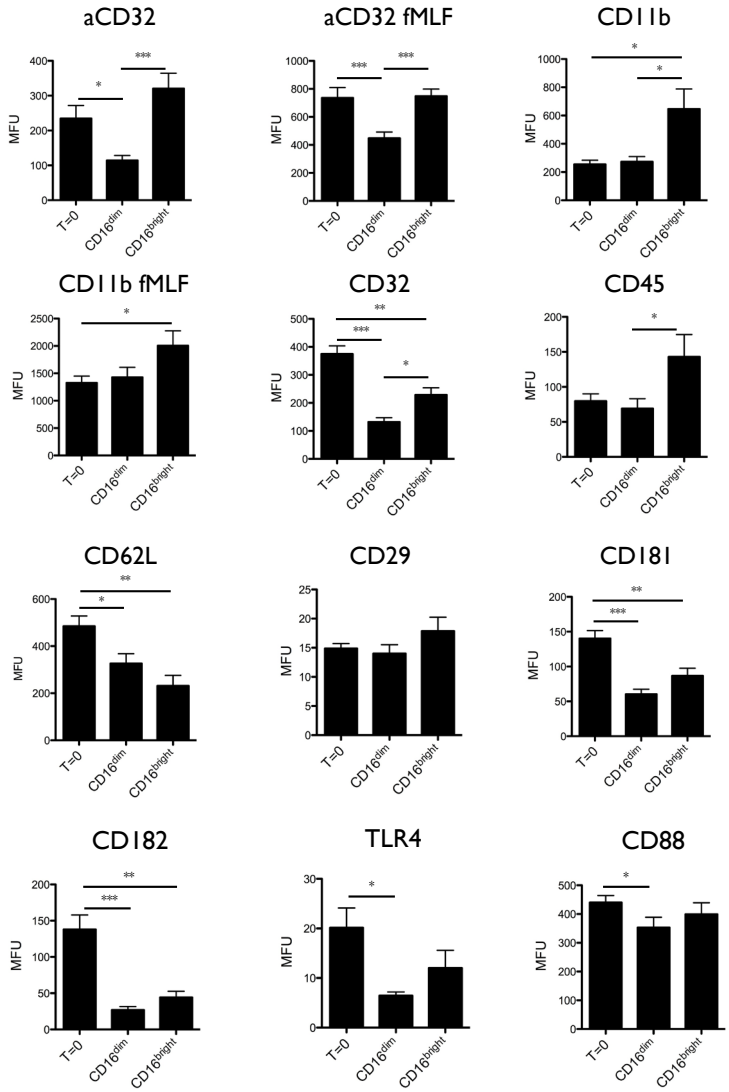

Figure 6. Receptor expression on CD16^{dim} and CD16^{bright} neutrophils. Blood was collected 180 minutes after LPS challenge. Erythrocytes were immediately lysed in NH_4Cl (see Materials and Methods). Hereafter, total leukocytes were stained with directly labeled antibodies and neutrophils were gated on their distinct forward and sideward scatter characteristics. Data are shown as means ± SEM (n=7). * P<0.05 ** p< 0.005 *** p<0.001.

on the CD16^{dim} neutrophils compared to neutrophils from T=0 (figure 6). In addition upregulation in response to fMLF of these markers was markedly lower in the CD16^{dim} neutrophils. Decreased expression of various innate immune receptors was stronger on CD16^{dim} neutrophils compared to CD16^{bright} neutrophils except for CD62L which is typically shed on activation (figure 6).

Oxygen radical production and interaction with S.epidermis of CD16^{bright} and CD16^{dim} neutrophils.

We compared the CD16^{bright} with the CD16^{dim} neutrophils in interaction with opsonized S.epidermis and generation of the respiratory burst. Neutrophils were double-stained with DHR and respiratory burst was measured after pre-incubation with fMLF. This revealed that CD16^{dim} neutrophils were less capable of generating a respiratory burst in comparison with CD16^{bright} neutrophils (figure 7A&B). However these CD16^{dim} neutrophils exhibited an enhanced fMLF-induced respiratory burst compared to control cells (figure 7A). In addition, sorted CD16^{dim} neutrophils exhibited a decreased interaction with opsonized S.epidermis compared to CD16^{bright} neutrophils (figure 7C).

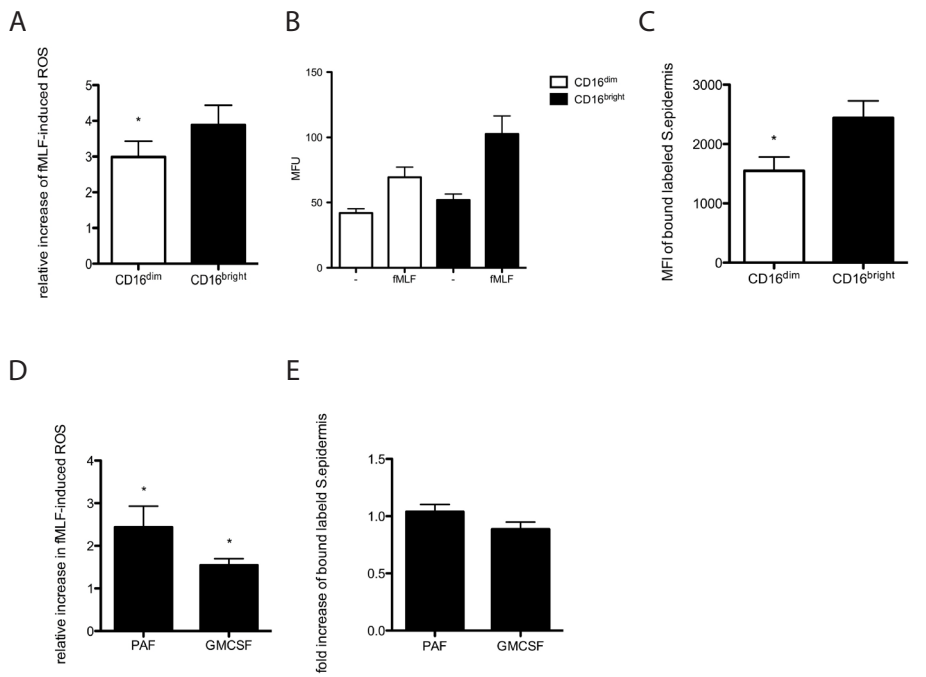

Figure 7. Functional characteristics of CD16^{dim} and CD16^{bright} neutrophils. (A) Cells were gated according as shown in figure 5 and the respiratory burst induced by fMLF (1 μM) was assayed by application of DHR. **(B)** Absolute fluorescence of DHR of neutrophil populations in presence and absence of fMLF. **(C)** CD16^{dim} and CD16^{bright} neutrophils were sorted by flow cytometry and interaction with opsonized S.epidermis was studied. **(D)** Relative increase of the fMLF induced respiratory burst by control cells after priming with PAF (1 uM) and GMCSF (10 nM) compared to non-primed cells. **(E)** The effect of priming on the interaction of neutrophils with opsonized S.Epidermis compared to non-primed cells. Data are presented as means ± SEM. * $p<0.05$ as analyzed by wilcoxon matched pairs test.

Although both interaction with S.epidermis and generation of a respiratory burst was lower in CD16^{dim} neutrophils, we could show that in control cells the generation of a respiratory burst was normally primed by PAF and GM-CSF (figure 7D). This is

comparable to the *in vivo* situation (figure 4), in which neutrophils are primed for an fMLF-induced respiratory burst after 3-6 hours. Bacterial interaction however could not be primed in control cells (figure 7E). Therefore, fully primed circulating cells would not compensate for a less functional CD16^{dim} neutrophil population.

Discussion

Clinical studies concerning the functionality of neutrophils in patients suffering from acute systemic inflammatory conditions have yielded conflicting results. Marked differences in functionality of the neutrophil compartment have been observed ranging from increased functionality to down regulated cellular function[30,31]. We have studied neutrophil responses during systemic inflammation using a controlled acute inflammatory model by challenging healthy individuals with systemic endotoxin. We showed in this human model that the circulating neutrophil pool displayed a paradoxical phenotype, exhibiting characteristics of both activated and suppressed functionality. These dysfunctional properties of the circulating neutrophil pool were characterized by: 1. down-regulation of essential innate immune receptors, 2. impaired responsiveness of receptors towards the innate immune activator FMLF and 3. decreased capacity to interact with opsonized bacteria. Much to our surprise we found, simultaneously priming and activation of the respiratory burst initiated by various agonists. We tested the hypothesis that these seemingly opposing functionalities of the circulating neutrophil pool could be explained by mobilization of neutrophils with functional heterogeneity[32]. It is known that systemic inflammation induces release of immature neutrophils from the bone marrow[19]. These cells display a banded nuclear morphology and have lower expression of FcγRIII (CD16) (figure 5). Neutrophil heterogeneity was studied previously by application of the 31D8 antibody, which was later found to recognize FcγRIII (CD16)[17]. It was shown that 31D8^{dim} cells from the bone marrow and in the circulation of healthy donors showed decreased functionality[18]. 31D8^{dim} neutrophils were found in a similar model of human endotoxemia, however, no data was published on the functional characteristics of these cells and the kinetics of activation and priming. These studies were performed more than 25 years ago and neutrophil heterogeneity in systemic inflammation has only attracted very limited attention since. In severe and persistent inflammation (sepsis) or after mobilization after G-CSF treatment, immature neutrophils have been proposed to be functionally different compared with mature neutrophils[30,33]. However, it is unclear whether these preparations contained neutrophil precursors such as metamyelocytes and promyelocytes, which might have influenced results.

LPS challenge also mobilized CD16^{dim} neutrophils (figure 5). We, therefore, stained our entire neutrophil pool with CD16 and sorted the dim and bright populations by flow cytometry. Our CD16^{dim} population mainly consisted of cells with a banded morphology but lacked neutrophil precursors. When comparing these two subsets we showed that the CD16^{dim} neutrophils were lower in receptor expression of in almost all measured innate

immune receptors, were less primed for ROS release and showed less interactions with opsonized bacteria.

The lower activation state of these CD16^{dim} neutrophils is reflected by their high expression of CD62L and low expression of CD11b and active FcγRII compared to competent CD16^{bright} neutrophils. Expression of various chemokine receptors such as CD181 and CD182 were down regulated on the whole neutrophil population upon LPS-challenge. The lowest expression was found in the CD16^{dim} neutrophils. CD181 and CD182 were shown to be actively and rapidly down regulated in inflammation by proteolitic cleavage[34]. However, it is tempting to speculate that immature neutrophils released from the bone marrow have an intrinsically lower expression of these receptors, as they have a less activated phenotype.

An intrinsic low functionality of immature neutrophils might also explain the reported decreased bacterial interaction of our CD16^{dim} neutrophils with opsonized S.epidermis. Decreased interaction has been reported in several studies of septic patients[14,30]. These reports focused on the entire neutrophil pool in septic patients and did not distinguish between progenitors, immature and mature neutrophils. Kaufmann et al. showed a decrease in phagocytosis and ROS production to unopsonized zymosan. In our study, however, ROS production to unopsonized zymosan was increased after LPS administration. This might be attributed to priming of the mature fraction of neutrophils shortly after LPS challenge. Taneja et al. reported decreased phagocytosis of serum opsonized zymosan by immature neutrophils from septic patients, however, failed to address the possibility of contaminating pro- and metamyelocytes[35]. Also other studies regarding functionality of the neutrophil compartment in severe inflammation are difficult to interpret because no data were presented regarding maturation states and their functional heterogeneity of neutrophils[8,13,36-38]. Wenische et al showed a decrease in phagocytosis of septic serum-opsonized E.coli, which can be restored by normal serum, thus concluding the impaired phagocytosis was due to serum factors[14]. In contrast, as we performed our assays with bacteria opsonized by normal human pooled serum, our results are the first to show that CD16^{dim} neutrophils have an intrinsic defect in the interaction with bacteria such as S. Epidermis.

In our model the entire neutrophil population was characterized by a slightly but significantly decreased bacterial interaction as soon as 3 hours after LPS administration (figure 3B). The intrinsic decrease in of the CD16^{dim} neutrophils could not be compensated by priming of the mature CD16^{bright} neutrophils. For neutrophils cannot easily increase their interaction with opsonized particles by priming or activation (figure 7D). This could explain the fact that persistent inflammation leads dysfunctional innate immune responses and increased susceptibility to microbial invasion, as this would be due to relative depletion of functionally mature CD16^{bright} neutrophils which are replaced by CD16^{dim} less functional immature neutrophils and eventually possibly by precursor cells[39]. In this study we show that as early as 3 hours after systemic inflammation the

first signs of decreased neutrophil functionality can be seen due to release of a $CD16^{dim}$ neutrophil population.

In light of the above decreased functionality is likely to be due to incomplete maturation. Neutrophil mediated immune dysfunction might therefore be due to at least two mechanisms. The first described by Hartl et al. in which proteolitic cleavage of essential chemokine receptors render previously competent neutrophils less functional [34]. The second is described in this manuscript, in which intrinsically less functional immature neutrophils are released from the bone marrow in severe inflammation. Restoration of adequate innate immune responses in the first case could be accomplished inhibiting proteolytic cleavage and in the second case innate immune dysfunction in systemic inflammation might be overcome by acceleration of cellular maturation. Further studies on neutrophil populations and their maturation are needed to clarify the underlying mechanisms.

Finally, although neutrophils cannot be easily primed for interaction with microbes, we show that *in vivo* and *ex vivo* priming with soluble stimuli such as PAF results in a marked increased fMLF-induced ROS release. This priming is apparent in mature and to a lesser extent in immature neutrophils. As a result, both mature and immature neutrophils remain capable of contributing to tissue damage.

Concluding, our study shows that systemic inflammation induced by intravenous endotoxin rapidly leads to the occurrence of a circulating neutrophil pool with a paradoxical functionality. This is due to selective priming of the respiratory burst in and the release of banded neutrophils displaying decreased anti-microbial capacity.

The resulting circulating neutrophil pool can simultaneously mediate tissue damage and be dysfunctional in anti-microbial defense. Importantly studying neutrophils as a heterogeneous population in inflammation might greatly enhance our understanding of neutrophil biology.

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A subset of neutrophils in human systemic inflammation inhibits T-cell responses through Mac-1 (αMβ2)

Janesh Pillay*
Vera Kamp*
Els van Hoffen
Tjaakje Visser
Tamar Tak
Jan-Willem Lammers
Laurien Ulfman
Luke Leenen
Peter Pickkers
Leo Koenderman

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** authors contributed equally*

submitted

Introduction

Immune suppression is essential in immune regulation, but can be detrimental in various pathological conditions. Classically, lymphoid cells, such as regulatory T-cells, have been implicated in balancing immune responses. Recently, however, a role for myeloid cells mediating immune suppression has gained much attention[1]. A heterogeneous group of cells referred to as myeloid-derived suppressor cells (MDSCs) has been shown to directly suppress T-cell functions in murine models of cancer, infectious diseases, bone marrow transplantation and autoimmune diseases[2-5]. Murine MDSCs consist of immature myeloid cells of monocytic and granulocytic lineages[5]. The majority of research concerning MDSCs has focused on murine tumor models. The immune suppressive characteristics of these MDSCs are thought to limit the effectiveness of anticancer vaccines[6]. Several mechanisms are described to regulate suppression of T-cell proliferation in these models. Classically arginase I is associated with MDSC suppressive function. Additionally also inhibition via ROS, TGFβ, and IL10 was described[7]. In mice MDSCs with a granulocytic origin suppressed T-cell proliferation mainly via a ROS mediated mechanism[8]. In humans, immature myeloid and granulocytic MDSCs have been identified in patients with metastatic cancer[9-11]. In agreement with murine models, these cells suppress T-cell functions mainly through an arginase I dependent mechanism[12]. Only few papers describe the involvement of ROS in human MDSCs[13]. As relatively little is known about human MDSCs compared to their murine counterpart this greatly limits our understanding of the mechanism of suppression in humans. Therefore it is essential to gain more insight in the occurrence and mechanisms of suppression by human MDSCs.

Apart from their occurrence in chronic inflammation, MDSCs have been shown to suppress lymphocyte responses in a murine model of sepsis[14]. In addition, in humans the concept is emerging that acute inflammation can result in immune suppression[15]. This suppression is characterized by an inadequate T-cell response, which can lead to opportunistic infections and reactivation of latent viruses[16,17]. So far, the role of human MDSCs in acute inflammation has not been evaluated.

Human acute inflammation results in the mobilization of large amounts of mature and immature neutrophils, which are traditionally viewed as effector cells important in combating infections. However as tumor associated neutrophils are detrimental[18,19], possibly through suppression of anti-tumor responses, we hypothesized that human neutrophils could also fulfill the role of MDSCs. To test this hypothesis we induced such inflammation by systemic challenge with lipopolysaccharide (by intravenous injection of 2 ng/kg E.Coli LPS) in healthy volunteers[20].

Results

Appearance of a neutrophil subset after i.v administration of LPS in humans

Firstly, we determined the phenotype of circulating neutrophils after LPS challenge. The left panel of figure 1A depicts a flowcytometer dotplot showing uniform expression of

CD16 and CD62L by normal granulocytes before LPS administration. The CD16 negative cells were eosinophils. After LPS challenge a marked decrease was seen in circulating eosinophils (figure 1A, right panel) in peripheral blood. As previously described a marked neutrophilia was induced. At three hours after LPS administration the pool of circulating neutrophils consisted of at least 3 morphological phenotypes (figure 1A, right panel). At this timepoint, banded ($CD16^{dim}$ / $CD62L^{bright}$) neutrophils appeared in the circulation, most likely released from the bone marrow. In parallel $CD16^{bright}$/$CD62L^{dim}$ neutrophils were found in the circulation displaying a hypersegmented morphology of the nucleus. The $CD16^{dim}$ / $CD62L^{bright}$ and $CD16^{bright}$/$CD62L^{dim}$ cells were not found in healthy donors before LPS administration (figure 1A left panel). These populations comprised 20-25% and 10-15% of total circulating neutrophils, respectively (figure 1B).

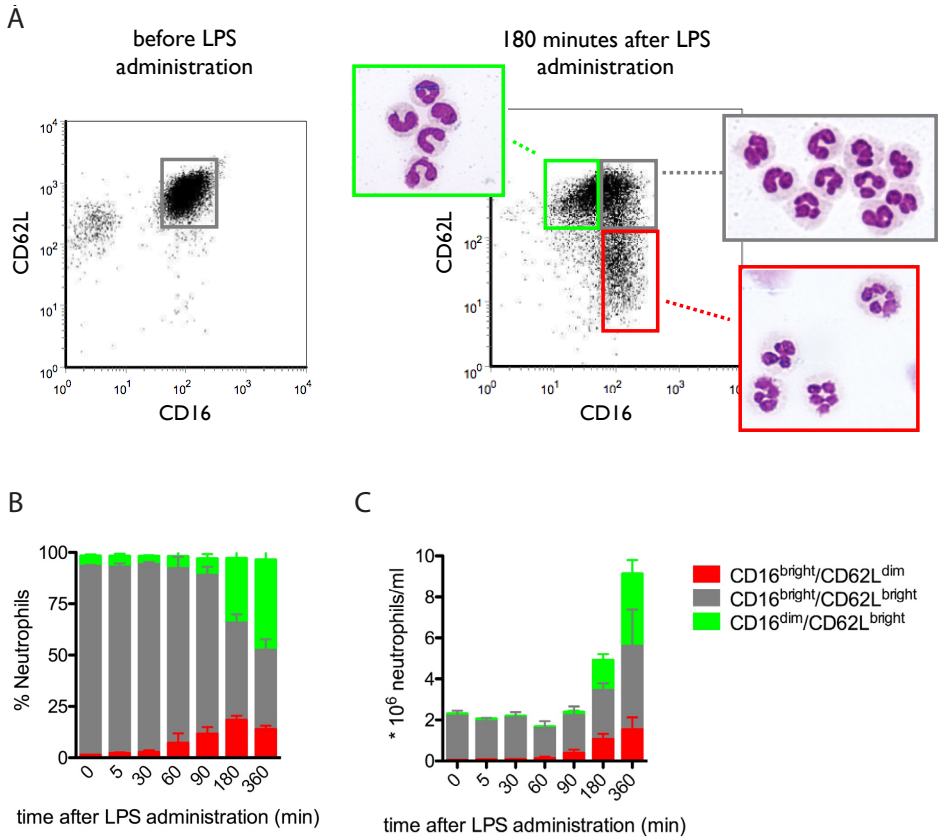

Figure 1. Neutrophil subsets after LPS administration. (A) Neutrophils were stained for CD16 and CD62L before and 180 minutes after LPS administration. Before LPS administration neutrophils form one population, the $CD16^{low}$ cells are eosinophils, at t=180min two distinct neutrophil subsets were identified. Subsets were FACS-sorted, cytospins were made, and stained with May-Grunwald Giemsa. **(B)** Neutrophils were stained for CD16 and CD62L at different timepoints, timepoint zero is before LPS challenge. Percentage of neutrophil subsets were calculated from flow cytometry data. Data are expressed as mean ± SEM n=7. **(C)** Absolute counts of neutrophil subsets were calculated using the percentage of the subsets and the absolute neutrophil counts, mean ± SEM n=7.

The third population CD16^{bright} / CD62L^{bright} cells fell in the same gate as the normal cells in the left panel of figure 1A. As a marked neutrophilia was present, the CD16^{dim} and CD62L^{dim} subsets comprised a substantial absolute number of circulating leukocytes (figure 1C).

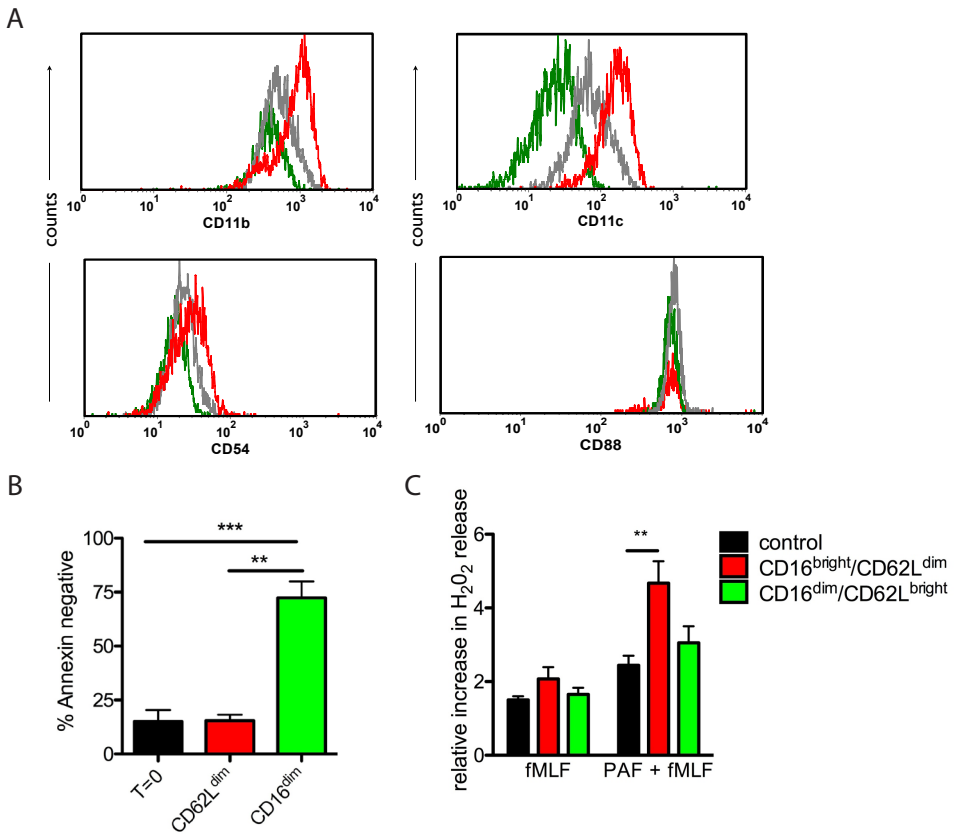

Figure 2. Neutrophil phenotype after LPS administration (A) Neutrophils were stained for CD16 and CD62L to discriminate between the subsets, additionally they were stained with CD11b, CD11c, CD54, CD88. Mean fluorescence intensity (MFI) is depicted. (▬)CD16^{bright}/CD62L^{dim} (▬)CD16^{bright}/ CD62L^{bright} (▬)CD16^{dim} / CD62L^{bright} Graphs show a representative figure of 5 experiments. **(B)** Neutrophil survival after 24 hours. Neutrophils subsets were FACS sorted, and cells were incubated for 24 hours at 37°C, 5% CO_2, after incubation they were stained with annexin-V PE for 15 min and measured by flow cytometry. Data are expressed as percentage annexin-V negative cells being the living cells, mean ± SEM n=5 **(C)** Relative increase in H_2O_2 in unsorted neutrophils measured by flow cytometry with dihidrorhodamine (DHR). Neutrophil subsets are visualized with CD16 and CD62L staining. Cells were stimulated with fMLF (10^{-6}M) and PAF (10^{-6} M) for 15 minutes. Neutrophils from healthy controls were used as a control. Graph shows relative increase in H_2O_2 release, mean ± SEM n=7.

Characterization of neutrophil subsets after i.v. LPS administration

The neutrophil subsets displayed marked differences in expression of surface markers. The $CD62L^{dim}$ neutrophils showed an increased expression of CD11b, CD11c and CD54, but an equal expression of CD88 compared to $CD16^{dim}$ neutrophils (figure 2A). Supplemental table 1 shows expression of 20 additional markers that were assayed. Expression of CD32 and CD45 was much higher on the $CD62L^{dim}$ neutrophil subset compared to the other subsets (supplemental table 1).

Up regulation of expression of CD11b on this subpopulation might be due to cellular activation induced by LPS administration, as this integrin is sensitive for, and is rapidly induced by soluble stimuli. A more stringent marker for activated cells is ICAM-1 (CD54), as this is mostly found on extravasated neutrophils[21] and is *in vitro* only up regulated after prolonged cytokine stimulation (data not shown).

Apart from the differences in expression of surface markers, these two distinct populations also displayed functional heterogeneity. $CD16^{dim}$ banded neutrophils showed a higher survival after 24 hours in culture compared to the $CD62L^{dim}$ neutrophils, which displayed a similar rapid apoptosis rate to normal (T=0) neutrophils (figure 2B). In contrast, activation of the NADPH-oxidase as measured by extracellular H_2O_2 production was approximately 3-fold higher in $CD62L^{dim}$ neutrophils compared to healthy control neutrophils and the $CD16^{dim}$ subset neutrophils after *ex vivo* stimulation with PAF and fMLF (figure 2C).

$CD62L^{dim}/CD11c^{bright}$ neutrophils suppress lymphocyte activation

As both mouse and human MDSC populations have been described to contain neutrophils[1], we examined the capacity of our distinct neutrophil populations to suppress PHA- and CD3/CD28-induced lymphocyte activation. Surprisingly, the $CD62L^{dim}$ hypersegmented neutrophils potently suppressed T-cell proliferation in a dose-dependent manner both upon PHA and upon CD3/CD28 stimulation (figure 3A & B). In marked contrast $CD16^{dim}$ banded neutrophils and neutrophils isolated from blood before LPS administration were not able to suppress lymphocyte proliferation (figure 3A & B). Around 80% of the co-culture neutrophils were apoptotic at 24 hours as assessed by Annexin V staining (supplemental figure 1).The $CD16^{dim}$ neutrophil subset shows less living cells in co-cultures (supplemental figure 1) compared to single cultures (figure 2B). As PHA and CD3/CD28 lymphocyte responses are model systems for T-cell proliferation, we tested a more physiological response using tetanus toxoid. For this we used isolated PBMCs from healthy volunteers who had received a tetanus booster vaccination less than 5 years ago. Responses induced by tetanus toxoid were suppressed to a similar extent as the PHA induced responses (figure 3C).

T-cell phenotype after $CD62L^{dim}$ neutrophil co-culture

Interferon-γ production after 4 days in the co-cultures with PHA stimulation and

CD62L^{dim} neutrophils was lower compared to the cultures in the presence of normal and CD16^{dim} neutrophils (figure 4A). This could be explained by specific inhibition of the IFNγ producing cells on the other hand inhibition of T-cell proliferation leads to less T-cells per well and could therefore lead to less total IFNγ production. Co-culture with CD62L^{dim} neutorphils did not influence the percentage of interferon-γ producing cells (figure 4B). These data suggest that neutrophils did not inhibit a subset of interferon-γ producing lymphocytes but rather inhibited total polyclonal proliferation. Also the percentage of IL-4 producing T-cells was not influenced (not shown). The concentration of the IL-4 was below the detection limit of our assay, IL-13 was reduced to a similar extent by coculture with CD62L^{dim} neutrophils as IFNγ (figure 4C)

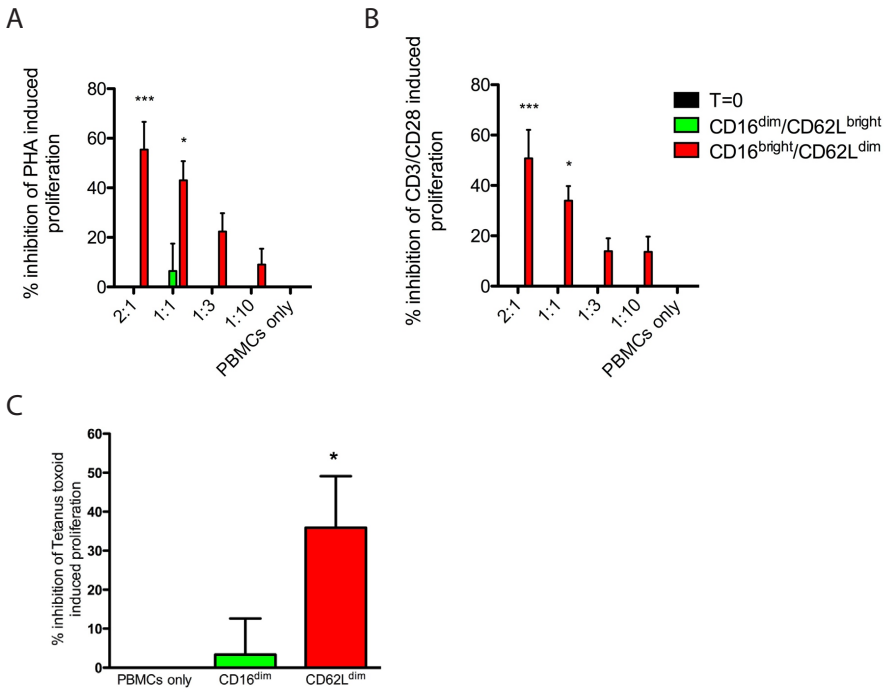

Figure 3. T-cell proliferation after incubation with neutrophil subsets. Blood was drawn 180 minutes after LPS administration, neutrophils were stained for CD16 and CD62L, and subsets were sorted. PBMCs were isolated from blood drawn before LPS administration and isolated by ficoll gradient separation. T-cell proliferation was measured by [^{3}H]thymidine incorporation. **(A)** PBMCs were stimulated with PHA (10µg/ml), and incubated with different concentrations of the neutrophil subsets for four days. Data are depicted as % inhibition of proliferation mean ± SEM n=7 **(B)** PBMCs were stimulated with CD3 (0.15 µg/m) / CD28 (1 µg/ml), and incubated with different concentrations of the neutrophil subsets for four days. Data are presented as mean ± SEM n=7 **(C)** PBMCs from healthy volunteers healthy volunteers who had received a tetanus booster vaccination less than 5 years age were stimulated with tetanus toxoid, and incubated with CD62L^{dim} or CD16^{dim} neutrophils added in a 2:1 neutrophil to lymphocyte ratio for four days. Data are depicted as % inhibition of proliferation mean ± SEM n=9.

CD62L^{dim}/CD11c^{bright} neutrophil induced T-cell suppression requires neutrophil Mac-1

In murine models MDSC T-cell suppression is mainly mediated by arginase I. L-arginine has been shown to inhibit the effect of arginase I mediated suppression[22]. However supra-physiological concentrations of L-arginine did not affect CD62L^{dim} neutrophil-induced suppression. This showed that arginase I was not utilized for T-cell suppression by this subset of CD62L^{dim} neutrophils (figure 5A). Neutrophils have also been shown to secrete IL-10 and TGFβ, which play an important role in T-cell suppression. However, antagonism of these factors revealed no role for these cytokines in our system (figure 5A). Next we investigated whether a direct contact between lymphocytes and CD62L^{dim} neutrophils was needed using a transwell system separating the cell suspensions. In this system neutrophils lost their suppressive capacity compared to neutrophils that were not separated, indicating that cellular proximity is needed between neutrophils and lymphocytes (figure 5B).

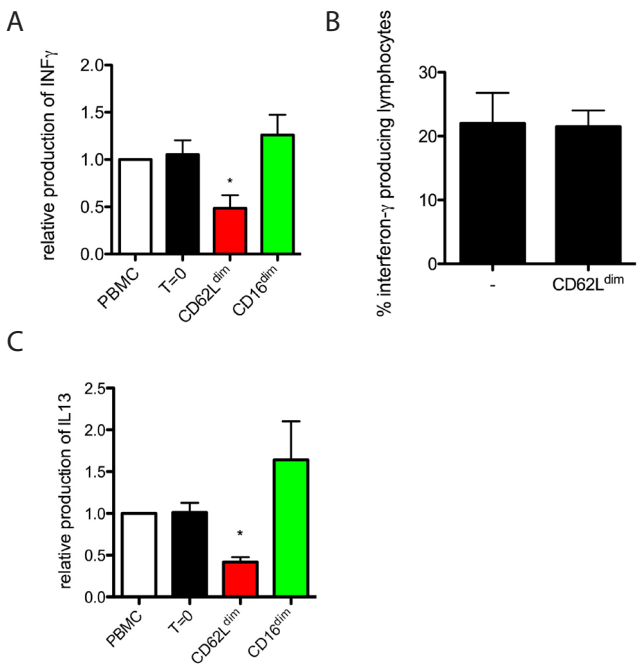

Figure 4. Cytokine production by T-Cells after co-culture. (A) IFNγ production after four days was measured in the supernatant of the co-culture using a sandwich ELISA. Data are presented as relative production of IFNγ compared to PBMCs only. Data are presented as mean ± SEM n=6. **(B)** The percentage IFNγ producing T-cells was measured by intracellular cytokine staining. PBMCs were stained for CD4 / CD8 and intracellular IFNγ. Data are presented as mean ± SEM n=4**. (C)** IFNγ production after four days was measured in the supernatant of the co-culture using a sandwich ELISA. Data are presented as relative production of IL13 compared to PBMCs only. Data are presented as mean ± SEM n=6

In addition to arginase I, reactive oxygen species (ROS) have been implicated in immune suppression by MDSCs[23]. The production of O_2^- by the membrane bound NADPH-oxidase is an essential anti-microbial function of neutrophils[24]. H_2O_2, which is a downstream metabolite of O_2^-, has been shown to modulate T-cell responses through various mechanisms[25,26]. Therefore, we examined whether H_2O_2 production contributed to the suppressive effects of this neutrophil subset.

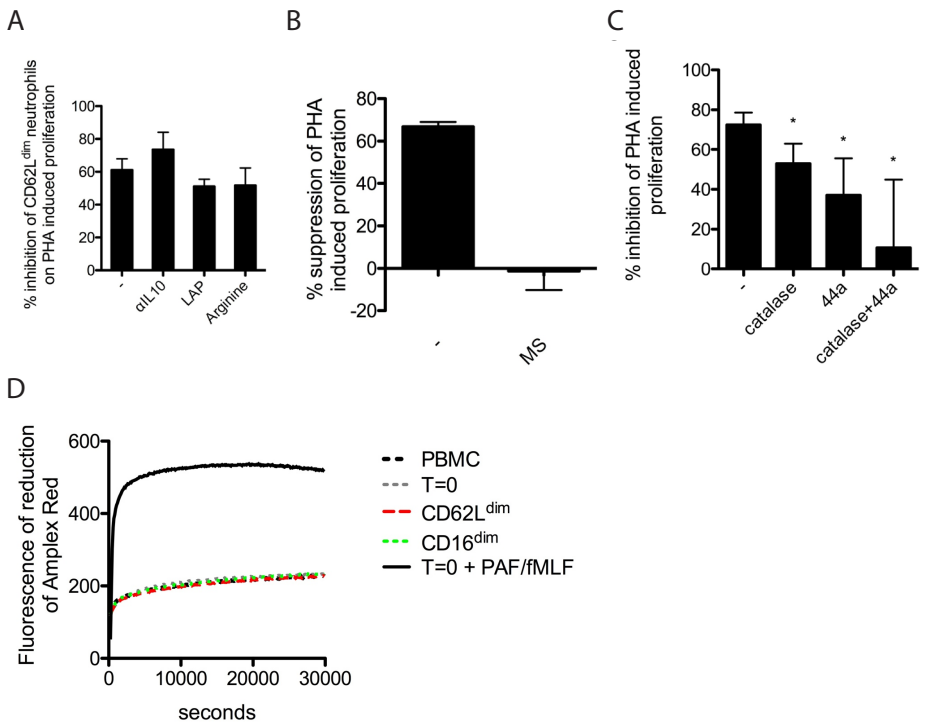

Figure 5. Mechanism of neutrophil-induced T-cell proliferation suppression. (A) Soluble mediators of T-cell inhibition. $CD62L^{dim}$ neutrophils were added in a 2:1 ratio to PHA (10µg/ml) stimulated PBMCs in the presence or absence of anti- IL10 (5 µg/ml), TGFβ inhibitor LAP (1 µg/ml), or L-Arginine (1mM). Data are presented as mean ± SEM of 5 independent experiments. **(B)** Separation of $CD62L^{dim}$ neutrophils and PBMCs. Neutrophils and T-cells were separated by a membrane using a Thinsert system (MS). Neutrophils were placed in the upper compartment in a 2:1 ratio and PBMCs in the lower. These data were compared to non-separated neutrophils and PBMCs (-). Data are expressed as mean ± SEM of 4 individual experiments. **(C)** $CD62L^{dim}$ neutrophils were added in a 2:1 ratio to PHA (10µg/ml) stimulated PBMCs in the presence or absence of Mac-1 blocking antibody 44a (10µg/ml) and/or catalase (4000 U/ml). As a control 44a and catalase were also added to PBMCs without adding neutrophils, data were corrected for this control. Data are presented as mean ± SEM of 7 independent experiments. **(D)** Co-cultures contained PBMCs with or without FACS sorted neutrophils subsets, or neutrophils from before LPS (t=0). Co-cultures were stimulated with PHA (10µg/ml), and were incubated in culture medium containing Amplex Red (10mM) and HRP (100 U/ml). Fluorescence after reduction of Amplex Red was measured over a period of 8 hours. As a positive control PAF (10^{-6}M) and fMLF (10^{-6}M) were added to the culture. One representative experiment of 3 is depicted.

Indeed, the H_2O_2 scavenger catalase was able to partially recover the inhibition of T-cell suppression by 20% when added to the co-cultures (figure 5C). Next, as neutrophils are capable of producing large quantities of hydrogen peroxide, we examined whether suppression of T-cell proliferation was due to massive release of H_2O_2. Co-cultures were supplemented with the fluorescent probe Amplex Red which is used to detect extra-cellular H_2O_2. In these cultures no extra-cellular release of H_2O_2 was observed (figure 5D). As a control, PAF/fMLF was added to the co-cultures, which showed that despite low numbers of neutrophils in the co-cultures, an extra-cellular release of H_2O_2 could be detected (figure 5D).

The necessity of cellular proximity of neutrophils and T-cells as observed in the transwell assay and the ability of catalase to prevent suppression combined with a lack of an overall oxidative environment led us to hypothesize that neutrophils locally deliver H_2O_2 to T-cells. Neutrophils are capable of creating immunological synapses using integrins for efficient phagocytosis of opsonized bacteria in which the integrin αMβ2 (Mac-1) plays an important role and H_2O_2 can be produced within these synapses[27]. In such synapses availability of catalase could have been limited, explaining the partial effect of catalase as described above. Leukocyte integrins are pivotal in mediating cellular contact creating platforms of cellular communication[28]. Indeed, blocking Mac-1 using monoclonal antibody 44a in our co-cultures resulted in 35% recovery of T-cell suppression (figure 5C). As T-cells require functional αLβ2 (LFA-1) integrin for effective proliferation, specifically blocking LFA1 or total β2 integrins could not be performed since T-cell proliferation was potently disrupted. Although our suppressive neutrophil subset was high in CD11c (αXβ2) expression, blocking CD11c in our co-cultures did not affect T-cell proliferation (data not shown). Combination of both catalase and 44a resulted in a 62% decrease of $CD62L^{dim}$ neutrophil induced inhibition of T-cell proliferation (figure 5C). This demonstrated that blocking of αM (CD11b/clone 44a) in combination with scavenging of H_2O_2 had an additive effect.

These data show that local delivery of H_2O_2 to T-cells results in decreased proliferation. The suppressive effect was not explained by induction of T-cell apoptosis through neutrophil cytotoxicity, as both suppressive ($CD62L^{dim}$) and non-suppressive ($CD16^{dim}$) neutrophil subsets showed similar amounts of T-cell apoptosis in our co-cultures (supplemental figure 2).

The immune suppressive effect could be mediated by monocytes as these cells have been reported to interact with neutrophils. Therefore, we depleted monocytes from the PBMC-fraction before adding neutrophils and PHA. This did not alter the suppressive characteristics of the $CD62L^{dim}$ neutrophils and demonstrated that monocytes were not required for the suppressive phenotype of these neutrophils (supplemental figure 3).

We next visualized the interaction between $CD62L^{dim}$ neutrophils and T-cells in a real time imaging set up using a culture medium containing the Amplex Red probe for visualization of H_2O_2. Monocytes were depleted from the PBMCs for these experiments.

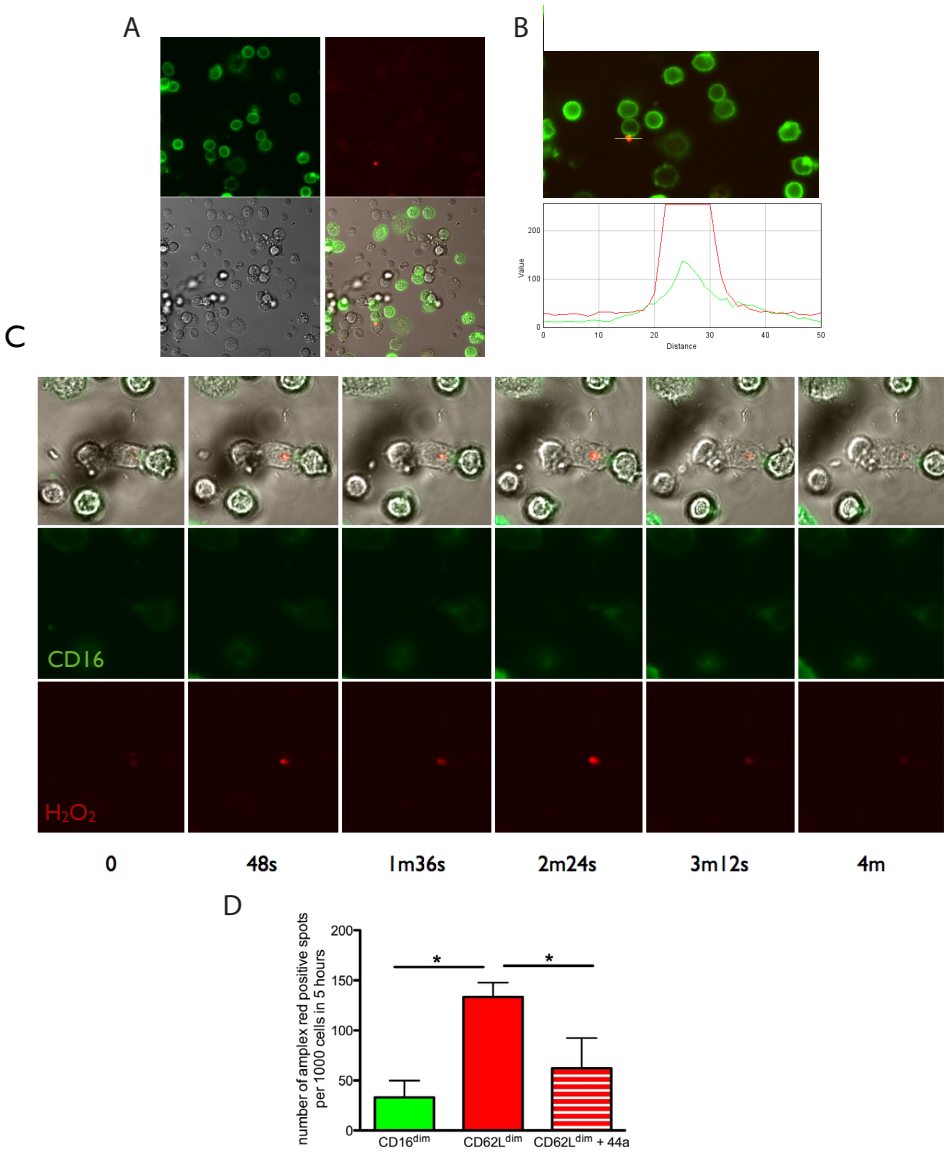

Figure 6. Visualization of neutrophil- lymphocyte interactions. $CD62L^{dim}$ sorted neutrophils stained with CD16 FITC were added in a 2:1 ratio to unlabeled PBMCs and stimulated with PHA (10µg/ml). Cells were incubated in culture medium containing Amplex Red (20mM) and HRP (200 U/ml). Images were acquired using a Zeiss LSM510 Meta microscope. **(A)** Neutrophil- lymphocyte interaction with a localized Amplex Red signal indicative for local production of H_2O_2. **(B)** Same interaction as in **(A)** showing that the amplex red signal co-localized with the CD16 labeled neutrophil membrane. **(C)** Time lapse image of a neutrophil- lymphocyte interation. Full video is available as supplemental movie. Representative examples are shown of 4 independent experiments. **(D)** Quantification of local production of H_2O_2 spots in time laps images. Time laps images were made by deconvolution microscopy using a DeltaVision microscope. $CD62L^{dim}$ and $CD16^{dim}$ sorted neutrophils stained with calcein were added in a 2:1 ratio to unlabeled lymphocytes and stimulated with PHA (10µg/ml). Cells were incubated in culture medium containing Amplex UltraRed (20mM) and HRP (200 U/ml). In one of the conditions Mac-1 blocking antibody 44a was added. The number of H_2O_2 positive spots were counted. Data are presented as mean ± SEM of 3 independent experiments.

Local release of H_2O_2 was observed at the sites of interaction between neutrophils and lymphocytes (figure 6A). Figure 6B shows the same interaction as figure 6A, further analysis shows that the Amplex Red signal co-localized with the CD16-labeled neutrophil membrane (figure 6B). These interactions were short and transient of nature lasting no longer than 4 minutes, as visualized in time-lapse recordings (figure 6C & supplemental movie 1). Interactions were quantified using time-lapse recordings of several hours.
The quantification is shown in figure 6D, $CD62L^{dim}$ neutrophils displayed more Amplex Red signals in 5 hours than the $CD16^{dim}$ neutrophils. The amount of interactions seen with $CD62L^{dim}$ cells was inhibited by 44a the Mac-1 blocking antibody.

Identification of suppressive neutrophils in human systemic inflammation induced by injury
Finally, because neutrophils are sensitive to ex-vivo manipulation, we minimized manipulation and assessed T-cell suppression in total leukocytes as a control. Three hours after LPS, total leukocytes were used containing 85% neutrophils, 15% lymphocytes and <1% monocytes. Addition of Mac-1 (αM/CD11b) blocking antibodies to total leukocytes increased T-cell proliferation after PHA stimulation. This showed that unmanipulated neutrophils were capable of suppressing T-cell proliferation in a Mac-1 dependent fashion (Supplemental figure 4). Healthy controls showed no increase in T-cell proliferation after addition of Mac-1 blocking antibodies (results not shown).
This total leukocyte model system allowed us to assess the occurrence of Mac-1 induced T-cell suppression in patients suffering from acute systemic inflammation. Severely injured patients showed the occurrence of a similar $CD62L^{dim}$ neutrophil subset immediately after admission (<12 hours) to our hospital (figure 7A). The presence of Mac-1 induced suppression was demonstrated by the increase in cell count and interferon-γ production induced by PHA upon addition of blocking antibody 44a (figure 7B & C). Blocking CD11c as a control did not induce increases in proliferation and interferon-γ production.

Discussion
This study reports the identification of a human neutrophil subset with a distinct functionality. A mature neutrophil subset released into the circulation during severe inflammation suppresses T-cell activation. This suppression requires specific delivery of H_2O_2 into the immunological synapse in a Mac-1 (CD11b/CD18) dependent manner.
Neutrophils are generally considered to be a homogenous population. Only few studies have addressed their potential heterogeneity, which has mainly been attributed to release of immature neutrophils from the bone marrow[29,30]. In inflammation this heterogeneity has only been described in the context of their generally accepted anti-microbial functions[31,32].
Our work is the first to show a distinct human neutrophil phenotype during acute inflammation with an immune suppressive capacity. These cells display a hypersegemented nuclear morphology, implying increased maturation compared to normal blood

neutrophils. In addition, this neutrophil subset is characterized by a unique phenotype ($CD62L^{dim}/CD16^{bright}$ /$CD11b^{bright}/CD54^{bright}$), which distinguishes them from classically short term activated neutrophils ($CD62L^{dim}/CD16^{bright}/CD11b^{bright}/CD54^{dim}$).
Importantly, we could not induce a similar Mac-1 dependent suppressive phenotype by *ex vivo* activation of normal neutrophils with various pro- and anti-inflammatory stimuli (supplemental figure 6). There was some suppression of proliferation after stimulation with fMLF, LPS, and IFNγ + TNF. However, this suppression was not dependent on Mac-1 as the blocking antibody 44a did not counteract the suppression. Most likely, the *in vitro* neutrophil activation led to an extracellular respiratory burst, resulting in T-cell suppression

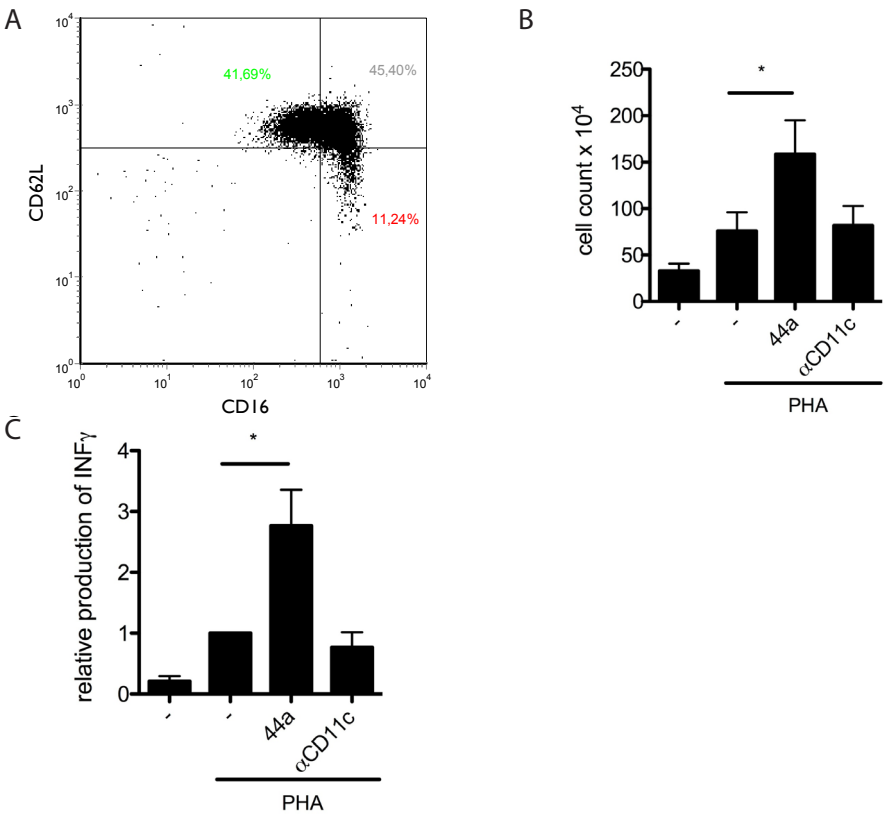

Figure 7. Suppression of T-cell proliferation in severely injured patients. (A) Blood was drawn of severely injured patients immediately after admission in the hospital. Total leukocytes were stained for CD16 and CD62L. A $CD62L^{dim}$ subset of neutrophils was seen similar to the subset found three hours after LPS. **(B)** Total leukocytes from severely injured patients were stimulated with PHA (10µg/ml) and incubated in the presence or absence of blocking antibodies to Mac-1 (44a) or CD11c (3.9) for 5 days. After 5 days of incubation total leukocytes were counted. Groups were compared using a paired samples t-test. Data is presented as mean ± SEM of 4 independent experiments. **(C)** Interferon-γ concentration was measured by ELISA in the supernatants of the 5 days cultures. Data are depicted as relative increase compared to PHA only. Groups were compared using a paired samples t-test. Data is presented as mean ± SEM of 5 independent experiments.

The finding that suppressive neutrophils could not be induced *in vitro* makes it tempting to speculate that homeostatic blood neutrophils lack a putative functional instruction induced in the tissues. This hypothesis is supported by the fact that inflammation induces immunological stress, and its mediators such as glucocorticoids and catecholamines can mobilize mature neutrophils[33]. It is not possible to mimic instruction and maturation in the tissues *in vitro*. In addition *in vivo* aged neutrophils might respond differently to circulating neutrophil aquired from healthy individuals. Studying the possibility of aged neutrophils is challenging as neutrophils *ex vivo* undergo rapid apoptosis within 24 hours. We have recently suggested that the half-life of circulating neutrophils is at least 3.75 days[34]. Neutrophil tissue half-life is estimated 6-15 times longer than their circulating half-life[35].

The neutrophils released during inflammation in this study display regulatory properties. This is in line with an emerging concept[36]. Recently, more regulatory functions of neutrophils have been described. Murine neutrophils have been shown to produce IL-10 impairing the anti-microbial defense[37]. In addition, in various murine models of cancer, sepsis and transplantation, neutrophils were found to be part of MDSCs. This heterogeneous group of cells, suppressing lymphocyte activation, consists mainly of immature neutrophils and monocytes. Suppression required arginase I[38,39]. In humans with metastatic cancer disease, arginase I –mediated suppression of lymphocytes was reported[10,11]. Arginase I depletes the micro-environment of L-arginine, which is essential for various T-cell functions. However the suppressive neutrophils in this study inhibited T-cell proliferation via local production of H_2O_2. Reactive oxygen species (ROS) can rapidly be scavenged by an abundance of molecules resulting in a very short half-life. The release of H_2O_2 in an immunological synapse, as reported in this study, might therefore increase the efficiency of H_2O_2 mediated immune regulation. This local release of reactive oxygen species onto the surface of T-cells, the lack of detectable neutrophil extracellular ROS release (figure 4C) and the absence of increased T-cell apoptosis after neutrophils induced suppression (supplemental figure 2), suggests that neutrophils are capable of releasing a limited amount of ROS for immune regulatory purposes. The role of ROS in immune tolerance has been shown previously in models of chronic inflammation[40]. However this latter study focused on macrophages because in contrast to neutrophils these cells were thought to release limited amounts of immune regulatory ROS. Additionaly studies on the importance of cellular contact have focused on the interaction between lymphocytes and antigen-presenting cells (APCs). Suppressive characteristics have been reported for dendritic cells, which prevent full T-cell activation through active Mac-1[41] A role for ROS in this study was not evaluated.

It is tempting to speculate that the mechanism mediated by Mac-1 and ROS in immune regulation is not restricted to neutrophils, and may represent a more generalized mechanism for the immune regulation by myeloid cells in humans as both ROS and Mac-1 have separately been implicated in immune regulation by APCs[40,41]. In addition, the

role of H_2O_2 in the suppression of T-cell activation described in this paper might in part explain the hyperinflammatory phenotype observed in patients suffering from chronic granulomatous disease which lack NADPH-oxidase[42].

Eventhough neutrophil – T-cell interactions have been described in lymphoid organs in mice[43,44], it is still unknown whether human neutrophils enter lympoid organs and come into close contact with T-cells. In many acute and chronic inflammatory conditions, however, neutrophils and T-cells are present in the tissues. As neutrophilic inflammation frequently results in tissue damage, neutrophil induced inhibition of T-cell proliferation might be essential to limit T-cell activation and, thereby, maintain tolerance in these inflammatory conditions. The exact role for neutrophils in immune tolerance in humans remains to be examined, however it is clear that neutrophil association with solid tumors is detrimental for disease outcome, possibly through suppressing immunogenic anti-tumor responses.

In conclusion, we provide evidence of a newly identified subset of human neutrophils, which can potently suppress T-cell responses. Identification of this neutrophil subset in human inflammation and the role of neutrophil Mac-1 integrin might provide novel therapeutic strategies to target immune suppression in inflammation and cancer.

Methods and Materials

Subjects and study design

The study protocol was approved by the Ethics Committee of the Radboud University Nijmegen Medical Centre and complies with the Declaration of Helsinki and the Good Clinical Practice guidelines. Male volunteers gave written informed consent. Experiments were part of several endotoxin trials (NCT00513110, NCT00783068, NCT00785018, NCT00916448 at www.clinicaltrials.gov). Subjects were enrolled after screening and prehydrated[43,44]. U.S. Reference E.coli endotoxin (lot Ec-5, Centre for Biologic Evaluation and Research, Food and Drug Administration, Bethesda, MD) was used in this study. Endotoxin was reconstituted in 5ml saline and injected as single intravenous bolus during 1 minute at t=0. Blood samples anti-coagulated with sodium heparin were taken from the arterial catheter. Patients with severe injuries were included with an expected Intensive Care stay of longer than 3 days. The protocol was approved by the Ethics Committee of the University Medical Centre Utrecht. Patients gave informed consent after sampling of the blood according to the research protocol.

Reagents

Human serum albumin (HSA) and pasteurized plasma solution was purchased from the Central Laboratory of the Netherlands (Sanquin, Amsterdam, the Netherlands). Isolation buffer contained PBS supplemented with pasteurized plasma solution (10%) and trisodium citrate (0.4% (w/v)). Incubation buffer contained 20mM HEPES, 132mM NaCl, 6mM KCl, 1mM MgSO4, 1.2mM KH2 PO4, supplemented with 5 mM glucose,

1.0mM CaCl2, and 0.5% (w/v) HSA.
Phytohemagglutinin (PHA-P) (1), latency associated peptide (LAP) (1) N-formylmethionyl- leucyl-phenylalanine (fMLF) (1), platelet-activating factor (PAF) (1), phorbol myristate acetate (PMA) (1), CD14 microbeads (3), Tetanus toxoïde (4), dihidrorhodamine (DHR) (1), Ionomycine (5), Brefeldin A (5), arginine (1), catalase (6), 1-MT (1), Fixation/Permeabilization Concentrate (7), Fixation/Permeabilization Diluent (7), Permeabilization Buffer (7), Horse Radish Peroxidase (HRP) (1) Amplex red (2) and Amplex UltraRed (2) were purchased from (1) Sigma (St. Louis, MO, USA), (2) Molecular Probes (Leiden, The Netherlands), (3) Miltenyi Biotec (Bergisch Gladbach, Germany) and (4) NVI (bilthoven, the Netherlands), (5) Invitrogen (Breda, the Netherlands), (6) Meridian Life Science, Inc. (BIODESIGN International, Saco, ME, USA), (7) eBioscience (San Diego, CA, USA). All other materials were reagent grade.

Antibodies
Unlabeled antibodies: Functionally blocking antibody 44a (anti αM integrin) was isolated from supernatant of the hybridoma obtained from the American Type Culture Collection (ATCC), Functionally blocking CD11c (clone 3.9) was obtained from Biolegend (San Diego, CA, USA), CD3 (clone CLB-T3/4E, 1XE) and CD28 (clone CLB-CD28/1, 15E8) were obtained from Sanquin, (Amsterdam, the Netherlands) and anti IL-10 (clone 12G8) from Schering-Plough Research (InstituteKenilworth, NJ, USA). Monoclonal antibodies used for flow cytometry: CD4 perCP (1) (clone L200), CD8 APC (1) (clone SK1), IFNγ FITC / IL4 PE (1) (clone 25723.11 / 3010.211), CD16 alexa 647 (1) (clone 3G8), CD16 FITC (4) (clone LNK16), CD62L PE (1) (SK11), CD62L FITC (1) (DREG56), CD11c APC (1) (clone S-HCL-3), CD11b PE (2) (clone 2LPM19c), Annexin V-PE (1) CD3 APC-alexa750 (5) (clone UCHT1), CD4 Krome orange (5) (clone 13B8.2), CD8 pacific blue (5) (clone B9.11), CD45RA FITC (1) (clone HI100), CD45RO (5) (clone UCHL1), granzyme B PE (6) (clone GB-11), CTLA4 PE (1) (BNI3), FoxP3 (7) (clone PCH101), CD8 APC (1) (clone SK1), CD25 PE-Cy7 (1) (clone M-A251), were purchased from: (1) BD (Franklin Lakes, NJ, USA), (2) DAKO (Heverlee, Belgium) (3) Biolegend, (San Diego, CA, USA) , (4) Serotec (Düsseldorf, Germany), (5) Beckman Coulter, Inc (Miami, FL, USA), (6) Sanquin, (Amsterdam, the Netherlands), (7) eBioscience (San Diego, CA, USA) respectively.

Neutrophil flow cytometry and cellcounts
Erythrocytes were lyzed in isotonic ice-cold NH4Cl solution followed by centrifugation at 4°C. After lysis total leukocytes were stained with antibodies for 30 min at 4°C in isolation buffer. Cells were washed before analysis on FACSCalibur or sorted on FACSAria, FACSVantage, (Becton Dickenson, San Jose, CA, USA) or MoFlo Astrios (Beckman Coulter, Inc, Miami, FL, USA). Total leukocyte counts were counted in whole blood using Cell-Dyn 1800 cellcounter (Abbot Diagnostics, Abbot Park, IL). Using the

percentages found by flow cytometry and the absolute leukocyte count, absolute number of circulating neutrophils subsets were calculated. For Annexin-V staining PBMCs were prestained with CD3. Afterward cells were stained with Annexin PE in annexin binding buffer.

Granulocyte and PBMC isolation
Cells were isolated as described previously. In short, cells were separated by centrifugation over Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) for 20 min at 900xg. The mononuclear cell layer was removed. The erythrocytes in the granulocyte layer were lyzed as described in flow cytometry.

Reactive Oxygen Species
Production of ROS was studied by staining total leukocytes with CD16 and CD62L and loading with dihidrorhodamine (DHR) for 15 minutes at 37°C. After stimulation with fMLF or PAF/fMLF the relative increase in ROS production was determined in both neutrophil subsets by flow cytometry. In addition isolated neutrophils and PBMCs were incubated in a 96 wells plate in the presence of PHA (10µg/ml), Amplex red 10mM and 100U/ml HRP. Production of fluorescent resorufin, formed from Amplex Red in the presence of HRP after peroxidation, was measured during 8 hours at 37°C using a fluoro-luminometer FluostarOptima (BMGLABTECH, Offenburg, Germany).

T-cell proliferation
Experiments were performed in IMDM (Gibco, invitrogen, Breda, the Netherlands) culture medium supplemented with 5% pooled human AB serum, penicillin (100 IU/ml), streptomycin (100mg/ml) and glutamine (1mM) (Gibco, invitrogen, Breda, the Netherlands). Proliferation at 96h was measured by [^{3}H]thymidine incorporation [1mCi/well (Amersham, Little Chalfont, UK)], which was added during the last 18 hours of culturing. Proliferative responses are expressed as the mean [^{3}H]thymidine incorporation as counts per minute (c.p.m.) of triplicate wells. For the solubility experiments thinserts, (Greiner, Alphen a/d Rijn, the Netherlands) were used, 24 well inserts with a pore size of 0.4µM. In experiments with inhibitors or blocking antibodies T-cell proliferation was always corrected for PBMCs alone with the same inhibitor or blocking antibody.

IFNγ ELISA
IFNγ production was measured by a sandwich ELISA according to the manufacturers recommendations (Sanquin, Amsterdam, The Netherlands). The detection limit was 2pg/ml.

T-cell flow cytometry stainings
T-cells were stimulated with either control medium or 20ng/ml PMA and 3µg/ml

ionomycin for 6 hours; 10µg/ml Brefeldin A was added after 1 hour. Cells were stained on ice for surface markers CD4 and CD8, fixed, permeabilized and stained with anti -IFNγ FITC and anti IL4 PE antibody at room temperature.

Microscopy

Co-cultures of sorted $CD62L^{dim}$ neutrophils (labeled with CD16 FITC) and unlabeled PBMCs in the presence of PHA (10µg/ml) were supplemented with Amplex Red 20mM and 200U/ml HRP. During the course of 2 hours, neutrophil – lymphocyte interactions were imaged with a Zeiss LSM510 Meta microscope. For quantification $CD62L^{dim}$ and $CD16^{dim}$ sorted neutrophils were stained with calcein 1 µM for 20 min at 37°C. Instead of Amplex Red, Amplex UltraRed (20mM) was used for these experiments. The interactions were imaged for an average of 3 hours using a Delta Vision microscope. Afterwards interactions were counted by hand; the person counting the interactions was blinded for the different conditions.

Statistical analysis

Data were analyzed using Graphpad Prism 4.0. Repeated measures ANOVA was used to compare different time points. A wilcoxon signed rank test or a paired samples t test was used to compare groups. A p-value <0.05 was considered significant.

* $p<0.05$ ** $p< 0.005$ *** $p<0.001$

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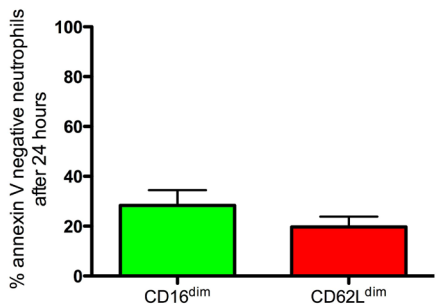
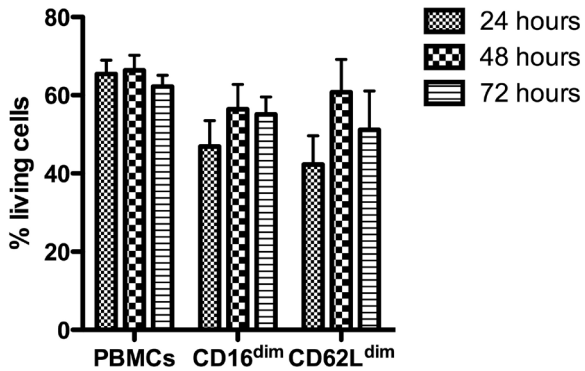
Supplementary Information

Table 1. Comparison by immunofluorescent staining of the levels of surface proteins on neutrophil populations 3 hours after LPS administration.

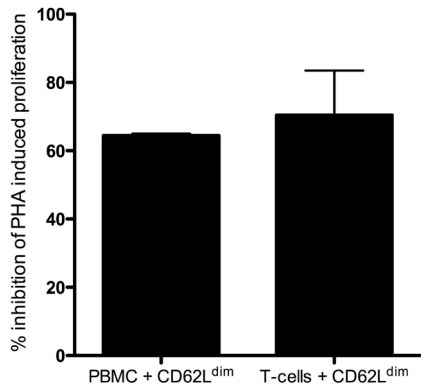
Antigen	$CD16^{dim}$/ $CD62L^{bright}$	$CD16^{bright}$/ $CD62L^{bright}$	$CD16^{bright}$/ $CD62L^{dim}$	Clone	Antibody supplier
CD11b	269.5 ± 35.6	364.1 ± 43.2	848.1 ± 179.4	2LPM19c	DAKO
CD11c	33.6 ± 10.0	83.4 ± 23.0	161.1 ± 3804	s-HCL-3	BD
CD29	43.0 ± 5.8	54.0 ± 7.8	60.0 ± 8.9	4B7R	Serotec
CD49d	1.9 ± 0.1	1.5 ± 0.1	3.9 ± 0.2	9F10	eBioscience
CD32	99.9 ± 6.0	185.2 ± 14.2	246.8 ± 17.7	FL18.26	BD
CD64	16.8 ± 2.3	13.8 ± 4.3	12.0 ± 0.9	10.1	Serotec
CD89	14.1 ± 3.2	11.2 ± 2.9	24.2 ± 4.6	A3	Santa cruz
CD33	24.6 ± 3.9	18.0 ± 4.1	34.5 ± 4.1	WM53	Serotec
CD66b	190.0 ± 28.6	118.5 ± 18.8	212.1 ± 23.5	80H3	Gene Tex
CD63	1.8 ± 0.1	1.5 ± 0.1	4.2 ± 0.2	H5C6	BD
CD14	9.9 ± 1.2	11.4 ± 0.9	15.3 ± 1.1	M5E2	BD
TLR4	8.3 ± 0.8	9.2 ± 0.7	11.7 ± 2.3	HTA 125	Imgenex
CD88	382.0 ± 169.9	424.1 ± 182.7	377.4± 165.7	P1 2/1	Serotec
CXCR1	33.7 ± 15.5	49.1 ± 22.2	47.7 ± 20.5	42705	R&D systems
CXCR2	18.8 ± 4.7	36.8 ± 8.7	39.4 ± 10.3	48311	R&D systems
CD54	10.7 ± 1.6	14.9 ± 3.4	20.0 ± 5.4	MEM-111	Invitrogen
CD35	25.4 ± 3.2	83.0 ± 22.4	201.0 ± 21.2	E11	BD
CD95	36.5 ± 1.1	37.2 ± 1.1	35.2 ± 0.9	DX2	DAKO
CD83	2.2 ± 0.5	2.0 ± 0.6	5.6 ± 1.5	HB15a	Santa cruz
CD45	5.5 ± 0.8	8.8 ± 1.9	36.4 ± 2.9	2D1	R&D systems



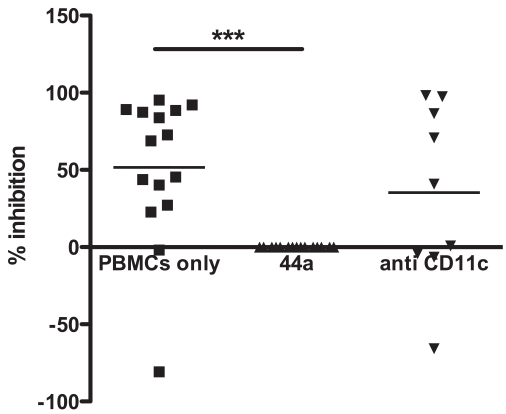
Supplemental figure 1. Neutrophil apoptosis after 24 hours in coculture. Neutrophils were added in a coculature with PBMCs in a 2:1 ratio and PHA (10µg/ml). After 24 hours, neutrophil apoptosis was assessed by staining for CD16, CD14, Annexin V and PI. n=6, data are presented as mean ± SEM.



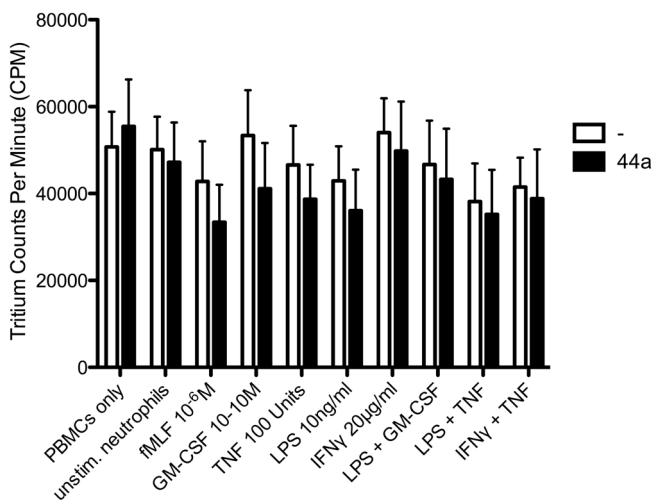
Supplemental figure 2. T-cell apoptosis in neutrophil co-cultures. Neutrophils were added in a 2:1 ratio in the culture conditions described for the T-cell proliferation cultures. At various timepoints cells were stained with CD3 FITC, Annexin V PE and apoptosis was measured by gating the CD3 positive cells by flow cytometry. Data shown are representative of 4 individual experiments mean ± SEM.



Supplemental figure 3. Inhibition of proliferation after monocyte depletion. Monocytes were depleted by magnetic beads coupled to CD14. A negative selection was performed, which resulted in >95% depletion of monocytes (not shown). Proliferation of T-cells was induced by PHA (10µg/ml). $CD62L^{dim}$ neutrophils were added in a 2:1 ratio. Data are expressed as mean ± SEM of n=3.



Supplemental figure 4. Whole blood assay. Shocked whole blood 3 hours after LPS administration was incubated with PHA (10µg/ml) and in the presence or absence of blocking antibodies. After 4 days, proliferation was measured by incorporation of [^{3}H]thymidine. n= 15.



Supplemental figure 5. T-cell proliferation with in vitro stimulated neutrophils. Neutrophils of healthy controls were incubated with stimuli for 1 hour at 37°C. After stimulation these neutrophils were added to PBMCs, with or without Mac-1 blocking antibody 44a, for a T-cell proliferation assay. Assays were performed as described in the material and methods. Data are expressed as mean ± SEM of n=6.

Acute phase concentrations of soluble fibrinogen inhibit neutrophil adhesion under flow conditions through interactions with ICAM-1 and Mac-1 (CD11b/CD18)

Janesh Pillay
Vera Kamp
Maarten Pennings
Erik-Jan Oudijk
Luke Leenen
Laurien Ulfman*
Leo Koenderman*

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** authors contributed equally*

submitted

Introduction

Neutrophil adherence to activated endothelium is a critical step in the multistep process of leukocyte extravasation[1]. This process is required for adequate migration of inflammatory cells to the tissues in response to infection or tissue damage. Aberrant activation of both neutrophils and the endothelium has been shown to be associated with endothelial damage[2]. Massive neutrophil influx can be detrimental in conditions of severe inflammation as activation of these cells can lead to excessive tissue damage and subsequent organ dysfunction[3].

Recently, several acute phase proteins have gained attention for their role in regulation of leukocyte rolling and adhesion[4,5]. Also, the acute phase protein fibrinogen has been implicated in this process. Immobilized fibrinogen and fibrin are well known ligands for neutrophil Mac-1 and mediate enhanced binding of leukocytes at sites of damaged endothelium[6]. Furthermore, studies have suggested that fibrinogen at low concentrations can form a bridge between leukocytes and endothelium thereby promoting leukocyte extravasation[7,8].

Fibrinogen is synthesized by the liver and in severe inflammatory conditions the plasma concentration of fibrinogen can increase 4-fold which equals up to 10 mg/ml[9]. The reason for this increase remains unknown as physiological concentrations of fibrinogen (1.5-2 mg/ml) are more than adequate for coagulation[10].

The role of fibrinogen in inflammation has mainly been described as pro-inflammatory[11-13]. However, these studies were performed in the context of immobilized fibrinogen and fibrin only. Indeed, Mac-1 –fibrin/fibrinogen interactions are important in various pro-inflammatory events such as experimental arthritis[13]. In addition, it has been shown that mice lacking fibrinogen have a delayed inflammatory response to intravenous endotoxin[12]. This suggests that physiological concentrations of fibrinogen are involved in the initiation of inflammation.

Little progress has been made in identifying endogenous factors that limit leukocyte adherence to activated endothelial cells. Recently, Choi et al reported that developmental endothelial locus–1 on the endothelial surface interferes with LFA-1 – ICAM-1 dependent leukocyte adhesion. This illustrates the potency of endogenous inhibitors to limit leukocyte adhesion[14]. However, apart from LFA-1, Mac-1 also plays a role in neutrophil adhesion to activated endothelium and subsequent intravascular crawling[15,16]. In addition, deposition of fibrin or expression of RAGE on the vascular endothelial surface in septic and acute inflammatory conditions provide additional ligands for leukocyte Mac-1[12,17].

In contrast to the pro-inflammatory properties of immobilized fibrinogen and fibrin, we and others have reported that soluble fibrinogen (sFg) is capable of inhibiting leukocyte and platelet interactions with fibrin, which is consistent with the hypothesis that sFg is capable of limiting leukocyte homing and, thereby, inflammation[18-20]. As sFg is increased after the initiation of inflammation, we tested the hypothesis that acute phase concentrations of fibrinogen are anti-inflammatory by regulating leukocyte endothelial

interactions and might thus contribute to the resolution of inflammation.

Material and Methods

Reagents and antibodies

Purified fibrinogen (FIB3) (Enzyme Research Laboratories, South Bend, USA) from human fresh frozen plasma and depleted of plasminogen and fibronectin was purchased from Kordia Life Sciences (Leiden, the Netherlands) and referred to as sFg throughout the manuscript. FIB1 and FIB (less pure fibrinogen preparations) were also purchased from Kordia Life Sciences. Human serum albumin (HSA) and pasteurized plasma solution were obtained from Sanquin (Amsterdam, The Netherlands). Hepes buffered RPMI 1640 medium with L-Glutamine was purchased from GIBCO, invitrogen (Carlsbad, California, USA). Ficoll Paque was from Pharmacia (Uppsala, Sweden). Monoclonal antibodies 44a (anti-αMβ2), IB4 (anti β2 integrin chain), W6/32 (anti-HLA), were isolated from the supernatant of a hybridoma obtained from the American Type Culture Collection (Rockvill, MD, USA). Monoclonal antibody NKI-L15 (anti-αLβ2) was kindly provided by Prof. Y van Kooyk, VUMC, Amsterdam, The Netherlands. Monoclonal antibody against α4 integrins (HP2/1) was obtained from Immunotech (Marseille, France). iC3b was purchased from Calbiochem, Merck Biosciences, Darmstadt, Germany. Heparin was obtained from LEO Pharma BV (Breda, The Netherlands).

HEPES incubation buffer contained 20mM HEPES, 132mM NaCl, 6mM KCl, 1mM $MgSO_4$, 1.2mM KH_2PO_4, supplemented with 5 mM glucose, 1.0mM $CaCl_2$, and 0.5% (w/v) HSA.

Isolation of human neutrophils and flow cytometry

Blood was obtained from healthy volunteers at the donor service of the University Medical Centre Utrecht (Utrecht, The Netherlands) and anti-coagulated with sodium-citrate. Neutrophils were isolated as described before[21]. In short, blood was diluted 2.5:1 with PBS containing 0.32% (weight/volume) sodium citrate and human pasteurized plasma solution (4 g/l). Mononuclear cells were removed by centrifugation over Ficoll-Paque for 20 minutes at 2000 rpm. Erythrocytes were lysed in isotonic ice-cold NH_4Cl solution followed by centrifugation at $4^{O}C$.

Flow cytometry

Isolated neutrophils were stimulated for 5 minutes at $37^{O}C$ in the presence and absence of sFg. After 2 washes neutrophils were stained using directly labeled antibodies according to the manufacturers' protocol. Cells were analyzed in a FACScalibur flow-cytometer (Becton & Dickinson, Mountain View, California). Neutrophils and lymphocytes were identified according to their specific forward- and side-scatter signals. Data from individual experiments are depicted as fluorescence intensity in arbitrary units (MFU) of at least 5000 events.

Assay to measure cytosolic free Ca^{2+} $[Ca^{2+}]_i$ in neutrophils.
Neutrophils (10^7 cells in 1 ml incubation buffer) were incubated with 1.5 μM Fura-2/AM for 45 minutes at 37°C in the dark. Subsequently, the cells were washed with HEPES medium and kept in the dark at room temperature until use. Before each measurement, the neutrophils were resuspended in HEPES medium (final concentration 2.5 x 10^6 cells/ml) and transferred to a stirred and thermostated (37°C) cuvette. Fura-2 fluorescence was measured in a fluorospectrophotometer (F-7000, Hitachi, Tokyo, Japan) at excitation wavelengths of 340 and 380 nm, and emission 510 nm. Concentration Ca^{2+} was calculated as previously described using 5 μM digitonin and 10 mM EGTA for F_{max} and F_{min}, respectively[22].

Zymosan-induced respiratory burst
H_2O_2 release by isolated neutrophils was measured for 30 minutes at 37°C. H_2O_2 was determined in a fluoro-luminometer (FluostarOptima/BMGLABTECH) by determination of fluorescent resorufin, which was formed from Amplex Red in the presence of H_2O_2 and horseradish peroxidase as described previously[23]. Unopsonized zymosan was added in the concentration of 1 mg/ml in the presence or absence of fibrinogen or blocking antibodies.

Static adhesion assays
Isolated neutrophils were resuspended in HEPES incubation buffer at a concentration of 2 *10^6 cells /ml. Neutrophils were labeled with calcein for 30 minutes at 37°C. 96 wells maxisorp plates (NUNC) were coated with biotin-SP-Affinipure goat anti-human Fc (γ) F(ab')2 (20 μg/ml) in PBS (1h, room temperature), washed twice with PBS and subsequently coated with Fc-ICAM-1 (10 μg/ml) (1h, room temperature). Plates were washed twice with PBS and incubated with incubation buffer until use. iC3b (2μg/ml) and heparin (100U/ml) were coated overnight at 4°C and washed with PBS before use. Calcein labeled neutrophils were allowed to adhere for 15-30 minutes at 37°C to coated plates in the presence or absence of sFib or blocking antibodies against Mac-1 (CD11b/44a), LFA-1 (CD11a/NKI-L15), β2 integrins (CD18, IB4) or control antibody against HLA-1 (W6/32). Total input of cells was measured in a fluoro-luminometer (FluostarOptima/BMGLABTECH). Non-adherent cells were removed by 5 washing steps for ICAM-1 plates and 2 washing steps for iC3b and heparin plates and measured again. Adhesion is depicted as percentage adhesion of input and relative to control treatment.

Fluorescent Beads adhesion assay
The fluorescent beads assay was performed as described as before[24]. In short, transfluorspheres (488/645 nm, 1.0 μm; Molecular Probes, invitrogen, Carlsbad, California, USA) were covalently coupled to streptavidin using 1-ethyl-3(-3-dimethylaminopropyl)-

carbodimide in MES buffer (pH 6). Then, beads were coated with biotin-SP-Affinipure goat anti-human Fc (γ) F(ab')2 and subsequently coated with Fc-ICAM-1 or Fc-VCAM. Total leukocytes acquired from erythrocyte-lysed blood were resuspended in HEPES incubation buffer. The ligand-coated beads were washed twice and added together with the cells (40,000/well) and Mn^{2+} and blocking antibodies (10μg/ml) or sFg (2mg/ml) in a 96-well V-shaped-bottom plate for 30 min at 37°C. Binding of the fluorescent beads to lymphocytes was determined by flow cytometry as described above and results reported as the percentage of cells positive for ICAM-1- or VCAM-1-coated beads.

Adhesion assay under flow conditions

L cells (Mouse fibroblast cell-line) were kindly provided by Dr. W. Smith (Baylor College of Medicine, Houston, Tex., USA). These L cells stably transfected with ICAM-1 and E-selectin were cultured in RPMI 1640 medium supplemented with glutamine and neomycin[25]. L cells coated on Thermanox coverslips (18 x 18 mm, confluent cell layer) were used in perfusion assays. Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from human umbilical cord veins[24]. HUVEC was activated by TNF-α (100 U/ml, 4h, 37°C) before the perfusion experiments. Perfusions under steady flow were performed in a modified form of transparent parallel plate perfusion chamber as previously described[26]. This microchamber has a slit height of 0.2 mm and width of 2 mm. The chamber contains a circular plug on which a coverslip (18mm x 18 mm) with confluent HUVEC was mounted.

Neutrophils (2 x 10^6 cells/ml) were pre-incubated with albumin or sFg (15 min, 37°C) and were aspirated from a reservoir through the perfusion chamber. The neutrophil suspension was perfused during 3 min at shear stress 1.5 dyn/cm^2 to obtain an endothelial surface with firmly adhering and rolling cells. For experiments in figure 5D HUVEC was pre-streamed with HSA (10 mg/ml) or sFg (6 mg/ml) (5 min, 50 μl/min, 37°C) and neutrophils were pre-incubated and either spun down before the experiment and incubated with buffer or not. During the whole procedure images were recorded. To automatically determine the velocity of rolling cells, custom-made software was developed in Optimas 6.1. Determination of total adherent cells (firmly adherent and rolling cells) and percentage rolling cells was performed as described previously[24]. In the first experiment using L-cells instead of HUVEC, IL-8 (10^{-8}M) was perfused over the rolling neutrophils cells to visualize chemokine-induced arrest.

Statistical analysis.

Data were analyzed using Graphpad Prism 4.0. Data are plotted as mean ± SE. Comparisons were analyzed by a wilcoxon signed ranks test, a paired samples t-test and a one-way ANOVA as stated in the figure legends. A p-value <0.05 was considered significant. (* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$).

Results

Soluble fibrinogen does not activate neutrophils

Previous literature suggests that sFg can activate neutrophils and induces signaling in neutrophils[27]. However, we did not observe activation of neutrophils by physiologically relevant concentrations of sFg, as incubation of neutrophils with sFg (2 mg/ml, 5 min at 37^0C) depleted of von Willebrand factor, fibronectin and plasminogen did not affect surface expression of CD11b and CD66b (figure 1A&C). These data were corroborated by the finding that no shedding of CD62L, another sign of neutrophil activation, occurred, as the expression of CD62L did not decrease (figure 1B). No signs of activation were observed even after prolonged (up to 45 min) incubation with sFg (not shown). Addition of platelet-activating factor (PAF, 10^{-6} M) was used as a positive control. This resulted in an increased expression of CD11b and shedding of CD62L (figure 1D&E) ($p<0.05$ and $p<0.005$ respectively). We next investigated the effect of sFg on primed neutrophils since it is well known that primed cells are more susceptible for activation. Cells were pretreated with PAF (1 μM) or GM-CSF (10 nM) for 5 minutes and 15 minutes at 37^0C, respectively. After 2 washes sFg 2 mg/ml was added to these primed cells for 5 minutes at 37^0C. sFg did not affect CD11b expression or CD62L expression on neutrophils primed with PAF or GM-CSF (figure 1D- G).

To exclude the possibility that different preparations of fibrinogen might be the reason for the different results observed on neutrophil activation we tested several fibrinogen preparations. FIB1 depleted of plasminogen, FIB2 depleted of plasminogen and von Willebrand factor (Kordia, Enzyme Research Laboratories) were tested in flow cytometry assays and did not result in activation of neutrophils (data not shown). This illustrates that von Willebrand factor and fibronectin are not likely to be the cause of activation of neutrophils by fibrinogen preparations used in various other studies[27,28].

A rise in cytosolic free Ca^{2+} is a sign of neutrophil activation induced by ligation of surface receptors and/or chemoattractant signaling. sFg (2mg/ml) did not cause an increase in intracellular Ca^{2+}, while the positive control fMLF induced a clear rise in intracellular Ca^{2+} (figure 1H).

Soluble fibrinogen inhibits Mac-1 dependent interactions and functions of neutrophils

Soluble fibrinogen is known to inhibit αMβ2-dependent neutrophil adhesion to fibrin and immobilized fibrinogen[6,20]. As this might be due to interactions between sFg and fibrin[20], we used other Mac-1 ligands such as ICAM-1, heparin, iC3b and zymosan to test the effect of sFg on neutrophil Mac-1 dependent interactions.

figure 2A shows that binding of resting, non-stimulated neutrophils to ICAM-1 coated plates is low. Activation of neutrophils with IL-8 induced adhesion from 10%±2 to 34%±2 binding. Incubation of neutrophils with blocking antibodies against β2 integrins (clone IB4) or the combination of anti-αM (clone 44a) and anti-αL integrin (clone NKI-L15) antibodies reduced adhesion to background levels. Furthermore, binding of neutrophils

to ICAM-1 coated plates was mainly αM integrin dependent since blocking αM only significantly inhibited adhesion compared to control antibody treatment whereas blocking αL did not. Next, the effect of sFg on αMβ2 dependent adhesion to ICAM-1 was studied. Figure 2B shows that sFg dose dependently inhibited binding of neutrophils to ICAM-1 whereas the control HSA treated neutrophils did not. The concentration sFg of 0.3 mg/ml was tested since previous studies showed an activating effect of this dosage on leukocyte binding to ICAM-1 surfaces[7]. We did, however, not observe an adhesion stimulatory effect.

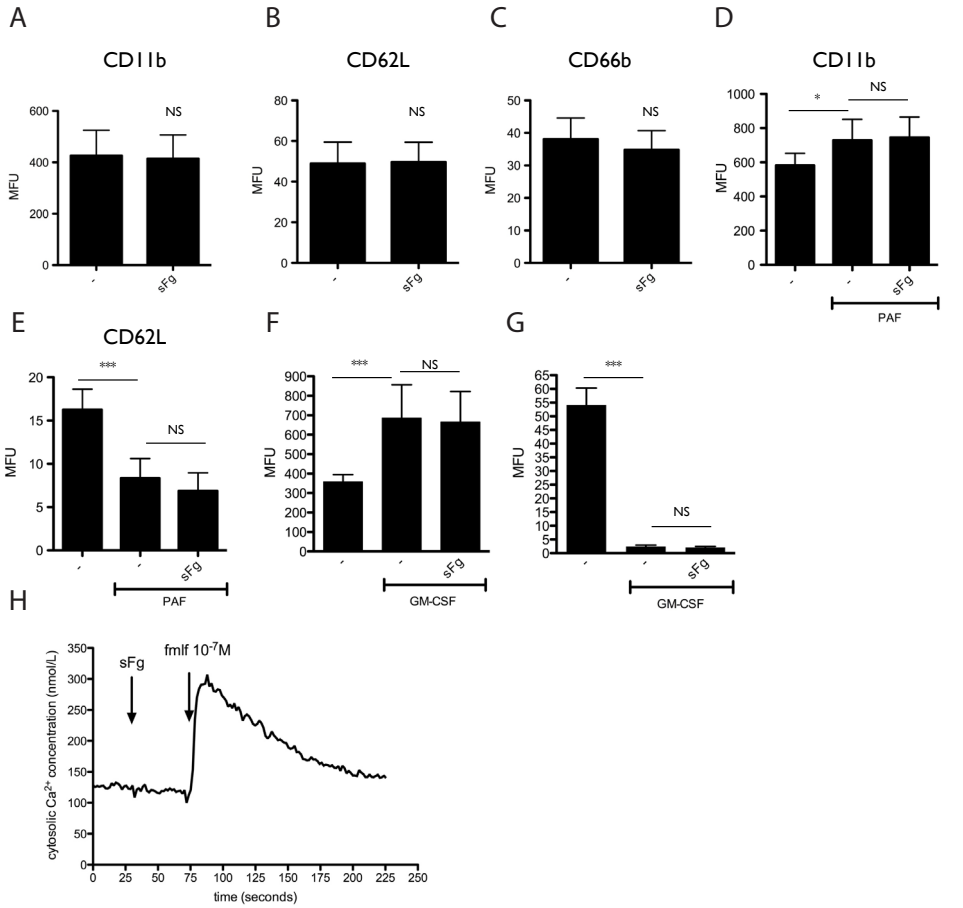

Figure 1. sFg does not activate neutrophils. (A,B & C) Isolated neutrophils were incubated with sFg (2 mg/ml, 5 min., 37°C) and washed. Then, neutrophils were stained with directly labeled antibodies against CD11b, CD62L and CD66b. (n=5) **(D-G)** Neutrophils were preincubated with PAF (1μM) or GM-CSF (10 nM) (5 min., 37°C), and washed. Then, these primed neutrophils were incubated with sFg (2 mg/ml, 5 min., 37°C). Cells were washed and stained with antibodies against CD11b and CD62L. Data are expressed as means ± SE of n=3. * P<0.05, *** p<0.001 as calculated by a paired samples t-test. **(H)** Neutrophils were incubated with 1.5 μM Fura-2/AM for 45 minutes Fura-2 fluorescence was measured in a fluorospectrophotometer (F-7000, Hitachi, Tokyo, Japan). Concentration Ca^{2+} was calculated as previously described using 5 μM digitonin and 10 mM EGTA for F_{max} and F_{min}, respectively[22]. Respresentative example of three independent experiments.

We next tested other αMβ2 integrin ligands heparin and C3bi. The percentage of neutrophils that bound to heparin compared with input after 30 min incubation was ~15%-20% and ~70-75% for C3bi, respectively.

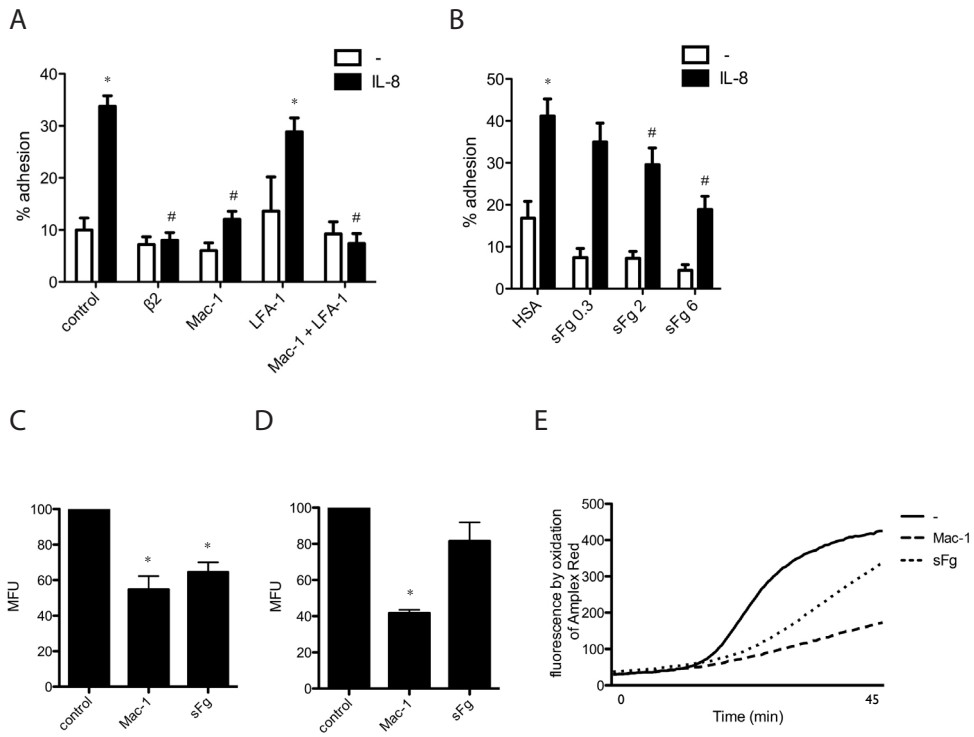

Figure 2. Fibrinogen inhibits Mac-1 dependent neutrophil functions. Calcein labeled neutrophils were allowed to adhere to ICAM-1-coated plates (15 min, 37ºC) in the presence or absence of blocking antibodies against αM, αL, β2 integrin chains (10 μg/ml) **(A)** or sFg (0.3, 2, 6 mg/ml) **(B)**. Percentage of neutrophils binding to the plate after 5 washes compared to total input before wash is depicted. * indicates $p<0.01$ of stimulated compared to non-stimulated condition and # indicates $p<0.01$ of treatment compared to control calculated using a One Way ANOVA with Tukey's post-hoc testing (n=5). Calcein labeled neutrophils were allowed to adhere to heparin **(C)** or iC3b **(D)** for 45 minutes in the presence of control anti HLA-1 antibody (W6//32), blocking αM antibody (44a, 10 μg/ml) or sFg (2 mg/ml). Hereafter, plates were washed twice and the amount of labeled neutrophils adhered to the plate was measured. Data are presented as percentage (means ± SE) with control W6/32 treated cells put on 100%. (n=6). * $P<0.05$ as calculated by a wilcoxon signed ranks test. **(E)** Unopsonized zymosan (1 mg/ml) was added to neutrophils in the presence or absence of 44a (10 μg/ml) or sFg (2 mg/ml). Hereafter, the cells were incubated under non-stirred conditions for 45 minutes at 37ºC in the presence of Amplex Red . Oxidation of the Amplex red was continuously measured for 45 minutes. A representative experiment of 3 independent experiments is shown.

Figure 2C and D show that blocking αMβ2 on neutrophils with monoclonal antibody (Mab) 44a inhibited adhesion to heparin and iC3b by 45±7.5% ($p<0.05$) and 59±1.9% ($p<0.05$) respectively in comparison to control antibody w632. sFg did significantly inhibit Mac-1 dependent neutrophil adhesion to heparin (35±13.7) ($p<0.05$) (figure 2C) but did not significantly affect the adhesion of neutrophils to iC3b coated surfaces (19±10.4%) ($p=0.11$) (figure 2D). Preincubation of the heparin coated plate with sFg (30 minutes 37^0C), followed by 2 washes, did not inhibit the adhesion of neutrophils to heparin indicating that the effect of sFg was not caused by a direct binding to heparin (not shown).

A functional response of neutrophils that is dependent on αMβ2 integrin is the respiratory burst in response to unopsonized zymosan[29]. Indeed, the αm blocking antibody 44a inhibited zymosan-induced burst (figure 2C). Furthermore, this respiratory burst was significantly inhibited by sFg over the time course of 45 minutes (figure 2C). Thus, these results show that sFg is capable of inhibiting αMβ2-dependent neutrophil functions.

Inhibition of leukocyte αLβ2 - ICAM-1 interactions by sFg.

Figure 2 showed that sFg inhibits αMβ2 mediated interactions. However, in the case of ICAM-1 binding it is not known whether sFg exerts its effect through interaction with αMβ2, ICAM-1 or both. To test this we used a static model system to assess αLβ2 integrin mediated lymphocyte binding to ICAM-1. The advantage of this assay is that lymphocytes were obtained by gating the individual leukocyte subset in a flowcytometry-based assay. Isolation of lymphocytes from total leukocyte population with beads prior to the adhesion assay was therefore not necessary. Lymphocytes were activated by Mn^{2+} (0.5 mM) and adhesion to ICAM-1 was assessed using an ICAM-coated beads assay [24]. Figure 3 shows that adhesion to ICAM-1 coated beads was not mediated by αMβ2, as blocking αMβ2 using Mab 44a did not reduce lymphocyte binding to ICAM-1 (figure 3). As expected, incubation of lymphocytes with the blocking antibody Mab NKI-L15 (CD11a) abrogated adhesion of lymphocytes to ICAM-beads ($p<0.05$). Next, leukocyte/ICAM-1 binding assays were performed in the presence of 2 mg/ml of sFg. Addition of sFg resulted in abrogation of lymphocyte adhesion to ICAM-1 ($p<0.05$) (figure 3A).

To test whether sFg bound to lymphocytes or to the beads, the lymphocytes were pre-incubated with sFg in the presence of Mn^{2+} followed by washing of the cells. This did not reduce leukocyte binding to ICAM-1 coated beads (figure 3B). Next we tested whether sFg would directly bind to ICAM-1 as has been previously suggested and, thereby, would function as an inhibitor[7]. Indeed, ICAM-1 beads, which were pre-incubated with sFg for 30 minutes, showed less adhesion of lymphocytes (figure 3C).

As a control, VCAM-1 coated beads, which are a ligand for lymphocyte α4 integrins did not show reduced binding after preincubation with fibrinogen (figure 3D). Blocking α4 integrin antibody HP2/1 abrogated binding of lymphocytes to VCAM-1 coated beads to background levels showing the specificity of the binding. We, therefore, concluded that

sFg is capable of attenuating leukocyte adhesion to ICAM-1 by direct binding to ICAM-1.

sFg inhibits neutrophil adherence under flow conditions

We next investigated whether sFg affects neutrophil adhesion under flow conditions. Neutrophils were perfused over L-cells expressing E-selectin and ICAM-1 at a shear of 2 dyn/cm^2. IL-8 (10^{-8}M) was used to induce integrin-mediated arrest of neutrophils to the L-cells. Neutrophils pre-incubated with a blocking antibody against Mac-1 prevented IL-8-induced arrest whereas blocking LFA-1 had no effect (figure 4). Interestingly, sFg (6mg/ml) inhibited neutrophil firm adhesion to a similar extent as the blocking antibody against Mac-1 ($p<0.05$).

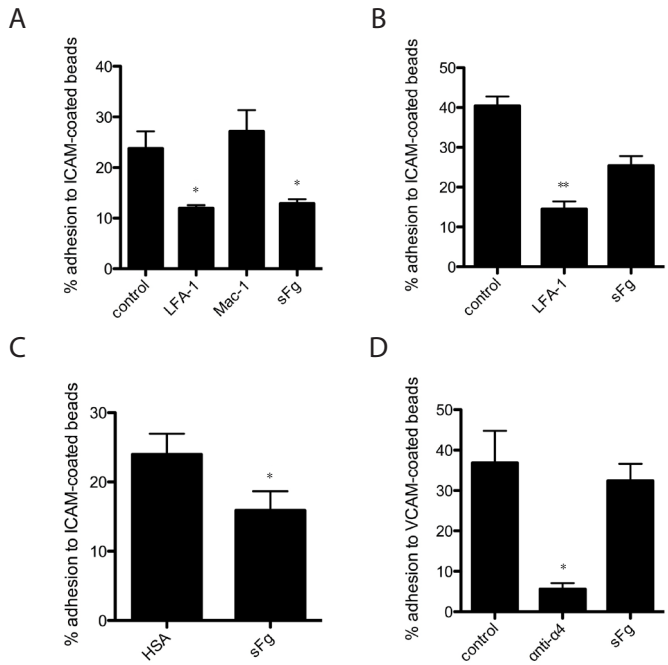

Figure 3. sFg reduces lymphocyte adhesion to ICAM-1 coated beads via ICAM-1. Lymphocytes were allowed to adhere to ICAM-1-**(A,B,C)** or VCAM-1-**(D)**-coated beads in the presence of Mn^{2+} (0.5mM) for 30 minutes at 37^{0}C. In **(A)** neutrophils were tested in the presence of control anti HLA-1 antibody (W6/32), blocking αM antibody (44a) or αL antibody (NKI-L15) or sFg (2 mg/ml). In **(B)** lymphocytes were pre-incubated with sFg (2 mg/ml) and after washing, cells were allowed to adhere to ICAM-1 coated beads in the presence of Mn^{2+} (0.5mM, 30 min. 37ºC) and mabs NKI-L15 or w6/32. In **(C)** ICAM-1 beads were pre-incubated with sFg (2 mg/ml), washed and incubated with lymphocytes in the presence of Mn^{2+} (0.5mM, 30 min. 37ºC). In **(D)** VCAM-1 coated beads were incubated with sFg (2 mg/ml) or lymphocytes with blocking antibody against α4 integrins (HP2/1) in the presence of Mn^{2+} (0.5mM, 30 min. 37ºC). Lymphocytes were gated based on forward/sideward scatter profiles. Data are depicted as the percentage (means ± SE) of lymphocytes adhered to beads as determined by flow cytometry, (n=6). * $P<0.05$ as calculated by a wilcoxon signed ranks test.

A more physiologically relevant experiment was performed by studying the role of sFg in leukocyte interactions with inflammatory endothelium under flow conditions. Neutrophils were perfused over TNFα-activated endothelial cells under flow conditions in the presence of sFg or HSA as a control. Figure 5A shows that sFg (2mg/ml) was not sufficient in inhibiting neutrophil-endothelial interactions; supporting the data that sFg is a low-affinity ligand for ICAM-1 and Mac-1[30]. Higher concentrations sFg (4-10 mg/ml), which are found in humans during an acute phase response, dose dependently decreased neutrophil firm adhesion to activated endothelium down to 40% ($p<0.005$ for sFg 10mg/ml) (figure 5A). This was accompanied by an increased rolling velocity (figure 5B). In addition, the total number of neutrophils interacting with the endothelial monolayer was unchanged (figure 5C) showing that capture of the neutrophils was not affected. To test whether sFg mainly acted on HUVEC, on neutrophils or on both we pre-streamed TNFα–activated HUVEC with either HSA (10 mg/ml) or sFg (6 mg/ml) for 5 minutes and subsequently perfused neutrophils incubated with either HSA (10 mg/ml) or sFg (6 mg/ml) for 3 minutes. Figure 5D shows that only in the continuous presence of sFg in solution during the perfusion the percentage rolling cells was significantly increased. Even neutrophils that were pre-incubated with sFg, spinned down and then perfused in incubation buffer over sFg pre-streamed HUVEC (last bar in graph) did not increase rolling behavior compared to control. This indicates that sFg needs to be continuously present to exert its effect and is in line with the hypothesis that the sFg exerts a low affinity interaction with αmβ2 and ICAM-1.

Phenotypically, neutrophils displayed a polarized morphology when incubated with control protein HSA (figure 6A). Strikingly, neutrophils displayed a round morphology (a sign of unactivated neutrophils) on activated endothelial cells when sFg was present (figure 6B). This strengthens our findings in figure 1 that our fibrinogen preparation does not activate neutrophils but rather prevents β2 integrin-ICAM-1 interactions.

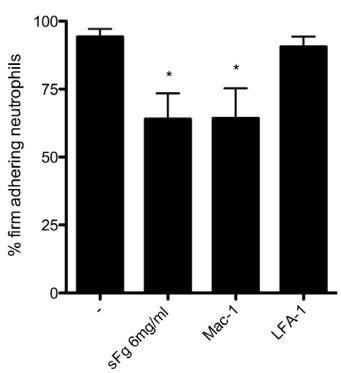

Figure 4. sFg reduces neutrophil firm adhesion under flow conditions (2 dyn/cm²). Neutrophils were pre-incubated with HSA 10mg/ml or sFg 6mg/ml and blocking or control antibodies. Neutrophils were perfused over L-cells expressing ICAM-1 and E-selectin for 3 minutes, whereafter of IL-8 was added (10^{-8}M). The percentage of firm adhering cells was determined 5 minutes after perfusion of IL-8 as described in the material and methods. Data are depicted as the percentage (means ± SE) of neutrophils firmly adhered to the L-cells (n=6). * $P<0.05$ as calculated a 1-way ANOVA.

Discussion

In this study we report a novel anti-adhesive role for soluble fibrinogen in regulating neutrophil–endothelial interactions. The role of fibrinogen in inflammation has mainly been described as pro-inflammatory. It has been shown in many studies that fibrin and immobilized fibrinogen are high affinity ligands for Mac-1 and can activate neutrophils and monocytes[28,31,32]. Here, we conclude that concentrations of sFg found in plasma in inflammatory conditions are anti-inflammatory rather than pro-inflammatory. This conclusion is based on several findings.

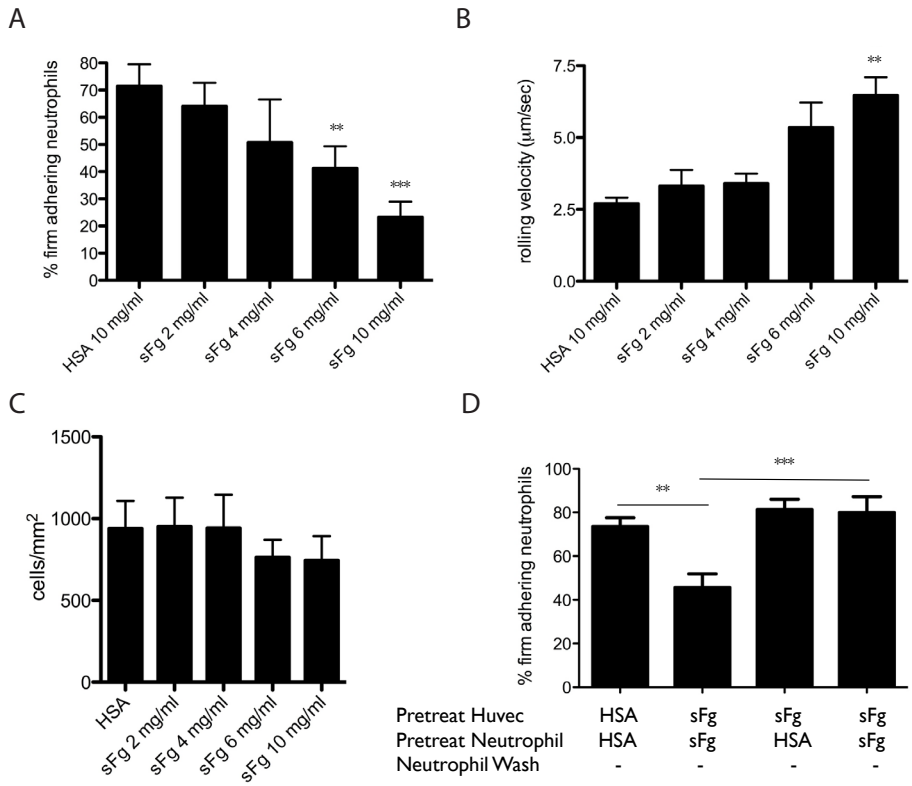

Figure 5. sFg reduces neutrophil firm adhesion on TNFα-activated HUVEC under flow conditions (2 dyn/cm²). **(A)** Neutrophils were pre-incubated with HSA 10 mg/ml or increasing concentrations of sFg (2-10 mg/ml). Cells (2 x 10^6/ml) were perfused over activated HUVECs for 3 min. The percentage of firm adhering cells **(A)** and the rolling velocity **(B)** of the rolling adherent cells were determined after 3 minutes. **(C)** Total cells (rolling and adhered) on the HUVEC surface was determined as described in the material and methods section. In **(D)** TNFα-activated HUVEC was pre-streamed with HSA (10 mg/ml) or sFg (6 mg/ml) (5 min, 0.5 dyn/cm²) and neutrophils were pre-incubated with HSA or sFg as indicated. In last bar an additional spun of neutrophils was performed to deplete sFg from the solution. Dare depicted as the percentage (means ± SE) of neutrophils firmly adhered to HUVEC (n=5). ** P<0.01, *** p<0.001 as calculated by a paired samples t-test or One Way ANOVA (# p<0.01).

First we showed that sFg inhibited neutrophil Mac-1 dependent functions (adhesion to ICAM-1, iC3b, heparin and the zymosan induced respiratory burst). Secondly, we showed that sFg inhibited lymphocyte binding to ICAM-1 coated beads by directly binding to ICAM-1. Finally we observed that sFg reduced neutrophil firm arrest on ICAM-1 expressing L-cells and on activated endothelium under flow conditions. Soluble fibrinogen is constantly present in the circulation, which might help to downregulate neutrophil activation via binding Mac-1 and ICAM-1, especially at acute phase concentrations. Interaction of sFg with Mac-1 did not inhibit all functions in a similar manner. E.g. adhesion to heparin was inhibited more effectively than adhesion to iC3b and the zymosan induced respiratory burst. The difference in the capacity of sFg to inhibit these various Mac-1 dependent functions can be explained by the presence of several binding epitopes on Mac-1.

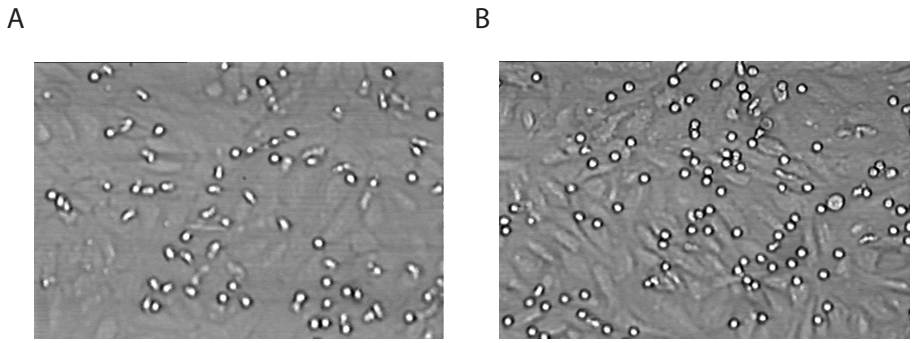

Figure 6. sFg prevents polarization of neutrophils on TNFα-activated HUVEC. Representative images from neutrophils on activated endothelial cells in the presence of HSA (10 mg/ml) **(A)** or soluble fibrinogen (6 mg/ml) **(B)** are shown.

The role of these different epitopes in mediating binding to heparin and iC3b has been elucidated by others using phage display antibodies directed to active Mac-1. Using an activation specific human anti-Mac-1 single chain antibody, a similar difference between heparin and iC3b was observed. This antibody termed MAN-1 recognizes Lys245-Arg261 in the αM I-domain[33]. MAN-1 inhibited Mac-1 binding to heparin but not to iC3b. As sFg inhibited heparin binding more effectively than iC3b binding in our hands, it is tempting to speculate that the site identified by MAN-1 is the binding site on Mac-1 for sFg.

Previous literature showed that sFg induced priming and signaling in neutrophils[28,34,35]. We could not reproduce these findings (figure 1A&B)[36], which could be due to several reasons. Firstly, the fibrinogen preparation we used was depleted of plasminogen, von Willebrand factor and fibronectin whereas Rubel et al. used fibrinogen depleted of plasminogen only[28]. Therefore we tested FIB1 and FIB2, which are depleted for plasminogen only or

plasminogen and von Willebrand Factor. However, these preparations did also not result in activation of the neutrophils (supplementary data). In other papers, the source and purity of the pyrogen free fibrinogen was not mentioned[27,37].
Secondly, activation status of the neutrophils might differ due to differences in isolation procedures and culture media used. Yet, performing our assays in up to 5% bovine serum as used by Rubel et al. did not prime for fibrinogen induced neutrophil activation in our assays (data not shown)[35]. Thirdly, lengthy static assays applying sFg might have allowed a certain degree of fibrinogen deposition. Both fibrin and fibrinogen immobilization results in potent activating ligands for Mac-1[19], whereas we used short incubations in solution and incubations under flow conditions with high amounts of sFg.
To further support the hypothesis that sFg can act as a physiological antagonist for neutrophil adhesion *in vivo* we applied an *ex vivo* flow system using TNFα-activated endothelium as a physiological substrate for neutrophil adhesion under flow conditions. Concentrations of sFg found in the plasma under acute phase conditions significantly reduced neutrophil arrest. Firm adhesion of leukocytes to activated endothelial cells is dependent on β2-integrins[38]. To explain the underlying mechanism of reduced neutrophil adherence we have to take into account that sFg was able to interact both with Mac-1 (figure 2) and ICAM-1 (figure 3C). In addition, incubation of neutrophils with sFg dose dependently inhibited neutrophil Mac-1 dependent adherence to ICAM-1 (figure 2B). As these experiments were all static, it was not known whether the interaction of sFg with either the endothelial cells (e.g. ICAM-1) or neutrophils (e.g. Mac-1) dominated. Therefore, we performed experiments under flow conditions that illustrated that sFg had to be continuously present to exert its depolarizing, anti-adhesive effect (figures 5D & 6). The pro-inflammatory properties of immobilized fibrinogen and fibrin could explain findings in models of arthritis and systemic endotoxemia[12,13]. In these models fibrin(ogen) deposition plays an important role in enhancement of disease severity. As neutrophil interactions with fibrin and immobilized fibrinogen are pro-inflammatory it will be important for the host to express mechanisms that limit these interactions. Indeed, we and others have reported that sFg is effective in limiting neutrophil interaction with fibrin and immobilized fibrinogen[6,20]. In the current study we now report that sFg also inhibits LFA-1 –ICAM-1 interactions through binding to ICAM-1 and/or Mac–1 and that sFg potently downregulates neutrophil adhesion under flow conditions. Furthermore, sFg interfered with Mac-1 dependent cytotoxic functions such as the respiratory burst.
The ability of fibrinogen to bind to ICAM-1 has been reported previously [7]. It was shown that low concentrations of fibrinogen (up to 0.3 mg/ml) dose dependently mediated bridging between leukocytes and endothelium leading to a pro-adhesive phenotype of neutrophils. Interestingly, in this study 1 mg/ml fibrinogen showed lower bridging than 0.3 mg/ml. Higher concentrations of fibrinogen were not evaluated in this study which we now show to have opposite, anti-adhesive effects. In our assay 0.3 mg/ml sFg did not have a stimulatory effect of neutrophil binding to ICAM-1 (figure 2B). The main difference

in experimental set up was the type of cells (THP-1 vs primary neutrophils) and surface (resting HUVEC vs purified ICAM-1). Thus we can conclude that low concentrations of sFg do not promote adhesion of freshly isolated neutrophils to ICAM surfaces, which is in line with all our observations that sFg acts de-adhesive instead of pro-adhesive for neutrophils.

sFg has been proposed to exhibit anti-inflammatory properties in various situations. In humans exhibiting the – 148 C/T fibrinogen gene polymorphism larger amounts of acute phase fibrinogen were present and this correlated with low concentrations of TNFα after experimental endotoxin administration[39]. In a large retrospective analysis with patients with major injuries showed less organ failure when administered cryoprecipitate in stead of fresh frozen plasma (FFPs). Interestingly, the cryoprecipitate is a source of concentrated fibrinogen whereas FFPs contains less fibrinogen[40]. It is tempting to speculate that cryoprecipitate provided an early normal or supraphysiological sFg concentration in these patients and, therefore, contributed to their resolution of inflammation. In addition, in a murine model of intravenous endotoxin, inflammatory responses were delayed in mice lacking fibrinogen, but peak values of cytokines and cell influxes into tissues were higher compared to WT mice[12]. This fits with the hypothesis that low physiological concentrations of sFg are important for the initiation of inflammation (through deposition of fibrin) and that the late increase of sFg as an acute phase protein contributes to the resolution of inflammation.

In conclusion, acute phase concentrations of sFg might limit collateral inflammatory damage by protecting endothelium from activated leukocytes and, therefore, contribute to the resolution of inflammation. This provides for the first time a physiological function for acute phase concentrations of fibrinogen in response to inflammation.

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C1-esterase inhibitor attenuates the inflammatory response during human endotoxemia

Mirrin Dorresteijn
Tjaakje Visser
Laura Cox
Martijn Bouw
Janesh Pillay
Anky Koenderman
Paul Strengers
Luke Leenen
Johannes van der Hoeven
Leo Koenderman
Peter Pickkers

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Introduction

Acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) are the leading causes of death in medical and surgical ICU patients[1-4]. The parenchymal damage and subsequent organ dysfunction are caused by an over-activated inflammatory response[5]. The systemic release of several humoral inflammatory mediators, such as tumour necrosis factor (TNF)-α, Interleukin (IL)-1β and IL-6 activate the vascular endothelium and modulate activation and tissue infiltration of circulating leukocytes[5-7].

A promising intervention to modulate the innate inflammatory response is treatment with a high concentration of C1-esterase inhibitor (C1INH). C1INH is an acute phase protein produced by the liver and important in regulating the activation of the complement and contact system, which play a role in opsonisation and the regulation of coagulation[8-10]. Currently, C1INH administration is applied in patients suffering from a deficiency of the protein causing hereditary angioedema[8].

Interestingly, several animal studies have demonstrated that supraphysiological levels of C1INH during models of acute inflammation (sepsis and (thermal) trauma), improve survival, preserve endothelial function and prevent the occurrence of capillary leak[11-14]. Furthermore, C1INH can inhibit adhesion of leukocytes to the endothelium and reduce tissue infiltration[11, 13, 15-19]. In septic patients, administration of C1INH reduced leukocyte activation and the release of cytotoxic mediators by degranulation[20]. The mechanisms by which C1INH exerts its actions are only partly understood but appear to be (partly) independent of its effects on the complement and contact system, since its beneficial effects in *in vitro* and animal studies remain intact after cleavage of the reactive centre[12, 21].

Up to now, only a few reports exist of the administration of C1INH to patients undergoing major surgery or suffering from severe sepsis or septic shock. Although these studies were only performed in very small patient groups and not always in a placebo-controlled fashion, their results were encouraging demonstrating a significant reduction of renal impairment and small case series suggest that the administration of C1INH is associated with less need for vasopressor therapy and a reduced hospital stay[8, 22-25].

However, to elucidate the mechanism by which C1INH exerts its anti-inflammatory effects, patient studies remain difficult to interpret due to heterogenenity of the underlying diseases. In contrast, human experimental endotoxemia provides an *in vivo* model with reproducible systemic inflammation[26]. During human endotoxemia, the release of several humoral mediators, activation of leukocytes and vascular endothelium occurs within a few hours after infusion. Interestingly, this cascade is independent of complement or contact system activation[27-29]. We hypothesized that C1INH can modulate the inflammatory response during human endotoxemia in the absence of contact or complement system activation.

Materials and methods

Subjects

This study was registered at ClinicalTrials.gov as # NCT00785018. After approval from the medical ethical committee, 20 healthy male subjects gave written informed consent to participate in the experiments in accordance with the Declaration of Helsinki. Subjects using any drugs were excluded. Screening of the subjects within 14 days before the test revealed no abnormalities in medical history and physical examination. Laboratory tests (including serology on HIV and hepatitis B) and ECG were normal. Ten hours before the experiment, subjects refrained from the intake of caffeine, alcohol, and food.

Study design

After admission to the research intensive care unit of the Radboud University Nijmegen Medical Centre, purified lipopolysaccharide (LPS) (U.S. Standard Reference Endotoxin *Escherichia coli* O:113) obtained from Pharmaceutical Development Section NIH (Bethesda, MD, USA), was administered at a dose of 2 ng/kg bodyweight at t=0h (hour). Thereafter, subjects were randomized by an independent research nurse to receive C1INH-concentrate (N=10, Cetor®, Sanquin, Amsterdam, The Netherlands, 100 U/kg bodyweight, infused in 30 minutes) or an equivalent volume of placebo (N=10, 0.9% saline, Baxter, Utrecht, The Netherlands) using the sealed envelope method. The dose of 100 U/kg C1INH was chosen based on a study performed in humans with acute myocardial infarction[30]. C1INH solution or placebo was prepared by the independent research nurse and given to the investigator in identical containers ensuring the double-blind fashion of the study. Intravenous infusion of C1INH or placebo was started by the investigators at 30 minutes after LPS to prevent binding to LPS (figure 1)[13, 28, 31, 32].

Hemodynamic and clinical response

For continuous monitoring of blood pressure and for blood sampling, the radial artery was cannulated with a 20 gauge arterial catheter. Heart rate monitoring was performed using a 5-lead ECG. A cannula was placed in an antecubital vein to permit infusion of prehydration fluid, endotoxin, C1INH or placebo and the continuous infusion of 150 ml/h 2.5% glucose/0.45% saline. Body temperature (FirstTemp Genius, Tyco Healthcare, Hampshire, UK) and symptoms were scored every 30 minutes. Subjective symptoms were scored using grades varying from 0 (symptoms: absent) to 5 (symptoms: worst ever experienced) in order to define the severity of nausea, headache, shivering, muscle and/or back pain. Thereafter, scores were added leading to an arbitrary 'total symptom score' with a maximum value of 25 points.

Assays

Measurements of C1INH antigen and activity complement levels C4, various cytokines, C-reactive protein (CRP), and soluble adhesion molecules were performed before LPS

and serially thereafter.

Objectives and hypothesis

The primary objective of the present study was to determine whether C1INH can modulate cytokine release during human endotoxemia. Secondary objectives include the effects of C1INH on CRP, hemodynamic and clinical response and the release of soluble adhesion molecules after LPS challenge.

Data analysis and statistics

Values are expressed as mean±SEM unless described otherwise. Kolmogorov-Smirnov tests indicated a normal distribution of almost all the data (a few exceptions of less relevant time points). Hence, a two-way repeated measures ANOVA was used to test variation over time, the variation between interventions, and the interaction between time and intervention (SPSS 16.0 software, SPSS, Chicago, IL, USA). Changes over time alone were analyzed by One-way ANOVA (Graphpad Prism 5, Graphpad Software, La Jolla, CA, USA). To compare differences between groups Student's t-tests were used, as appropriate (SPSS 16.0 software, SPSS, Chicago, IL, USA). As the symptom score is a discontinuous variable, we used non-parametric Friedman ANOVA for changes over time and Mann-Whitney U tests for differences between groups (SPSS 16.0 software, SPSS, Chicago, IL, USA). These data are expressed as median and ranges.

A p-value <0.05 was considered to indicate significance. Given the explorative 'proof of concept' nature of this study, no formal sample size calculation was performed. Furthermore, no subgroup analyses were made.

Table 1. Demographic characteristics

	Placebo	C1INH	Total group
Age, yrs	21.6±2.6	22.6±3.6	22.1±3.1
Height, cm	187±7	181±7	184±8
Weight, kg	80±10	71±6	75±9
BMI, kg/m²	23±2	22±1	22±2
HR, bpm	66±4	70±12	68±9
MAP, mmHg	103±10	98±8	101±9

Data are presented as mean ± SD. BMI, body mass index; HR, heart rate; MAP, mean arterial blood pressure

Results

Baseline characteristics

After screening 23 healthy volunteers, 20 subjects were enrolled in the study protocol and randomized to receive C1INH or placebo (figure 1). Besides a difference in body weight (Students *t*-test p=0.03), there were no significant differences in baseline characteristics between both groups. Demographic data are shown in Table 1.

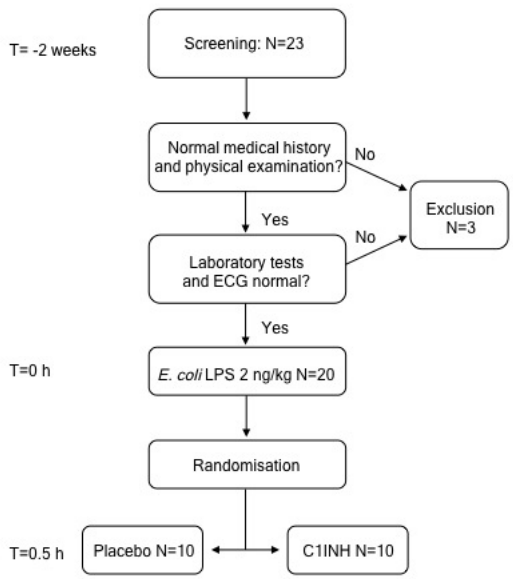

Figure 1: Flow diagram of subjects included in the study. After screening of 23 subjects, 20 subjects were included in the experiments. All received 2 ng/kg *Escherichia coli* lipopolysaccharide. Thereafter subjects were randomized to receive C1-esterase inhibitor (100 U/kg) or placebo.

Safety

No serious adverse events occurred during the experiments. The symptoms observed during the experiments could be related to the administration of LPS and are discussed below.

Clinical and hematological response

As summarized in Table 2, endotoxin infusion resulted in the expected changes in clinical and hemodynamic parameters in both groups. All endotoxin-induced changes were statistically significant (over time analysis using one-way ANOVA).

Endotoxin-induced symptoms typically started with headache approximately 1h after LPS administration. Symptoms, as scored by the subjects, peaked at t=1.5h at a median value of 5.5 (range 3-10) (out of a maximum of 25), for subjects receiving C1INH versus 6.5 (range 1-11) in subjects receiving placebo (over time $p<0.001$ using Friedman, no significant difference between groups using Mann Whitney U test (p=0.579)). Blood pressure dropped by 19±3% in the placebo group compared to 18±2% in the C1INH group (no significant difference between groups (p=0.392)). A comparable compensatory

rise in heart frequency was observed in both groups. Also, the maximum increase in temperature of 1.7±0.2 °C (C1INH) and 1.9±0.2 °C (placebo) was similar in both groups (Table 2).

Table 2: Hemodynamic parameters, symptoms, hematological and biochemical laboratory data during human endotoxemia in the absence and presence of C1INH.

		T=0	T=1	T=2	T=3	T=4	T=8	T=24	*P-value*
MAP, (mmHg)	Pl	103±4	104±2	93±2	85±4	86±3	83±6	ND	0.392
	C1	98±3	97±3	86±3	83±3	83±3	83±3	ND	
HR, (bpm)	Pl	66±2	73±3	81±3	90±3	91±2	81±4	ND	0.787
	C1	70±5	74±5	87±4	91±3	93±3	77±4	ND	
Total symptoms	Pl	0±1[a]	0±2[a]	3.5±6[a]	2.5±9[a]	1±8[a]	0±1[a]	ND	0.579[b]
	C1	0±3[a]	0±03[a]	3±5[a]	2±6[a]	1±4[a]	0±3[a]	ND	
ΔTemp., (°C)	Pl	NA	0.6±0.1	1.3±0.2	1.9±0.2	1.8±0.3	1.0±0.3	ND	0.826
	C1	NA	0.5±0.2	1.2±0.2	1.7±0.2	1.3±0.2	0.8±0.2	ND	
Hb, (mmol/l)	Pl	8.4±0.1	8.5±0.2	ND	8.3±0.1	ND	8.3±0.1	8.4±0.2	0.519
	C1	8.4±0.1	8.6±0.1	ND	8.5±01	ND	8.4±0.1	8.5±0.1	
Leuko., ($x10^9$/l)	Pl	5.3±0.6	3.1±0.5	ND	6.3±0.7	ND	11.4±0.7	5.5±0.5	0.801
	C1	4.6±0.3	2.2±0.4	ND	7.0±0.5	ND	11.0±0.7	5.4±0.4	
Thromb., ($x10^9$/l)	Pl	187±10	181±9	ND	189±13	ND	190±10	196±12	0.426
	C1	167±10	159±11	ND	179±8	ND	181±12	184±12	
CRP, (mg/l)	Pl	<5	ND	ND	ND	ND	9±1	39±4	0.026
	C1	< 5	ND	ND	ND	ND	7±1	29±2	

Time (T) expressed in hours after LPS administration. Pl: Placebo, C1: C1INH, MAP: mean arterial blood pressure, HR: heart rate, Hb: haemoglobin, Leuko: leukocytes, Thromb.: thrombocytes, CRP: C-reactive protein. ND: not determined, NA: not applicable. Data are expressed as mean±SEM. *p* values are comparisons between groups over time and were determined by Two-way repeated measures ANOVA.

[a] Total symptoms are expressed as median±range. [b] *p* value signifies difference between groups at t=1.5h determined by Mann-Whitney U test

C1INH antigen, activity and levels of complement factor 4

After LPS infusion, levels of C1INH antigen and activity demonstrated a modest increase in the placebo group (figure 2, over time: $p<0.01$). After administration of C1INH at 30 minutes after endotoxin infusion, levels of C1INH antigen increased from 0.20 ±0.01

g/l to 0.54 ± 0.03 g/l at t= 1 h after LPS administration (over time: $p<0.001$, between both groups: $p<0.001$) and remained high throughout the experiment. Also, levels of C1INH activity increased from 0.94 ± 0.03 U/ml to 2.42 ± 0.13 U/ml (over time: $p<0.001$, between groups: $p<0.001$, figure 2). The levels of C4 remained low in both groups (not significantly different over time ($p=0.585$) in both groups, or between groups ($p=0.735$)).

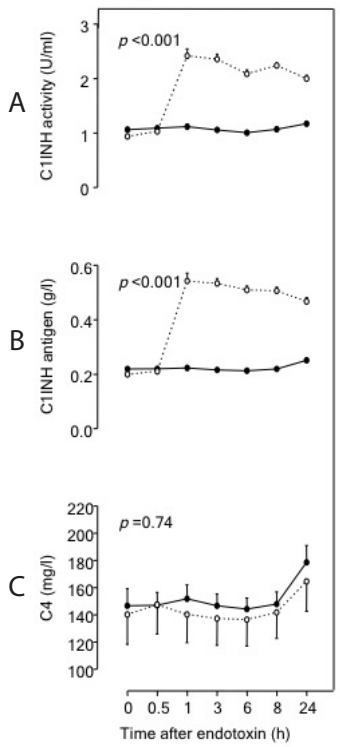

Figure 2: Levels of C1-esterase inhibitor activity (panel A), antigen (panel B) and Complement factor 4 (C4) (panel C) after administration of 2ng/kg *Escherichia coli* lipopolysaccharide at t=0h. At t=0.5h C1-esterase inhibitor at a dose of 100 U/kg intravenously over 30 minutes(o) or placebo (•) was infused. Data are expressed as mean ± SEM. *p* values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

After the administration of endotoxin, all measured cytokines showed a marked increase as illustrated in figure 3 (over time: all $p<0.001$). The administration of C1INH attenuated the release of all pro-inflammatory cytokines. Compared to subjects receiving placebo, peak levels of IL-6 were reduced by 39%. TNF-α peak levels were abrogated by 32%. Concentrations of MCP-1 and IL-1β decreased by 45% and 32% compared to placebo respectively. Conversely, the release of the anti-inflammatory IL-10 was increased in subjects receiving C1INH by 66%. The increase in IL-1RA tended to be less in the C1INH group, but this effect did not reach statistical significance ($p=0.07$). CRP was significantly reduced in the C1INH group compared to placebo ($p=0.03$, Table 2).

Markers for endothelial activation

Endotoxin administration is known to cause a release of soluble adhesion molecules,

suggesting activation of endothelial cells[33, 34]. As demonstrated in figure 4, all measured markers for endothelial activation were significantly induced by LPS infusion except for vWF. vWF was not significantly induced after LPS infusion in subjects receiving placebo (over time p=0.09). No significant difference in concentrations of circulating endothelial markers was observed between both groups.

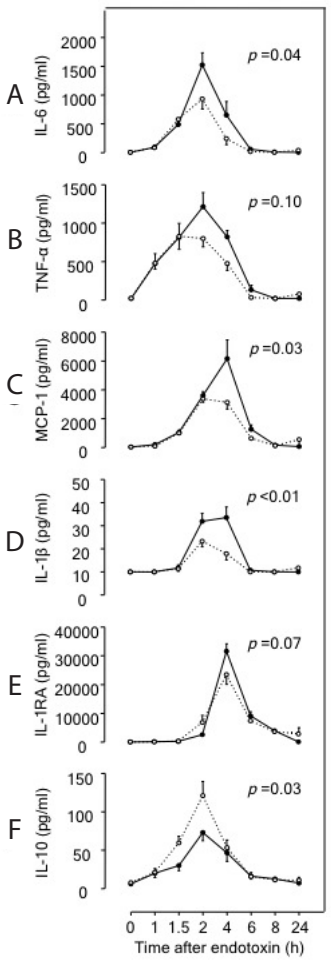

Figure 3: Cytokine concentration in the absence (•) and presence (o) of C1-esterase inhibitor after administration of 2ng/kg *Escherichia coli* lipopolysaccharide at t=0h. IL-6: Interleukin-6 **(A)**, TNF-α: Tumor Necrosis Factor-α **(B)**, MCP-1: Monocyte Chemotactic Protein-1 **(C)**, IL-1β: Interleukin-1β **(D)**, IL-1RA: Interleuking-1 receptor antagonist **(E)**, IL-10: Interleukin 10 **(F)**. Data are expressed as mean ± SEM. *p* values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

Discussion

The present study is the first to demonstrate that administration of a high dose of C1INH can modulate a controlled inflammatory response in humans, as elicited by *in vivo* infusion of endotoxin. During experimental endotoxemia, the release of pro-inflammatory cytokines was attenuated by C1INH, whereas the release of the anti-inflammatory cytokine IL-10 was potentiated. This C1INH-mediated shift in the pattern of the inflammatory response occurred in the absence of activation of the complement

system and could not be explained by binding to endotoxin.

After administration of C1INH, concentrations of antigen and activity were increased throughout the entire experiment. An increase of C1INH antigen and activity of 340 mg/L and 1.5 U/ml respectively is expected after an average gift of 7500 U. This higher plasma concentration is similar to that found in time during infectious diseases[8]. It seems that C1INH in acute phase protein concentration has a clear immunoregulating function.

The observed early increase of plasma IL-10 in the C1INH group compared to the placebo group is most remarkable. While the IL-10 levels were significantly increased by C1INH at 1.5h after LPS infusion, the levels of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and MCP-1 did not differ between groups until 2h after LPS infusion. Our findings are in agreement with data published by Storini et al[35], who showed a more pronounced increase of IL-10 mRNA expression after ischemia-reperfusion brain injury in mice treated with C1INH. They also found a concurrent smaller increase of mRNA levels of TNF-α and IL-6 in the C1INH treated group.

Interestingly, in our study the production of the anti-inflammatory cytokine IL-1RA was not potentiated, but even moderately blunted by C1INH in our study. This indicates that C1INH does not induce a general anti-inflammatory response. It is, therefore, tempting to speculate that C1INH acts as an anti-inflammatory mediator in humans by enhancing IL-10 production. To our knowledge, the direct effects of C1INH on IL-10 production have not been studied previously. IL-10 can block the release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 *in vitro*[36, 37]. In addition, studies *in vivo* have demonstrated that IL-10 can protect mice from lethal endotoxemia[38, 39]. Apart from increased levels of inflammatory mediators, IL-10 knockout mice were also characterized by an enhanced cellular inflammatory response in tissue[40]. These results imply a protective role for IL-10 in leukocyte driven inflammation, but the exact mechanism by which C1INH increases IL-10 production remains to be elucidated.

In our study IL-10 levels reached maximum concentrations concomitant with TNF-α at 2 hours after LPS infusion. In previous experiments IL-10 peaked later than TNF-α, between 2 to 3 hours after LPS infusion. No measurements were performed at 3 hours after LPS in these experiments and therefore maximum concentrations of IL-10 on this time point could have been missed. This could explain why it seems that peak values of TNF-α and IL-10 seems to occur at the same time point.

Earlier studies have suggested that the LPS-induced inflammatory reaction can be diminished due to scavenging of LPS by C1INH. This hypothesis was based on *in vitro* studies demonstrating the ability of C1INH to bind LPS and to reduce LPS binding to endothelial cells[13, 31]. However, it seems unlikely that the binding of LPS to C1INH explains the anti-inflammatory effects found after administration of C1INH concentrate in our study. Plasma concentrations of LPS are known to decrease rapidly to undetectable levels within 15 to 20 minutes after administration[28, 32]. In our study, C1INH infusion was started 30 minutes after LPS infusion, well after complete plasma clearance of LPS.

Furthermore, treatment with C1INH caused enhanced levels of LPS-induced IL-10. Scavenging of LPS due to an interaction with C1INH would have resulted in decreased levels of all cytokines, including IL-10 [41]. Therefore, it can be concluded that C1INH has direct immune modulating effects irrespective of a scavenging effect on LPS.
The observation that C1INH has a vast anti-inflammatory effect, even when it is administered after the induction of inflammation, is in agreement with several animal studies demonstrating beneficial effects of C1INH administration well after the onset of inflammation[12, 42]. In a murine cecal ligation and puncture (CLP) model, C1INH even increased survival when administered up till 6 hours after CLP, but not as much as when administered directly or at 3 hours after CLP[12].

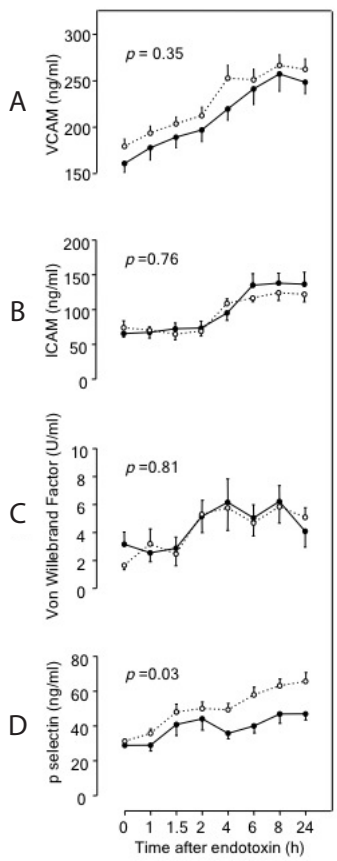

Figure 4: Concentrations of soluble adhesion molecules in the absence (•) and presence (o) of C1-esterase inhibitor after administration of 2ng/kg *Escherichia coli* lipopolysaccharide at t=0h. ICAM: Inter-Cellular Adhesion Molecule **(A)**, VCAM: Vascular Cell Adhesion Molecule **(B)**, P-selectin: Platelet selectin **(C)**. Data are expressed as mean ± SEM. *p* values in the figure express differences between groups obtained by repeated measures ANOVA over the complete curve.

The anti-inflammatory effects of C1INH seem, at least in part, independent of its function as a serpin, for in our study no signs of activation of the classical pathway of the complement system were found. We only measured serum concentrations of C4, and therefore can not rule out possible activation of the classical pathway via complement

components bound to micro particles and a possible effect hereon by C1INH[43]. Although some studies with higher doses of LPS have demonstrated complement activation[44], no studies with a similar low-dose of LPS have shown activation of the final common pathway of the complement system so far[28, 29].

The concept that C1INH has immunomodulatory effects independent of its role as a serpin, has been demonstrated by a seminal study performed by Liu et al[12]. They have showed that serpin-inactive C1INH was at least as effective as active C1INH to prevent mortality in a murine CLP model for sepsis. This indicates that C1INH does not rely on its serpin-dependent properties to evoke its anti-inflammatory effects.

Some animal studies indicate that C1INH can inhibit margination of tissue leukocytes[8, 14, 18]. However, these studies did not evaluate simultaneous increases in leukocyte counts in the peripheral blood. In our study, we did not find any effect of C1INH on peripheral leukocyte counts (Table 2), which is in agreement with data from an endotoxemia model in rats where similar results were observed[16.] However, CRP was significantly lower at t=24 hours in the C1INH group, indicating that these subjects have apparently endured less severe inflammation.

As demonstrated in our study, the levels of soluble adhesion molecules ICAM, VCAM and P-selectin as well as vWF increased after induction of systemic inflammation by LPS. Increases in these soluble adhesion molecules are thought to reflect activation of endothelial cells[33].

However, no differences were found between subjects receiving placebo or C1INH. This may point at the inability of C1INH to antagonize activation of endothelial cells after LPS challenge. Apparently, C1INH had no effect on the sheddases which cause the quick release of these molecules from the endothelial surface. This is in contrast to animal studies in which an attenuation of inflammation-induced mRNA synthesis of ICAM-1, VCAM-1 and P-selectin by C1INH was demonstrated[15, 39]. However, mRNA synthesis does not always reflect the levels of the circulating adhesion molecules[45].

In our study no difference in clinical response such as hemodynamic changes or symptoms could be demonstrated. Obviously, human endotoxemia, being a very useful tool to study the innate immune response, can by no means mimic the cascade of events occurring in ICU patients suffering from inflammatory disorders such as septic shock or severe trauma. As an enhanced cytokine release during severe inflammation is associated with development of ARDS and MODS and eventually death[3, 46, 47], it is tempting to speculate that the ability of C1INH to shift of the inflammatory response to a more anti-inflammatory pattern, especially early in the disease process, could be beneficial in patients suffering from these acute inflammatory syndromes.

A limitation of our study is the fact that only healthy young male subjects were included. As this study should be viewed as a 'proof of concept study', to demonstrate the effects of C1INH on the innate immune response, we aimed to create a homogeneous study population. Therefore, the extrapolation of the effects of C1INH on LPS challenge to

putative clinical effects should be taken with caution. Although a few studies have been reported applying C1INH in sepsis or trauma patients, future randomized controlled patient studies are of pivotal importance to determine the effects of this protein in the clinical setting.

Conclusions

In the present study, C1INH has potentiated the release of the anti-inflammatory cytokine IL-10 and simultaneously reduced the release of pro-inflammatory cytokines during human experimental endotoxemia. This shift in the pattern of the inflammatory response occurred in the absence of activation of the complement component C4 and could not be explained by binding of C1INH to LPS.

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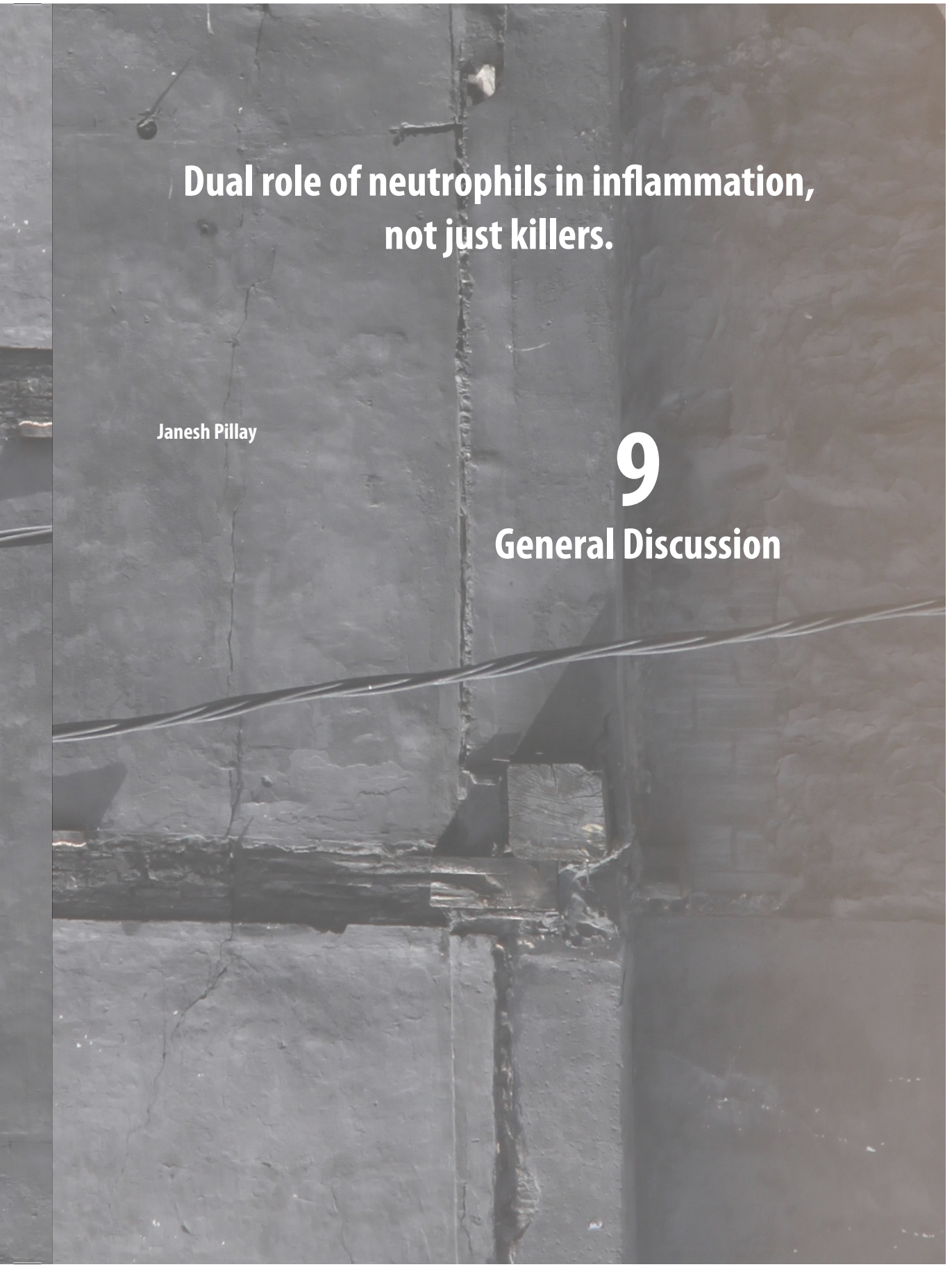

Dual role of neutrophils in inflammation, not just killers.

Janesh Pillay

9

General Discussion

Reappraisal of the circulating neutrophil lifespan

The short *in vivo* circulatory neutrophil half-life previously reported (8-12 hours) seems perfectly in line with their classical antimicrobial functions[1]. A short lifespan and tightly regulated apoptosis is thought to limit tissue damage, which can be caused by cytotoxicity due to hyperactivation of neutrophils.

The half-life of human neutrophils *in vivo* has previously been studied using different labeling techniques requiring *ex vivo* cell manipulation. Neutrophils were labeled *in vivo* by ^{3}H-thymidine, then isolated and re-infused into a recipient[1]. Others used *in vivo* and *ex vivo* ^{32}P-DFP or ^{3}H-DFP labeling, a procedure known to activate neutrophils and markedly affect the distribution of these cells[2-4] (figure 1). Also neutrophil priming and activation can greatly influence their homing characteristics. This will affect the localization of exit to tissues and possibly their speed of clearance from the circulation[5,6]. These labeling techniques have revealed a circulatory neutrophil half-life of 8-12 hours in humans, which has not been debated since. In chapter 3, we applied an *in vivo* labeling technique to label neutrophils in humans and mice in situ without affecting their functionality. Based on these data, we estimated a circulating half-life of murine neutrophils that is in agreement with the data previously reported in other murine *in vivo* labeling experiments[7]. Murine neutrophil half-life was measured by *in vivo* BrdU-labeling in previous studies. These studies did not require *ex vivo* manipulation of neutrophils. This shows that previous studies using *in vivo* labeling techniques yielded similar neutrophil half-lives to our *in vivo* labeling study (chapter 3). However, the corresponding human neutrophil half-life was estimated to be 3.4 days, which is 8-10 times longer than previously reported[1]. Several observations question the previously reported short half-life:

(1) *In vivo* labeling techniques in mice applying BrdU incorporation into dividing cells resulted in a neutrophil half-life similar to the situation found in humans (8-12 hours)[7]. Despite different labeling techniques it has been assumed for years that murine and human neutrophils have a similar lifespan, a feature not seen for other leukocytes. For example, Vrisekoop et al. showed that human naïve CD4+ and CD8+ T- cells had a 40-fold longer lifespan than their murine counterparts[8].

Interestingly, when murine neutrophils were labeled *ex vivo* and re-infused the circulating half-life dropped to 1.5 hours[6]. This shows that *ex vivo* manipulation of neutrophils has the potential of shortening the measured circulatory neutrophil lifespan. In humans it was observed that isolated neutrophils additionally primed with GM-CSF or PAF were retained in the lung. Eventually these radio labeled neutrophils exited the lung, however the fate of the neutrophils after leaving the lung was not reported[9].

(2) *In vivo* manipulation of the host can result in a shortening of circulating lifespan of neutrophils. In calves surgically joined for cross-circulation, labeled neutrophils that had disappeared from the circulation could be remobilized after administration of corticosteroids[10]. Surgery induced inflammation/stress might have caused rapid sequestration of neutrophils. Surprisingly, steroid induced mobilization of labeled

neutrophil persisted in the circulation for several days[10]. This suggests that subtle activation of neutrophils can lead to a rapid sequestration from the circulation into a pool of cells that can be rapidly remobilized. Unfortunately, little is known regarding this pool of cells. It is possible that this pool represents the poorly defined 'marginated pool'. (3) In a model of murine marrow ablation and reinfusion of labeled neutrophils it was shown that these neutrophils firstly home to tissues such as lung and spleen. Surprisingly the neutrophil half-life in these tissues was found to be 5-7 days[11].

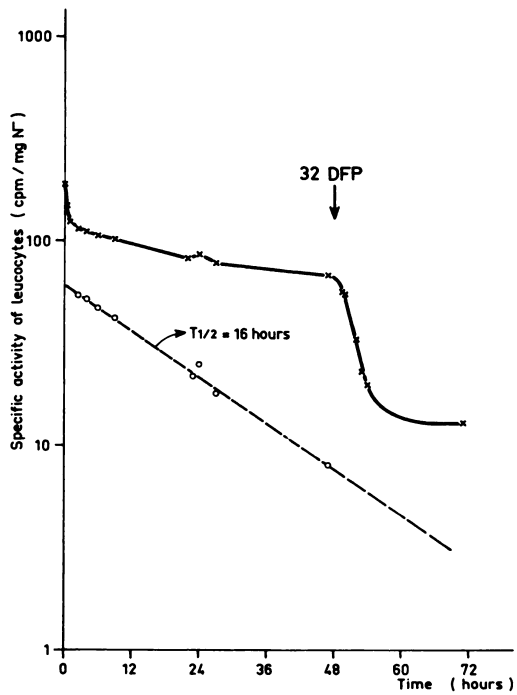

Figure 1. Influence of i.v ^{32}DFP on ^{51}Cr labeled neutrophils. Figure 9 reprinted by permission of the society of Nuclear Medicine from Dresch et al[4]. Neutrophils were labeled *ex vivo* with ^{51}Cr and reinjected into the homologous donor. Several hours thereafter ^{32}DFP was injected intravenously. Notice the sharp drop in ^{51}Cr-specific activity directly after injection of ^{32}DFP.

These observations show that *ex vivo* and *in vivo* activation of neutrophils can result in aberrant homing kinetics and can lead to rapid neutrophil sequestration into a rapidly mobilizable pool[6]. In addition, it shows that neutrophils that have exited the circulation under these conditions do not go into apoptosis, but remain associated with the tissues and can be readily remobilized and circulate for days[10]. This last observation shows that the intrinsic lifespan of neutrophils is in the order of days and supports our data that the circulatory lifespan of neutrophils is longer than previously appreciated.

The mathematical model that we used to calculate the circulating lifespan of neutrophils was based on a number of assumptions made in previous *ex vivo* labeling models. These assumptions apply to homeostatic conditions: 1. neutrophils are a kinetically homogeneous population, 2. neutrophils do not recirculate and 3. neutrophils are formed in the bone marrow (BM).
Important for the interpretation of our data is the issue raised by Li et al[12]. In a response to our study they argued that an alternative interpretation of our data is that the circulating half-life of neutrophils is much shorter than 3.5 days, and that the slow labeling kinetics observed in peripheral neutrophils is in fact a reflection of the slow kinetics of neutrophil precursors in the BM. Before discussing this explanation, a detailed discussion regarding the neutrophil life cycle is necessary.

Neutrophil lifecycle

Production

Neutrophils are produced in the BM by sequential divisions of neutrophil precursors (figure 2). The granulocyte lineage starts at the myeloblast, the common granulocyte precursor. During neutrophil production this myeloblast divides into pro-myelocytes. Promyelocytes divide into myelocytes, which divide into metamyelocytes[13]. Importantly no divisions have been detected from myelocyte to myelocyte in the cultures of BM aspirates[13]. All myelocyte and promyelocyte divisions could be described as maturing divisions. Metamyelocytes do not undergo cell division anymore but mature into banded and finally segmented neutrophils. This maturation normally occurs in the BM and these maturing cells are generally referred to as the post-mitotic pool.
In our study, label is incorporated into the cells of the mitotic pool, i.e myeloblasts, promyelocytes and myelocytes. We have corrected for the delay of neutrophils in the PMP in our model (chapter 3). However, slow down-labeling of the the mitotic pool after cessation of labeling could complicate the interpretation of our labeling curves, because the slow speed at with which label is lost among peripheral neutrophils could in fact reflect the slow speed at which cells in the mitotic pool are turning over. In other words, if the half-life of cells in the mitotic pool were to be as long as 3.5 days, the circulating neutrophil half-life could in fact be infinitely short[12]. Although the *in vivo* labeling kinetics of the mitotic pool are not known, there are a number of reasons why we think a half-life of 3.5 days of the mitotic pool is unlikely:

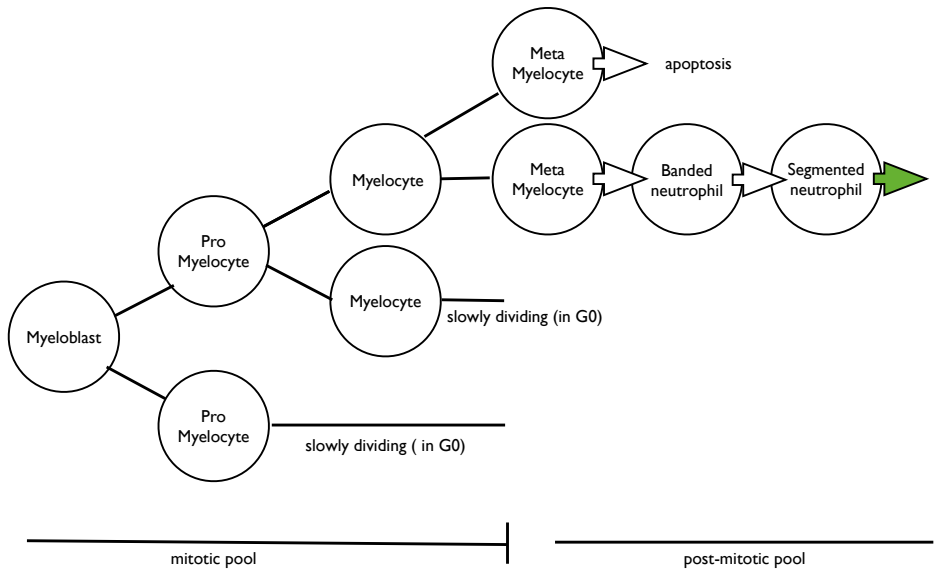

Figure 2. Neutrophil precursors and their divisions in the BM as adapted from Boll et al.[13], 55% of the cells entering the post-mitotic pool go into apoptosis[14]. Green arrow, exit to the circulation.

The myelocyte pool consists of two distinct pools. A rapidly dividing pool, which contributes to homeostatic granulopoiesis and a slowly dividing pool, which is reserved for emergency granulopoiesis. Mitotic indices (the number of cells in mitosis/total number of cells) of promyelocytes and myelocytes in healthy human BM are very low (1.4 % and 1.9 %, respectively)[13]. The slowly dividing pool influences these mitotic indexes, as only a small percentage of these cells are in mitosis. It has been reported that this slowly-dividing pool of myelocytes has a generation time of 70 days[13]. The generation time includes G0, G1, G2 and M phases. As the labeling duration in our study was 63 days, labeling of this pool of slowly dividing cells is expected to have occurred. The contribution of this slowly dividing pool to homeostatic granulopoeisis is minimal as they have an estimated G0 of 69 days[13]. The rapidly dividing myelocytes divide rapidly with a short G0 phase and a cell cycle time of 30 hours, and therefore have a rapid turnover rate. Therefore the slow dividing pool minimally contributes to the labeling of peripheral blood neutrophils in our experiments. This is confirmed by our mathematical modeling in chapter 3 in which no suggestions for multiple down-labeling slopes are found. Based on these results, in homeostatic conditions, the myelocyte pool with a rapid turnover is responsible for granulopoiesis and a delay of 3.5 days in this compartment is unlikely.
In vitro culture of pro-myelocytes and myelocytes revealed a cell doubling time of 63 hours

and 55 hours respectively (2-2.5 days). Simplified, this cell doubling time represents the half-life of the label in the mitotic pool. When the population doubles due to maturating divisions in the absence of label, the label enrichment of the total population halves. In the study by Boll et al. cell doubling time was calculated by dividing the observed mitotic duration by the observed mitotic index[13]. This observed mitotic index was also based on the slow dividing population of myelocytes, which are labeled to a lesser extent and have a much longer G0 time than the fast dividing pool. The actively dividing and labeled cells in the fast pool require multiple divisions to 'double' the entire myelocyte pool. It is therefore probable that the observed doubling time does not represent the half-life of label in the mitotic pool, but is biased towards the fast pool, whose half life in BM is in fact much shorter. This suggests that the half-life in the MP is much shorter than 2-2.5 days.

The calculations by Li et al., show that when the circulating half-life is 3.5 days the label enrichment in the marrow is higher than that of the blood neutrophils (figure 3). This might reflect the reported apoptosis of the post-mitotic pool. Mackey et al. show that in homeostatic conditions 14% of cells in the post mitotic pool are apoptotic[14]. They used data from Dancey et al. to calculate that 55% of myelocytes entering the PMP eventually go into apoptosis. In this study post-mitotic metamyelocytes and bands were assessed. This study is the only study assessing BM apoptosis. It cannot be excluded that the fraction of apoptotic neutrophils were circulating neutrophil returning to the BM[15]. Additional studies should be performed with special interest to the morphology of the apoptotic cells, to determine whether apoptosis of mature or immature neutrophils occurs. Our murine results show similar enrichment $^{2}H_{2}O$ between BM and blood. In mice apoptosis of the post mitotic pool is reported to be 10 times lower than in men[16]. Our measured murine BM compartment consists of post-mitotic neutrophils only, whereas the assumption made by Li et al.' for humans includes the entire BM (mitotic and post-mitotic pool). The curve in which the BM enrichment is 50-75% higher than the blood enrichment seems more logical when post-mitotic apoptosis is taken into consideration (figure 3).

The arguments above suggest that the mitotic pool has a shorter *in vivo* half-life than the 3.5 days required to reconcile our labeling data with a circulatory neutrophil half-life on the order of a few hours. However to definitively exclude the possibility that the mitotic pool is the kinetically important step influencing our measured half-life of 3.5 days, additional *in vivo* labeling studies will be performed.

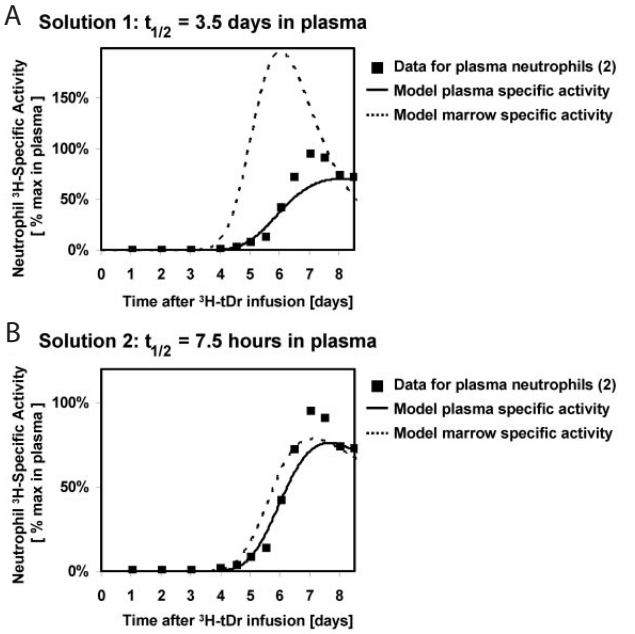

Figure 3. Distribution of labeled neutrophils between marrow and blood assuming blood half-lives of **(A)** 3.5 days and **(B)** 7.5 hours. In both cases, the model fits the specific activity in the plasma neutrophils to published specific activity measurements for thymidine-tagged neutrophils in circulation. Reprinted with permission from the American Society of Hematology from Li et al[12].

Estimation of neutrophil production

Another way to estimate neutrophil circulatory lifespan is to calculate neutrophil production. In homeostatic conditions neutrophil production and circulatory lifespan are balanced. In this section neutrophil production will be estimated independently of labeling data. The number of neutrophils produced per day will be used to estimate neutrophil circulatory lifespan.

Neutrophil production has been mathematically calculated using neutrophil peripheral turnover data, in other words the circulating neutrophil half-life. Based on ^{3}H-Thymidine labeling *in vivo* calculations have resulted in an estimated daily production of neutrophils of $0.85x10^9$/kg/day in healthy individuals[1]. Applying *in vivo* BM labeling their calculations regarding the number of neutrophils in the post-mitotic pool (PMP) matched strikingly with their data mentioned above: an estimated production of $0.87x10^9$/kg/day. Estimating the number of post-mitotic neutrophils in the BM poses several problems. Firstly, it has recently been reported that mature neutrophils are homeostatically removed from the circulation and cleared in the BM[17]. The exact number of cells per day that are removed by the BM is unknown. Secondly, it has been suggested that post-mitotic metamyelocytes and banded neutrophils undergo apoptosis in the BM. This results in only 55% of PM pool

cells entering the circulation as mature neutrophils[14]. Thirdly, BM is well vascularised and BM aspirates might result in a significant number of normal circulating neutrophils being mistaken as part of the PMP.
To circumvent the problems in assessing the size of the PMP, neutrophil production can be calculated based on the mitotic pool. Importantly these data are independent of *in vivo* or *ex vivo* labeling studies:

Firstly the percentage of dividing myelocytes per day must be calculated **(1)**.

Myelocytes are the last dividing cell in the neutrophil lineage (figure 2). Proliferation index (PI) comprises the percentage of cells in the S-, G1, G2- or M-phase.

The percentage of myelocytes in G2, S and M-phase can be derived from a study published by Matarraz et al.[18]. They showed that the PI of a population containing both myelocytes and the non-dividing metamyelocytes was 4%..
The contribution of myelocytes and metamyelocytes to all bone marrow cells is 23.5 and 14%, respectively [13]. The percentage of this combined pool is 23.5 + 14 = 37.5% of total bone marrow cells. The percentage of myelocytes in this combined pool is 23.5:37.5 x100%= 63%

Therefore, the percentage of myelocytes in G2, S and M –phase can be calculated by combining data by Matarraz et al. and Boll et al. from:

4%/0.63 = 6.4% of myelocytes in G2, S and M-phase

Myelocytes in G1-phase cannot be observed and need to be calculated. Boll et al. report that the cycle time of myelocytes is 30 hours, and that the S-,G2- an M-Phase is 18 hours. This results in a G1 time of 12 hours. The proliferation index including the percentage myelocytes in G1-phase can be estimated as 30/18 times the percentage of myelocytes in G2, S and M-phase.

Therefore the PI of myelocytes is (after correction for G1-phase)
6.4x 30/18= 11% .

The percentage of myelocytes dividing per day can be calculated based on the cell cycle time of 30 hours and equals 11 x 24/30 = **8.5% of myelocytes divide/day (1)**

Next the size of the myelocyte pool needs to be calculated **(2)**.

The size of the mitotic pool can be taken from Dancey et al., and comprises $2.11x10^9$/kg.

The percentage of myelocytes is 23.5% of total marrow cells [13]. The percentage of the mitotic pool is 39.5% of total marrow cells. This 39.5% is calculated by adding the percentage myelocytes (23.5), pro-myelocytes (12) and myelobasts (4) from Boll et al.

The ratio of myelocytes in the mitotic pool is 23.5 : 39.5.
Therefore, the percentage myelocytes is 59%

The absolute number of myelocytes can be calculated by using the estimate of the mitotic pool size from Dancey et al.

This results in $2.11x10^9$/kg x 0.59= **$1.13 x10^9$/kg myelocytes (2)**

The neutrophil production per day can be calculated using **(1) and (2)**, the absolute number of myelocytes/kg ($1.13 x10^9$) and the percentage of myelocytes dividing per day (8.5%)

Based on these data the number of myelocyte-divisions 1.13 x0.085 = $0.096 x10^9$/kg/day.

As every myelocyte divides into two metamyelocytes, which ultimately mature to two neutrophils, the estimated production is 0.096 x 2 = $0.192 x10^9$/kg/day

An average person, who weighs 70kg would produce $13.4x10^9$ cells per day entering the post-mitotic pool.
On average humans have $25x10^9$ circulating neutrophils (chapter 5). As the removed cells are replaced with the BM output of $13.4x10^9$ neutrophils/day circulating lifespan can be calculated.

$25x10^9$ / $13.4x10^9$ = 1.87 days

These calculations do not include a marginated neutrophil pool, which is thought to be of equal size. This would result in a total of 25 x 2 = 50 $x10^9$ total neutrophils (circulating and marginated), and a lifespan of 3.73 days.
As the existence and size of the marginated pool is unknown, the lifespan based on these calculations is 1.87-3.73 days.

These calculations are based on the assumption that no apoptosis occurs in the myelocyte stage or during the maturation in the post-mitotic pool. There are reports that suggest considerable apoptosis in the myelocyte stage and PMP[14,19]. Mackey et al. showed that up to 55% of maturing neutrophils became apoptotic[14]. More directly measured experimental

data on neutrophil BM apoptosis is needed. However we can say that in the presence of apoptosis the calculated lifespan is *somewhere between* 1.87 days (no margination/no apoptosis) – 8.29 days (50% of the cells in marginated pool and 55% apoptosis in PMP).

This is the same order of magnitude as the lifespan of 5.4 days described in chapter 3.

This rough estimate of neutrophil production is not based on labeling data. The subsequent calculated half-life is much larger than the previously reported 6-12 hours and is line with our *in vivo* labeling study. Importantly, this would also mean that the production of neutrophils in the bone marrow is much lower than previously anticipated.

(Re)Circulation/Margination

Circulating neutrophils exist in a free circulating state and in a state of margination (figure 4)[2]. Marginated neutrophils are neutrophils that interact with the vessel wall of the microvasculature. It has been shown that around 50% of neutrophils in mice display loose interactions with the capillary wall, termed 'rolling', in homeostatic conditions[20]. It is thought that free circulating and marginated neutrophils are not specific populations but that the two states are interchangeable and in equilibrium. The concept of margination is derived from ex –vivo labeling studies and reinfusion of labeled neutrophils. In these studies it was observed that after injection of ^{32}P-DFP labeled neutrophils, 50-56% of injected activity disappeared immediately[1,2]. This percentage was found to be dependent on labeling technique as when ^{3}H-Th was used only 43% of injected activity disappeared immediately[1]. Immediate disappearance of ~50% of label was explained by a hypothetical marginated pool in equilibrium with the circulating pool. The possibility remains however that the rapid removal of label was due to *ex vivo* manipulation and labeling of neutrophils, which results in neutrophil sequestration or retention.

The microvasculature of the lung is thought to be a major site of neutrophil sequestration due to the large capillary surface and the narrow microvasculature[5,21,22]. Neutrophil activation increases transit time through the lung[21]. Important for this increased transit time is neutrophil rigidity through actin polymerization by activation[21,23-25].

This sequestration in the lung also occurs in primed labeled neutrophils, which are reinjected in to human subjects. Neutrophils isolated in the presence of autologous plasma to prevent activation showed far less retention in the lung (<5%)[9].

Although the concept of homeostatic margination of neutrophils remains unproven, and might simply be due to cellular activation or priming in early studies, subsequent reports addressed the possible mobilization of this marginated pool.

It is possible to increase circulating neutrophils counts in humans and mice through stress, exercise and administration of noradrenaline and glucocorticoids[26,27]. This increases the mature neutrophil count in peripheral blood. It has been proposed that this increase in circulating mature neutrophils is due to de-margination. In the light of the recent

discovery of homing of senescent neutrophils to the BM, it would be interesting to study whether these cells are (re)mobilized by glucocorticoids.

Such remobilized neutrophils have been suggested to circula te for days after a single injection of corticosteroids[10]. In this study neutrophils were labeled *in vivo* in a calf, which was then surgically anastomosed with its' twin for cross circulation[10]. Labeled neutrophils rapidly disappeared with a half-life of 7-9 hours and could be remobilized with glucocorticoids. Two possible interpretations are interesting. (1) As described above, surgically induced immunological stress could result in rapid neutrophil sequestration and thus underestimation of neutrophil circulating lifespan. (2) If a marginated pool exists, this pool is not in equilibrium with the circulating pool. This would imply that neutrophils enter the marginated pool and reappear days later. Reappearance can be accelerated by administration of glucocorticoids. Studies, such as performed by Dancey et al. and Cartwright et al. applying pulse-labeling or the equivalent of reinjecting labeled cells, might not have sampled long enough for these cells to reappear and might have underestimated neutrophil circulatory half-life[1,2] In our labeling studies, labeling of up to 63 days would have labeled this hypothetical marginated pool completely, thus providing a more accurate circulating half-life.

It is possible that neutrophils released from the BM or activated neutrophils rapidly leave the circulating pool into a marginating pool or extravasate into the tissues, to gradually return to the circulation[6,11]. Examples of extravasated neutrophils returning to the circulation have recently been shown in studies addressing reverse migration, i.e. migration back to the bloodstream from the tissues. *In vitro* experiments showed that neutrophils were able to reverse migrate back over vascular endothelial cells. In humans, *in vivo*, under homeostatic conditions only 0.25% of circulating neutrophil displayed the phenotype of reverse transmigrated neutrophils (CD54 high, CXCR1 low)[28]. In inflammation induced by wounds in zebrafish or ischemia/reperfusion in mice a much larger percentage of neutrophils was seen to reverse migrate[29,30].

Clearance of circulating neutrophils

Several sites of clearance for neutrophils have been proposed, including BM, liver, spleen and loss to extracorporeal fluids (sputum, stool)[6,31,32]. Recently it has been shown that up to 30% of murine neutrophils are cleared in the BM in a CXCR4- SDF1α dependent manner[17]. In this model neutrophils were isolated and cultured overnight before reinfusion into a recipient mouse. This isolation and culturing results in neutrophil activation and, therefore, might not represent neutrophil homeostatic clearance. In addition a study has shown that murine neutrophils isolated from different sites show different clearance sites[6]. Isolated and labeled peripheral blood neutrophils were found in the spleen (20%), liver (30%) and BM (30%) 4 hours after infusion. Whereas neutrophils isolated from the BM were cleared in the spleen (10%), liver (15%) and BM (60%). Neutrophils isolated from thyoglycolate induced peritonitis were predominantly cleared in the liver (40%),

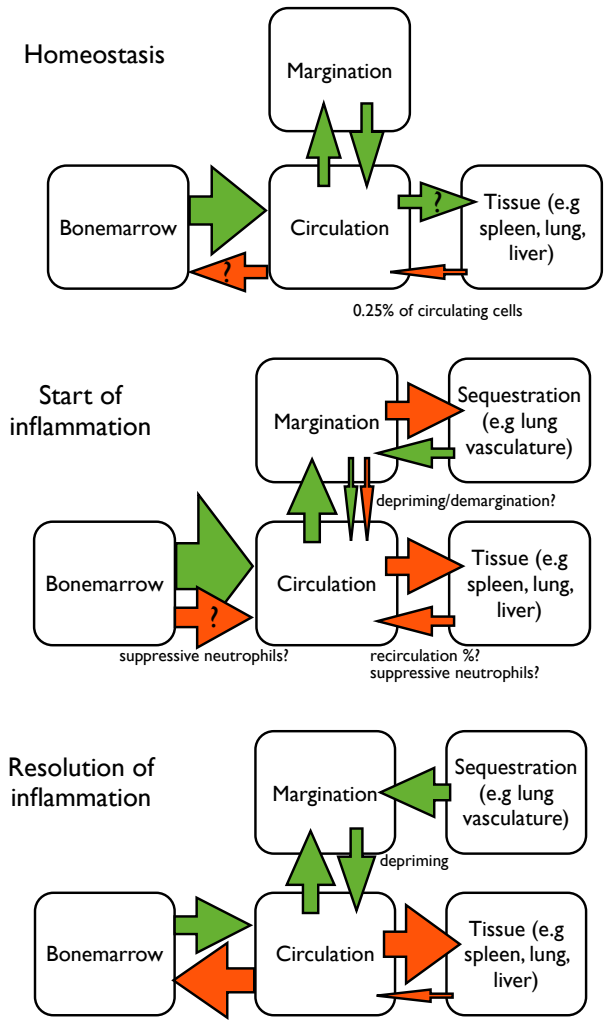

Figure 4. Hypothetical kinetics of neutrophil distribution in homeostasis, inflammation and resolution of inflammation. Green arrows depict non-activated neutrophils, orange arrows depict activated neutrophils. **In homeostasis** neutrophils are released from the BM into the circulating pool and a hypothetical marginated pool. A small number of cells are thought to recirculate from the tissues[28]. The site of removal of neutrophils from the circulation is the BM, spleen and liver[33]. **In inflammation** a large increase is seen in neutrophils released from the BM. In addition, neutrophils previously removed from the circulation may be mobilized from the BM. More neutrophils shift from the circulating to the marginating pool and sequester in the microvasculature. A larger percentage of neutrophils exiting the circulating pool to the tissues is suggested to recirculate back to the blood[28]. Both the previously senescent neutrophils remobilized from the BM and the recirculating neutrophils may comprise our observed suppressive neutrophils subset (chapter 6). **Resolution of inflammation** warrants the removal of large numbers of circulating neutrophils. Sequestrated neutrophils firstly enter the hypothetical marginated pool after being deprimed or deactivated[9,34]. Studies using the injection of labeled neutrophils best represent this phase of inflammation. Activated neutrophils are removed by the spleen and liver, and less activated neutrophils are removed by the BM[33].

with spleen en BM contributing 10 and 20% respectively. This neutrophil homing to BM was shown to be CXCR4 dependent, as administration of a blocking antibody limited infused neutrophil homing to the BM but not to liver and spleen[33]. In this study it was shown that neutrophil activation decreased sensitivity to SDF1α, which limited neutrophil homing to the BM. All of these studies were performed by injection of isolated neutrophils. Increased neutrophils activation i.e BM-derived vs periferal blood neutrophil vs peritoneal exudates suggest that minimally activated neutrophils neutrophils are cleared largely in the BM, whereas activated neutrophils are removed from the circulation by the liver and spleen[32,33].

The true homeostatic removal of neutrophils from the circulation has not been adequately addressed, although it is likely that the BM is a major site for senescent neutrophils. It would be interesting to study the dependency on CXCR4 of true homeostatic neutrophil clearance. A murine model implementing an *in vivo* labeling technique in combination with a timely infusion of a CXCR4 blocking antibody might provide some insight whether homeostatic neutrophil clearance is indeed CXCR4 dependent.

Functional neutrophil heterogeneity

Under homeostatic conditions neutrophils are generally considered to be a homogenous population of circulating cells. As briefly discussed in chapter 5 early studies that used Mab 31D8 (later identified as binding to FcγRIII (CD16)) identified a functional neutrophil heterogeneity related to cell maturity[35-37]. In inflammation these maturation related characteristics of neutrophil heterogeneity are much more pronounced as we have shown in chapter 5. Release of banded neutrophils is observed in all acute inflammatory conditions, such as induced by tissue injury and infections[38,39]. In chapter 5 these banded cells ($CD16^{dim}$) isolated by cell sorting from peripheral blood during experimental systemic human endotoxemia showed specific characteristics: decreased expression of several receptors (CD11b, CD45, CD32) and a decreased anti-microbial functionality *ex vivo* (e.g. phagocytosis of S. epidermis and production of reactive oxygen species) compared to $CD16^{bright}$ neutrophils. In both neutrophil populations the respiratory burst was primed (figure 7, chapter 5) three hours after LPS administration. This resulted in rapid priming of the respiratory burst in the entire circulating neutrophil pool. In contrast, the entire circulating neutrophil pool showed a decrease in interaction with s.epidermis three hours after LPS administration. This was mainly due to the release of $CD16^{dim}$ neutrophils. Antimicrobial function of the $CD16^{bright}$ population was not increased three hours after LPS and did therefore not compensate for the decreased function of the $CD16^{dim}$ neutrophils.

Banded $CD16^{dim}$ neutrophils showed increased chemotaxis towards fMLF and C5a (figure 5) compared to the $CD16^{bright}$ neutrophils. This indicates that although the capacity to bind bacteria may be decreased *in vitro*, increased chemotaxis towards microbes may compensate for this decreased binding of bacteria. Therefore, it would be interesting

to compare antimicrobial properties between homeostatic circulating neutrophils and banded neutrophils in an anti-microbial assay including chemotaxis and phagocytosis[40]. Apart from the release of CD16^{dim} banded neutrophils from the BM, other neutrophil populations in inflammatory conditions have been reported.

As discussed above, neutrophil 'reverse migration' back into the bloodstream after resolution of inflammation has been observed in several species (zebrafish and mice, and has been proposed in humans)[28-30]. This subset of neutrophils expressing high levels of CD54 (ICAM-1) en low levels of CXCR-1 is seen in human chronic inflammation (e.g such as rheumatoid arthritis) and in mice after ischemia-reperfusion[28,29]. However, unfortunately no reports have followed up on functionality of reverse migrated neutrophils.

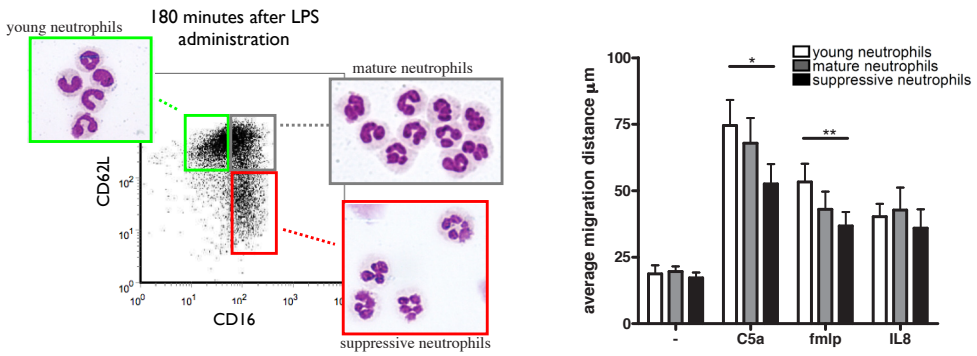

Figure 5. Chemotaxis of neutrophil subsets. Neutrophil subsets were sorted and put in a boyden chamber and allowed to migrate towards the chemoattractant gradient for 90min. Afterwards filters were stained and average migration distance was calculated, mean ± SEM n=6-7

A distinct neutrophil population, which displays different functional properties, has been observed in septic patients. In these patients it was shown that 15-20% of circulating neutrophils are characterized by adhesive functionality that can be blocked by blocking antibodies directed against α4- integrins[41]. Neutrophils from healthy subjects do not express α4-integrins. It was shown in that neutrophils from septic patients adhered to a VCAM-1 coated surface, but unfortunately this study did not show direct expression of the against α4- integrins on neutrophils by flow cytometry or western blot analysis. In our model of systemic inflammation we did not observe expression of α4- integrins on neutrophils. In contrast to our model, however, sepsis results in circulating neutrophil progenitors[42]. Therefore, the observed population of cells adhering in an a4-integrin-dependent manner may by due to circulating neutrophil progenitors.

The above-mentioned heterogeneity of neutrophils other than the banded cells and

progenitors has mainly focused on receptor expression and has thus far revealed only minor functional differences. In chapter 6 we show that in systemic inflammation a subset of neutrophils is found in the circulation displaying an additional non-antimicrobial functionality. These neutrophils are able to suppress T-cell responses *ex vivo* through cellular interactions requiring neutrophil Mac-1.

Origin of suppressive neutrophils

The appearance of these suppressive neutrophils in the circulation after trauma and LPS administration is surprising, as this neutrophil population has not previously been described. In many ways this population displays an activated phenotype: expression of high CD11b, and low CD62L, and a primed respiratory burst (chapter 6). However these neutrophils effectively suppress T-cell proliferation *in vitro*. This unique suppressive phenotype could not be induced *in vitro* by prolonged incubation of neutrophils from healthy controls with various cytokines. A possibility remains that in order to acquire the observed suppressive phenotype 'normal' neutrophil require multiple signals such as cytokines, 'danger'-signals (such as LPS) and interaction with vascular endothelium as will be present *in vivo*.

For example, suppressive neutrophils might originate from cells that have migrated through the vascular endothelium during experimental endotoxemia, but could not pass the basal membrane because of absence of proper homing signals. This might have lead to reverse migration and occurrence of suppressive neutrophils in the circulation. Indeed, the neutrophil subset described in chapter 6 showed similarities to the reverse migrated neutrophils: low expression of CXCR1 (CD181) and high expression of ICAM-1 (CD54)[28].

Interaction with the endothelium might occur in a more 'loose' way such as rolling. In the concept of margination, these neutrophils are in constant equilibrium with circulating neutrophils. Systemic inflammation may activate marginated neutrophils differently to circulating neutrophils resulting in a suppressive subset. This might be due to expression of chemokines bound to the vascular endothelial surface activating marginated neutrophils[43,44]. Another possibility is that engagement of neutrophil selectins and/or integrins during margination in combination with circulating cytokines play a role in differential activation resulting in a suppressive neutrophil phenotype[45].

Finally our suppressive subset might be a novel population of neutrophils residing in spleen or BM[17,46]. We observed nuclear hypersegmentation of the suppressive neutrophil subset, which might be a sign of neutrophil maturity. It is tempting to speculate that aged neutrophils, previously homeostatically removed from the circulation can be remobilized. This would fit data that noradrenaline and glucocorticoids can mobilize mature neutrophils, and that BM is an important site of neutrophil clearance (figure 4)[10,17,26,33].

In vivo neutrophil (pulse-) labeling studies combined with methods of neutrophil

mobilization (glucocorticoids, strenuous exercise or LPS administration), will provide an answer whether the suppressive neutrophil subset is an unique subset or is induced by activation of circulating neutrophils.

Neutrophil suppression *in vivo*

Do neutrophils suppress T-cell responses *in vivo*? In recent years it has become clear that neutrophils and T-cells can interact in various inflammatory environments. Neutrophil and T-cells infiltrate inflammatory foci during sterile and/or microbial inflammation. In addition, it has been shown that neutrophils can home to lymph nodes in mice in response to various stimuli. In mice infected with mycobacterium bovis, neutrophils phagocytose bacteria and migrate to the draining lymphnode in a CCR7 dependent fashion[47,48]. Phagocytosis of pathogens is not a requirement for migration to lymphodes as inflammation induced by intraperitoneal injection of LPS also caused neutrophils to migrate to lympnodes and the marginal zone of the spleen[49,50]. The function of neutrophils in the lymphodes remains to be established and likely depends on the stimulus or pathogen. For instance neutrophils induce a TH1 response in response to various pathogens such as candida albicans and legionella pneumophila, e.g. through production of IL-12[51,52]. In contrast, in mycobacterial infection they suppress immunity though production of IL10[53]. We and others have shown that neutrophils can inhibit responses to OVA *in vivo* (figure 6)[54]. The mechanism of their suppression is not completely understood and is still under investigation.

The relevance of this suppression is not entirely clear. It is a challenge to study this concept as neutrophils might participate in microbial clearance, tissue damage and suppression. Inhibiting general suppressive mechanisms such as IL-10 or depletion of neutrophils is likely to result in decreased microbial clearance, with an increased microbial load. This increases pathogen load leads to increased inflammation. Therefore, it is of great importance to characterize the different phenotypes of neutrophils in these different responses, before adequate experiments *in vivo* can be designed.

It is tempting to speculate why neutrophils should have the ability to suppress immune responses. In inflammation, cell death and necrosis can result in a release of auto-antigens. Auto-antigens and autoreactivity are a hallmark of many chronic inflammatory diseases, such as rheumatoid arthritis and wegeners granulomatosis[55-57]. In cancer it has been suggested that mammalian chitinase-like proteins function as auto-antigen resulting in antibody formation, deposition and subsequent persistent inflammation[58]. Neutrophils performing their anti-microbial functions might cause tissue damage, which can result in a release large amounts of autoantigens. The presence of suppressive neutrophils would limit T-cell responses to autoantigens released by neutrophil-induced tissue destruction. In addition, neutrophil elastase, an important antimicrobial protein, can act as an auto-antigen[55]. Therefore, in an inflammatory environment accompanied by release of autoantigens, neutrophils might play an important role in limiting T-cell

responses to autoantigens.

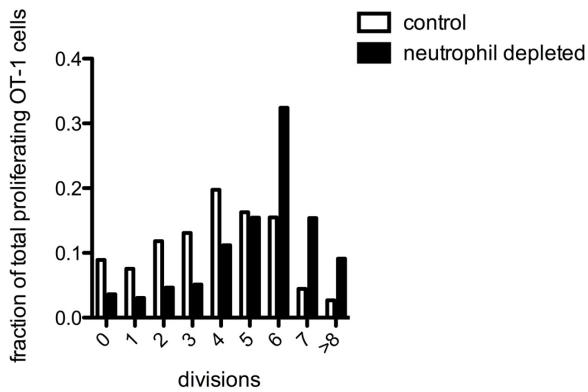

Figure 6. The role of neutrophils *in vivo* in a murine model of OVA induced T-cell proliferation. WT mice were injected with CFSE- labeled OT-1 (OVA specific) expressing splenocytes from a donor mouse. After 24 hours, neutrophils were depleted using an Ly6G specific antibody and an i.p injection of OVA was administered. 3 days later mesenterial and thoracic lympnodes were collected and T-cell proliferation was assessed by flow cytometry. Data is depicted as the number of proliferating (KI-67 positive), OVA- specific, CD3 positive cells. Data is depicted as mean of at least 5 mice. Representative example of 2 independent experiments.

Although neutrophil migration to lymph nodes has been reported, neutrophils may also play an important role in limiting T-cell proliferation in peripheral tissues. In conditions in which large numbers of T-cells and neutrophils are present, suppression of T-cell responses might become apparent. In influenza and respiratory syncytial virus (RSV) infection, the lung is firstly infiltrated by large numbers of neutrophils. Days later when neutrophil numbers subside, T-cell numbers increase[59]. Apart from proliferation in lymphnodes and migraction to the lung, influenza infection results in a large number of T-cells proliferating in the lung[60]. Another hallmark of influenza infection is a large bystander response of non-specific T-cells proliferating in response to cytokines such as type 1 interferons[61].

Bystander T-cell proliferation in the lung could contribute to excessive inflammation in the lung and influenza driven pathology. It is tempting to speculate that neutrophils control this bystander T-cell response in the early phase. Depletion of neutrophils has resulted more severe pathology in influenza infection[62]. The mechanism for this observation is still unknown. It is too simple to assume that this is entirely due to neutrophils suppressing T-cell responses. Although neutrophils do not directly clear influenza, they can contribute to control of influenza by inducing the production of type 1 interferons by dendritic cells[63]. Therefore depletion of neutrophils does not seem to be a useful tool in studying their role in limiting T-cell proliferation in influenza.

This demonstrates that the choice of murine disease models and the methods of studying neutrophil-mediated suppression should be made carefully. The dual role of neutrophils

inflammation is nicely illustrated by tumor-associated neutrophils. Neutrophils associated with murine lung tumors in mice facilitate tumor growth and limit intratumoral CD8+ T-cell responses. After blocking TGF-β signaling, neutrophils were more cytoxic towards the tumor and stimulated CD8+ T-cell activation[64].

Dual role of hydrogenperoxide (H_2O_2) in inflammation

In chapter 6 we have shown that neutrophils produce H_2O_2 in an immunological synapse and can inhibit T-cell proliferation. H_2O_2 can suppress lymphocyte proliferation through various mechanisms such as inducing apoptosis, oxidation of surface thiols and oxidation of cofilin[65,66]. This requires high concentrations of H_2O_2. Hydrogen peroxide is unstable and is rapidly converted to H_2O and O_2. Therefore, release into a synapse would potentiate and concentrate local concentrations of H_2O_2, and result a specific use of this oxidant in immune suppression. Apart from neutrophils, H_2O_2 can be produced by macrophages and dendritic cells during antigen presentation to T-cells and results in decreased lymphocyte activation[67,68].

Hydrogen peroxide has several reported functions besides immune suppression. In severe inflammation H_2O_2 may also cause tissue damage and vascular leakage[69]. This is demonstrated by improved outcome in various models of severe acute inflammation such as influenza, sepsis, and ischemia/reperfusion injury after administration of anti-oxidants such as catalase[70]. The antimicrobial role of H_2O_2 is not clarified, as it may simply be a byproduct of antimicrobial O_2^- (chapter 1). However, it has recently been shown that H_2O_2 is a potent inducer of chemotaxis in a model of tissue injury for neutrophil-like immune cells in zebrafish[71]. Hydrogen peroxide might therefore indirectly contribute to microbial clearance by attracting immune cells.

These diverse biological functions of H_2O_2 make it an attractive target to limit inflammation and immune suppression in acute inflammation. With the knowledge available this might lead to decreased tissue damage and decreased inflammation-induced immune suppression. Recently, however, it was shown that intracellular H_2O_2 acts as a signaling molecule limiting NF-κB transcription and thus inflammatory cytokine production. This resulted in increased LPS-induced lung injury when catalase coupled to PEG was administered[72]. It was later suggested that when neutrophil mitochondrial H_2O_2 production was increased, it resulted in decreased inflammatory cytokine production. This could be reversed by the addition of PEG-catalase[73]. This data is controversial as it is in contrast with the generally accepted view that oxidative stress increases NF-κB transcription[74]. A distinct difference is that previous studies focused on mainly extracellular administered H_2O_2, which increased NF-κB transcription. It would be interesting to compare the non-membrane permeable catalase to the membrane permeable PEG-catalase in LPS-induced lung injury or other inflammatory models. It is tempting to speculate that catalase administration could be beneficial in various inflammatory models, whereas PEG-catalase may have detrimental effects by increasing inflammation.

Limiting inflammation, limiting immune suppression?

Reducing acute inflammation is challenging as few effective pharmacological agents have been described. Inhibition of the immune system in conditions of trauma and sepsis has not been achieved sufficiently using glucocorticoids[75]. This may be attributed to the observations that glucocorticoids have mainly proven to inhibit the adaptive immune system, and may even activate innate immune cells[76,77]. The concentration of endogenous glucocorticoids, such as cortisol, is increased as part of the 'stress' response to injury and infection. Many other proteins are increased in inflammation, as part of the acute phase response or during resolution of inflammation. As briefly discussed in chapter 1, some of these proteins may be promising as anti-inflammatory drugs targeting the innate immune system[78,79]. Proteins of the coagulation system have been shown to have an anti-inflammatory effect apart from their role in coagulation. Activated Protein C (APC) has been shown to limit mortality in septic mice, independent of its role in limiting coagulation[80]. Some evidence is available that it can reduce mortality in humans, in a subgroup of septic patients[81,82]. The anti-inflammatory effects of APC were could be attributed to interactions with various innate immune cells: reduced integrin-dependent migration of neutrophils, and reduced dendritic cell and macrophage activation[83,84]. In chapter 7 and 8 we examine the anti-inflammatory role of two proteins, which are upregulated during inflammation, fibrinogen and C1-esterase inhibitor (C1INH). Fibrinogen is upregulated hours after the start of inflammation, and can reach a threefold higher concentration[85]. Normal plasma concentrations are 1.5-2.5 mg/ml, which is more than sufficient for coagulation. In chapter 7 we show that soluble fibrinogen in acute phase concentrations limits neutrophil adhesion to vascular endothelium. The damage to vascular endothelium by adhering neutrophils is in organ failure after trauma[86]. It is tempting to speculate that fibrinogen binds with a low affinity to neutrophil Mac-1 and endothelial ICAM-1, thus limiting interactions. In addition, by inhibiting neutrophil Mac-1 fibrinogen might also reduce adhesion to ligands deposited on inflammatory and damaged endothelial cells like RAGE and fibrin[87,88] (figure 7). In several trauma patients acute phase concentrations of soluble fibrinogen were measured up to 8mg/ml several days post-trauma (results not shown). It is tempting to speculate that massive transfusion and resuscitation necessary to compensate for blood loss, which not necessarily considers plasma fibrinogen concentration, will result in low fibrinogen levels directly post-trauma. This might result in an increased inflammatory response because of a longer time to reach adequate acute-phase concentrations. Studies correlating post-resuscitation fibrinogen levels to inflammatory complications should be performed. These studies might eventually result in fibrinogen-guided resuscitation of trauma patients.

In chapter 8 we describe the anti-inflammatory properties of C1INH. C1INH is present in a concentration of 250mg/l in the circulation and increases up to 2-fold in inflammation. C1INH inhibits activation of the classical and lectin pathway of the complement system. In additions it has been suggested that C1inh inhibited neutrophil adhesion to the

vascular endothelium and thus has anti-inflammatory properties besides complement inhibition[89]. In the human endotoxemia model we were able to inhibit pro-inflammatory cytokine release and increase IL-10 by administering C1INH 30 minutes after LPS infusion. The mechanism of this increased IL-10 production and the suppression of pro-inflammatory cytokines is still under investigation.

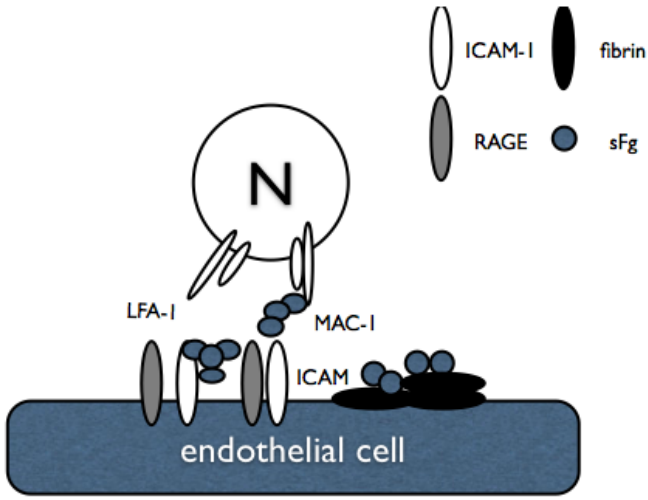

Figure 7. Model of neutrophil – endothelial adhesion inhibition by soluble fibrinogen. Soluble fibrinogen binds with low affinity to endothelial ICAM-1 and neutrophil Mac-1. Binding neutrophil Mac-1 may inhibit interaction with additional ligands on endothelial cells such as RAGE and fibrin.

Inflammation can lead to immune suppression through various mechanisms such as exhaustion of neutrophils, occurrence of regulatory T-lymphocytes, anergy of monocytes, exhaustion of neutrophils and probably lymphocyte suppression by neutrophils[90,91] (chapter 6). It would be interesting to observe whether limiting inflammation would limit immune suppression. A reduction in the inflammatory response, might lead to less compensatory immune suppressive mechanisms. This was not seen in chapter 8. Attenuating inflammation in the human endotoxemia model led to a decrease in pro-inflammatory cytokines, however increased the IL-10 concentration (chapter 8). In addition, we observed a similar number of suppressive $CD62L^{dim}$ neutrophils in the volunteers receiving C1INH and LPS. This could be due to the fact that C1INH was administered 30 minutes after LPS. This could have resulted in (1.) LPS directly leads to suppressive neutrophils (2.) Cytokine levels were reduced in the volunteers receiving C1INH, although remaining levels were sufficient to generate or release suppressive neutrophils. For instance TNFα was only marginally (3.) Clinical symptoms did not differ between the volunteers receiving C1INH and placebo, the occurrence of suppressive

neutrophils might be due to a stress response in combination with LPS. These hypotheses can be partially addressed by administering C1INH before LPS, as this might more effectively reduce cytokines and clinical symptoms.
To address the question whether limiting inflammation would limit immune suppression, it should be kept in mind that multiple compensatory mechanisms exist underlying immune suppression. In addition limiting inflammation itself might reduce the potential of microbial clearance. Immune suppression induced by sepsis illustrates this duality. Limiting lymphocyte apoptosis in sepsis by blocking PD-1 results in increased bacterial clearance and reduced murine mortality[92,93]. In contrast, blocking lymphocyte apoptosis, albeit through a different mechanism, in mice with cancer and sepsis increases mortality due to sepsis[94]. This example stresses the importance of immune monitoring before immune intervention in complex inflammatory states such as sepsis and severe inflammation. This monitoring might provide insight in to the suppressive or pro-inflammatory state of an individual. Trauma induces immune suppression, likely through compensatory mechanisms after inflammation induced by DAMPs. Limiting this immune suppression might be attempted without detrimental side effects seen in sepsis due to decreased microbial clearance. As the initial stimulus is not microbial, little danger exists for an increased pathogen load and detrimental outcome when limiting initial inflammation. More non-lethal trauma models should be combined with infectious models to study interventions limiting susceptibility to secondary infections.

In conclusion, the dual role of neutrophils in inflammation described in this thesis highlights the complexity of their role inflammatory diseases. It will prove challenging to dissect the exact role of immune suppression by neutrophils for different diseases. However due to their abundance, limiting their suppressive and damaging activity might prove invaluable in chronic and acute inflammatory diseases such as after trauma, in sepsis and in influenza. Finally, although seemingly paradoxical, suppressing inflammation might limit immune suppression.

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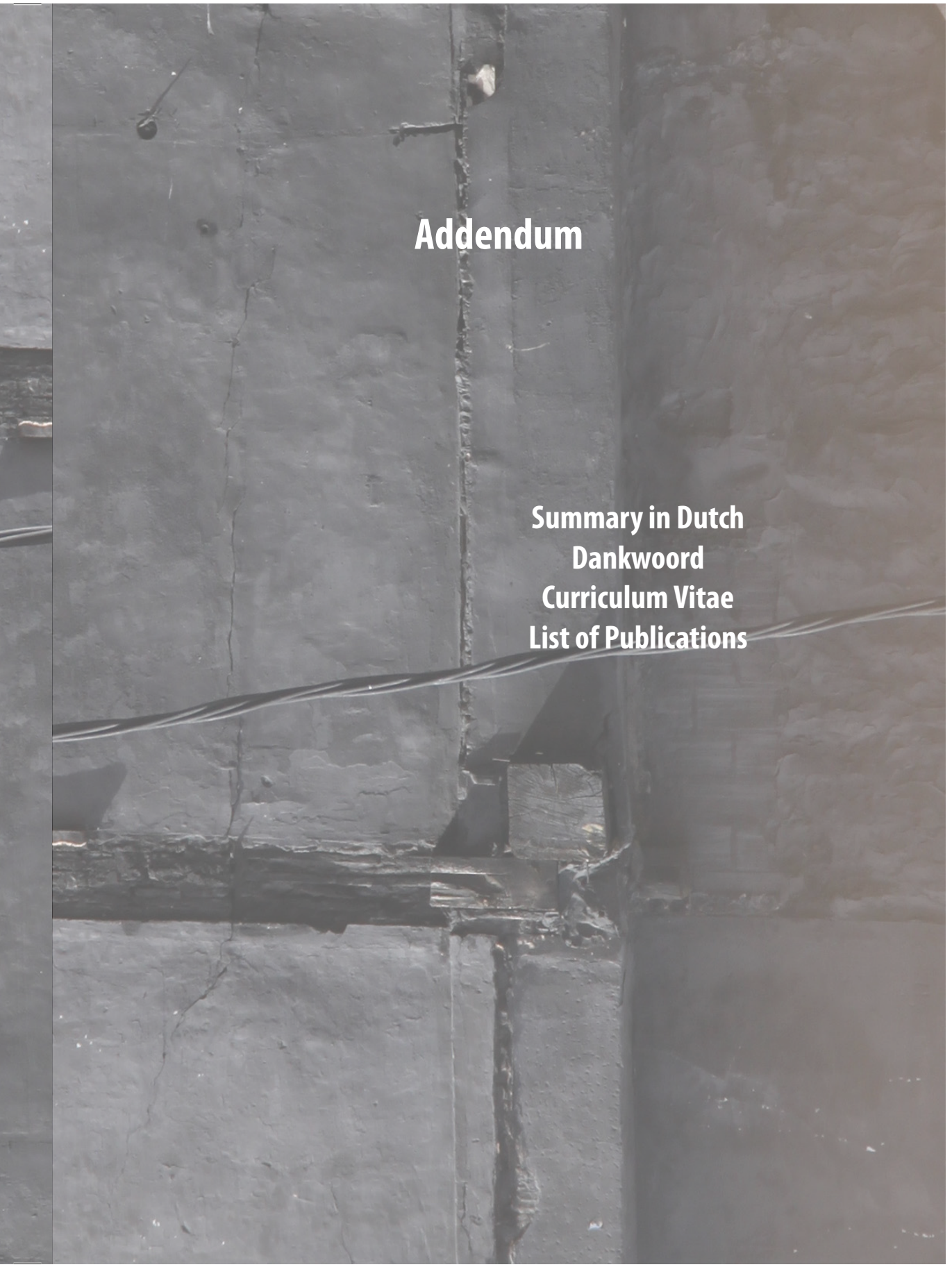

Addendum

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Nederlandse Samenvatting

Algehele onstekingsreactie (Inflammatie)

De natuurlijke reactie van het lichaam op weefselschade (door chirurgie, brandwonden of door verwondingen) en ernstige infecties is het tot stand brengen van een algehele onstekingsreactie. Dit gaat gepaard met activatie van het immuun systeem en het vrijkomen van onstekingsmediatoren (bijv. cytokinen) en onstekingscellen. Inflammatie bevordert de wondgenezing en verbetert de bescherming tegen bacteriën.

Een ernstige algehele onstekingsreactie kan ook leiden tot complicaties. Dit kan het gevolg zijn van een hyper-activatie van het immuun systeem waardoor weefselschade optreedt en organen beschadigd raken. In ernstige vormen leidt dit tot (multiple) orgaan falen.

Naast een hyper-activatie van het immuun systeem, kan een algehele onstekingsreactie leiden tot een verminderde afweer. Het immuun systeem probeert zich zelf via allerlei reguleringsmechanismen af te remmen en hyper-activatie te voorkomen. Wanneer deze reguleringsmechanismen te ver doorschieten kan dit dit het leiden tot een verminder afweer. Hierdoor worden patiënten gevoeliger voor infecties of kunnen ze bestaande infecties niet onder controle houden. Infecties kunnen weer leiden tot een hyperactatie van de algehele onstekingsreactie met als gevolg weefselschade en orgaan falen.

Neutrofiel

Een belangrijke cel voor de afweer tegen onder andere bacteriën is de neutrofiel. Bij een ernstige algehele onstekingsreactie neemt het aantal van deze cellen in de bloedbaan toe van 50% tot wel 90% van alle witte bloedcellen. Neutrofielen hebben een groot arsenaal aan schadelijke mechanismen en stoffen om het lichaam te beschermen tegen bacteriën en parasieten (Hoofdstuk 1). Het ongecontroleerd vrijkomen van deze stoffen kan ook weefselschade in het eigen lichaam veroorzaken. Foutieve of hyper-activatie van neutrofielen door onstekingsmediatoren kan leiden tot een productie van zuurstofradicalen en 'degranulatie' (het uitscheiden van cel-schadelijke stoffen).

Te gebruiken als voorspeller?

Neutrofielen reageren op hun omgeving (met activerende en remmende ontstekingstoffen) door receptoren op het oppervlak te vermeerderen, dan wel te verminderen. Hierdoor lijken ze de ideale kandidaat om gebruikt te worden voor het zogenaamde 'immuun monitoring'.

Complicaties bij trauma patiënten zijn lastig te voorspellen. De huidige scoringssystemen zijn tot op zekere hoogte toereikend voor het voorspellen van de mortaliteit, maar niet voor het voorspellen van complicaties zoals orgaanfalen of sepsis. Aan deze complicaties ligt het immuun systeem ten grondslag. In Hoofdstuk 2 zijn er mogelijkheden besproken

om o.a receptor expressie op neutrofielen te gebruiken om complicaties bij trauma patiënten te voorspellen. Uit literatuuronderzoek blijkt dat er veel tegenstrijdige gegevens zijn over de receptor expressie op neutrofielen na trauma. Dit kan verklaard worden door heterogene patiënten populaties en heterogeniteit tussen de studies in de tijd van bloedafname of medicijngebruik van de patiënten. Belangrijk is ook de dynamiek van deze cellen in het bloed direct na inflammatie; Met name het verdwijnen van geactiveerde cellen naar de weefsels en de influx van 'jonge' neutrofielen uit het beenmerg, kunnen de metingen van receptoren op hele populatie sterk beïnvloeden.
Om de dynamiek van de neutrofielen goed te bestuderen hebben we ervoor gekozen om te werken met een humaan model van inflammatie. Het toedienen van lipopolysachhariden (LPS) van de E.coli bacterie aan gezonde vrijwilligers resulteert in een kortdurende algehele ontstekingreactie. In Hoofdstuk 4 beschrijven we dat de reactie van neutrofielen op LPS in grote mate lijkt op de reactie van deze cellen bij een onstekingsreactie geïnduceerd door weefselschade. Derhalve is het een zeer waardevol gecontroleerd model om neutrofielen te bestuderen.

Levensduur van een neutrofiel
Neutrofielen werden verondersteld kort levende cellen te zijn met een levensduur in het bloed van slechts 8-12 uur. Deze studies waren gebaseerd op onderzoekstechnieken waarbij de cellen gemanipuleerd werden. Neutrofielen zijn hier erg gevoelig voor en geactiveerde cellen verdwijnen razendsnel uit de circulatie. Dit zou tot onderschatting van de werkelijke levensduur kunnen hebben geleid. In Hoofdstuk 3 laten we zien dat neutrofielen veel langer leven dat 8-12 uur, door een in vivo labelings-techniek te gebruiken. In mensen verblijven deze cellen tot wel 5.4 dagen in de bloedbaan. Dit heeft grote implicaties voor het toekomstig onderzoek naar deze cellen. Door hun nu langere levensduur zou manipulatie van neutrofielen voor therapeutische doeleinden meer effect hebben.

Meerdere functioneel verschillende neutrofiel populaties
Neutrofielen worden ook verondersteld een homogene populatie cellen te zijn, dit in tegenstelling tot bijna alle andere immuuncellen.
In Hoofdstuk 5 & 6 beschrijven we dat er meerdere neutrofiel populaties te zien zijn tijdens de ontstekingsreactie. Jonge neutrofielen uit het beenmerg zijn minder goed in het opruimen van bacteriën. Dit zou kunnen verklaren dat bij langdurig ontstekingsreacties met veel 'jonge' neutrofielen, patiënten gevoeliger zijn voor het krijgen van infecties. Zowel de jonge neutrofielen als de normale neutrofielen zijn nog steeds even potent in het uitscheiden van schadelijke stoffen. Dit zou dus kunnen verklaren dat patiënten met een langdurige onstekingsreactie gevoeliger zijn voor infecties, maar ook nog steeds het risico lopen op weefselschade en orgaanfalen door het eigen immuunsysteem.

Neutrofielen onderdrukken het immuunsysteem

In Hoofdstuk 6 beschrijven we dat er tijdens een onstekingsreactie naast jonge en normale neutrofielen, een derde soort neutrofielen is. Deze neutrofielen zien er ouder uit en lijken een actiever receptoren profiel op hun oppervlak te hebben. In tegenstelling tot de andere neutrofielen in het bloed, heeft deze populatie een bijzondere interactie met de rest van het immuunsysteem.

Deze neutrofielen remmen de deling van geactiveerde T-cellen. T-cellen zijn belangrijke immuun cellen van het verworven immuunsysteem. Deze cellen vermeerderen zich als ze in aanraking komen met een specifiek antigen. In Hoofdstuk 6 gebruiken we hiervoor tetanus toxoid. Een verminderde T-cel respons resulteert, in patiënten met een persisterende onstekingreactie, in opvlammingen krijgen van virussen (zoals het Herpes Simplex virus) welke het immuunsysteem normaal onder controle houdt. Daarnaast draagt een verminderde T-cel respons bij aan de gevoeligheid voor opportunistische infecties.

De subset van neutrofielen die T-cellen onderdrukken doen dit door direct cel contact te maken en waterstofperoxide op het membraan van de T-cel de deponeren. Voor het direct cel contact lijkt de integrine CD11b/CD18 (Mac-1) belangrijk te zijn. Dit biedt mogelijkheden voor therapeutische interventie om de immuun suppressie te voorkomen. Deze T-cel remmende neutrofielen zouden ook een belangrijke rol spelen in de afweer reactie tegen kanker. Neutrofielen in de buurt van tumoren blijken de T-cel reactie (de bedoeld is om de tumor op te ruimen) te remmen. Meer onderzoek naar deze bijzondere populatie neutrofielen levert wellicht interessante therapeutische mogelijkheden om infecties na een onstekingsreactie (trauma, operaties, sepsis) te verminderen en om de afweerreactie tegen kanker te verbeteren.

Remmen van de algehele onstekingsreactie

Concluderend induceert inflammatie neutrofielen die weefselschade kunnen veroorzaken, neutrofielen die minder goed zijn in het opruimen van bacteriën en neutrofielen het immuunsysteem kunnen onderdrukken. Het verminderen van inflammatie zou dus zowel weefselschade kunnen voorkomen als de afweerreactie op peil kunnen houden.

In Hoofdstuk 7 & 8 beschrijven we 2 eiwitten die gebruikt zouden kunnen worden om inflammatie te remmen. In Hoofdstuk 7 laten we zien dat het stollingseiwit fibrinogeen de hechting van neutrofielen aan endotheelcellen remt. Deze hechting is nodig zodat neutrofielen om de bloedbaan kunnen verlaten en weefselschade kunnen veroorzaken. Het is noemenswaardig dat dit vooral te zien was bij hoge concentraties fibrinogeen (4-8 mg/ml). Deze concentraties worden ook gezien tijdens ontstekingsreacties. De normale concentratie fibrinogeen is 2mg/ml. Dit is ruimschoots genoeg is voor de functie binnen het stollingsysteem.

In hoofdstuk 8 geven we C1-esterase inhibitor (CINH) aan gezonde vrijwilligers die

daarna LPS toegediend krijgen. C1INH resulteert in een stijging van een remmende onstekingsmediator (IL-10) en dalende activerende cytokinen. Doordat C1INH veilig is voor menselijke toediening kan toekomstig onderzoek snel antwoord geven op de vraag of het remmen van inflammatie ook leidt tot minder complicaties.

Dankwoord

Allereerst wil ik graag iedereen bedanken die bij dit gedeelte van het proefschrift is aangekomen. Ik hoop dat jullie ook de voorgaande 184 bladzijden interessant vonden om te lezen.....

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Dank je voor de vrijheid die je met hebt gegeven zowel binnen als buiten het onderzoek.

Loek, trauma is inflammatie en ook nog een beetje chirurgie. Ik wil je bedanken voor je enthousiasme de afgelopen jaren en de mogelijkheden die je me gaf. Ik ben blij dat het je niet verbaasde dat ik toch geen chirurg wilde worden. Desondanks ik hoop de komende jaren veel van je te kunnen leren, en met je te kunnen samenwerken.

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De Intensive Care research groep in Nijmegen onder bezielende leiding van Peter Pickkers. Dank jullie voor alles; de gezelligheid (in Nijmegen, Keulen en Munchen), de Bossche Bol op -80°C, en het mooiste humane inflammatie model op aarde.

Charlie, koffie?

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Darsin and Zoran, my big little brothers.

Puck, het mooiste meisje ter wereld, omdat je er was en omdat je er bent....

Curriculum Vitae

Janesh Pillay was born on the 23 of november 1980 in Rotterdam, The Netherlands. After completing his secondary education at Erasmiaans Gymnasium, he entered Medical School at the University of Utrecht in 1999.

As a part of his studies enrolled in several research electives at the departments of Trauma (Prof. dr. L.P. Leenen) and the departments of Respiratory Medicine (Prof dr. L. Koenderman) and studied the inflammatory response following trauma. Special focus was on neutrophil responses to acute inflammation.

After graduation from medical school, the author was granted the opportunity to attain his PhD. The project concerned the neutrophil responses in severe acute inflammation and the role of various neutrophil populations. The results of which are described in this thesis.

From july 2010 to july 2011 the author worked as a resident in the department of Surgery at the Meander Medical Center in Amersfoort (dr. A.J. van Overbeeke). As of 2012 he is pleased to start his training in Anesthesiology at the University Medical Center Utrecht under supervision of Prof dr. J.T. Knape.

List of Publications

Bastian O, Pillay J, Alblas J, Leenen L, Koenderman L, Blokhuis T. Systemic inflammation and fracture healing. J Leukoc Biol. 2011 May;89(5):669-73. Epub 2011 Jan 4. Review.

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Stellingen

Science thrives on a certain amount of chaos

An organized desk is a sign of an organized mind

Neutrophils can suppress inflammatory responses
(This thesis)

It's no measure of good health to be well adjusted to a profoundly sick society
(Jiddu Krishnamurti)

Limiting inflammation will limit immune suppression
(This thesis)

Humata, Hukhta, Huveshta, " to think good, to speak good, to act good"
(Avesta)

Neutrophil circulating lifespan is 5.4 days
(This thesis)

Wetenschap is ook maar een mening
(Henk & Ingrid)