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Sanne Mol

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Neutrophil activation in disease: An unexpected journey

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. P.P.C.C. Verbeek
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
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A-a-aye, I'm on vacation
Every single day 'cause I love my occupation
A-a-aye, I'm on vacation
If you don't like your life, then you should go and change it

Vacation, the dirty heads

CHAPTER 1

General Introduction

The immune system is a complex network that is tasked with protecting the human body against invading pathogens and malignant diseases. It maintains a delicate balance between eliminating harmful substances and malignant own cells while tolerating harmless foreign substances and healthy cells. The immune system is classically divided into the innate and adaptive immune system. Neutrophils are a type of white blood cells that are a vital component of the innate immune response and are the first line of defense against invading pathogens[1]. In addition to their role in infection control, neutrophils also play a key role in regulating inflammation and influencing adaptive immune responses[2]. Neutrophils are present in high numbers in the synovial fluid (SF) and synovial tissue of patients with chronic inflammatory joint diseases, such as rheumatoid arthritis (RA) and spondyloarthritis (SpA)[3]. In addition, the over-activation of specific effector T helper cell subsets is a characteristic of these diseases and in recent years it has been demonstrated that neutrophils play a role in the process of T helper cell subset polarization[4,5]. Thus, activated neutrophils are considered crucial players in the pathogenesis of chronic inflammatory joint diseases. Given the critical importance of neutrophils in the immune system, understanding their mechanisms of action and the factors that regulate their function is essential for developing new strategies to prevent and treat inflammatory joint diseases. However, how neutrophils play a role in shaping adaptive immunity is poorly understood. This thesis aims to enhance our understanding of neutrophils and how they shape adaptive immunity in chronic inflammatory joint diseases. Specifically by addressing the following questions:

1. How do different stimuli affect neutrophil activation and neutrophil-derived extracellular vesicles (EVs)?
2. What components present in SF from RA and SpA patients are associated with neutrophil activation and neutrophil-derived EVs?
3. How is DC function altered upon encountering neutrophil-derived EVs?

Unexpectedly, in the middle of conducting research for this thesis the coronavirus disease 2019 (COVID-19) pandemic started. The pandemic had a major influence on our research since contact with the clinic was limited for over a year and thus limiting our access to patient material. As it was demonstrated that neutrophils also contribute to the pathogenesis of COVID-19[6,7], we broadened the scope of this thesis by answering the following question:

4. How do immunoregulatory drugs, that are used to treat COVID-19 patients, influence neutrophil activation?

Although the diseases that stood central in this thesis are very different, inflammatory joint diseases are autoimmune diseases, while COVID-19 is a viral disease, in the pathology of both diseases, neutrophils play a critical role. In this chapter, the subjects that are central to this thesis are introduced.

The Immune System

The main task of the immune system is to defend the body against any kind of infection or cancer cells. The body is constantly exposed to harmful pathogens, such as bacteria, viruses, fungi, and parasites. Our immune system attacks and eliminates these harmful pathogens. At the same time, our immune system must tolerate unharmful foreign substances and thus maintain a complex balance between tolerating and attacking. To make matters even more complex, the immune system is also tasked with eliminating malignant cells, without causing harm to healthy cells. These tasks are performed by both the innate immune system and the adaptive immune system. In general, the innate immune system provides a rapid response to invading pathogens. It acts as the first line of defense and its response is largely non-specific. It consists of various types of cells, including neutrophils, monocytes, macrophages, dendritic cells (DCs), basophils, eosinophils, and innate lymphoid cells[8]. Some of these cells, and especially DCs, serve as a bridge between the innate and the adaptive immune system and are important communicators between the two systems[9,10]. The adaptive immune system is also referred to as the specific immune system. Its response is slower than that of the innate immune system but has a specific response to each pathogen. Furthermore, it is capable of generating a memory response and when a pathogen is encountered a second time, it can quickly reactivate its specific response against that pathogen, allowing for faster clearance. B cells and T cells are crucial components of the adaptive immune system[11]. Although the immune system is classified into two main components they do not act alone, as they need each other to optimally perform their tasks. When the innate immune system is triggered, cells from the innate immune system communicate with cells from the adaptive immune system to activate them.

Neutrophils

Neutrophils are part of the innate immune system and are the most abundant leukocyte. Each day around 10^{11} neutrophils are produced by the bone marrow. Neutrophils represent 40-60% of the immune cells in our bloodstream[12,13].

Neutrophils are generally considered to be cells with a short life span, with a maximum of approximately 24 hours[14]. Although, around a decade ago evidence was published that neutrophils possibly have a longer life span of around 5 days[15]. Upon activation neutrophils are equipped to kill pathogens, and they have developed an impressive repertoire for this. These strategies are described below and summarized in **figure 1**.

The first weapon in their arsenal is degranulation. Neutrophils contain four different types of granules and upon activation, neutrophils can release the content of these granules in a sequential fashion. The four granules are primary granules, secondary granules, tertiary granules, and secretory vesicles. These granules differ in content, and one explanation for this is that some proteins cannot exist together and will degrade one other[16,17]. Each type of granules contains multiple substances that help eliminate pathogens. Some of their contents overlap, but each has its prominent substances. Primary granules, also known as azurophilic granules, contain many toxic mediators, including neutrophil elastase (NE) and myeloperoxidase (MPO). Secondary granules, also known as specific granules, contain lactoferrin. Tertiary granules, also known as gelatinase granules contain gelatinase B. Secretory vesicles contain serum albumin[18,19].

The second mechanism neutrophils use for killing pathogens is phagocytosis, in which neutrophils can ingest and thereafter eliminate pathogens within the neutrophil. Besides the clearance of pathogens, it is also an effective mechanism to clear dead cells and tissue debris[20].

The third mechanism is the formation of neutrophil extracellular traps (NETs). In this intriguing mechanism, also known as NETosis, neutrophils release their nuclear content together with granular content to form a 'net' of chromatin, consisting of citrullinated histones, and antimicrobial molecules, such as MPO and NE[21,22]. In these NETs, pathogens are trapped and killed. This process is sometimes described as an ultimate kamikaze action since it should be impossible for neutrophils to survive the release of their nuclear content. However, recent studies show that neutrophils that have undergone NETosis; this process is termed vital NETosis[23,24].

Another mechanism neutrophils use to kill pathogens is by the production and release of reactive oxygen species (ROS). ROS can kill pathogens by damaging their nucleic acids, proteins, and cell membranes. ROS can be released both extracellularly into the environment where pathogens are present, and intracellular into the phagosome

where it can help the killing of phagocytosed pathogens[25,26]. Furthermore, ROS production by neutrophils influences the overall antimicrobial response of neutrophils since it induces NET formation[27].

Also, neutrophils can release mediators, that exert various effects. The release of mediators is not only useful for eliminating pathogens but is also essential for communicating with other cell types, including those of the adaptive immune system. Next to the release of mediators for cell communication, neutrophils can release EVs that is another mechanism for cellular communication. Neutrophil-derived EVs were an important research topic in this thesis and we will introduce them further in this introduction.

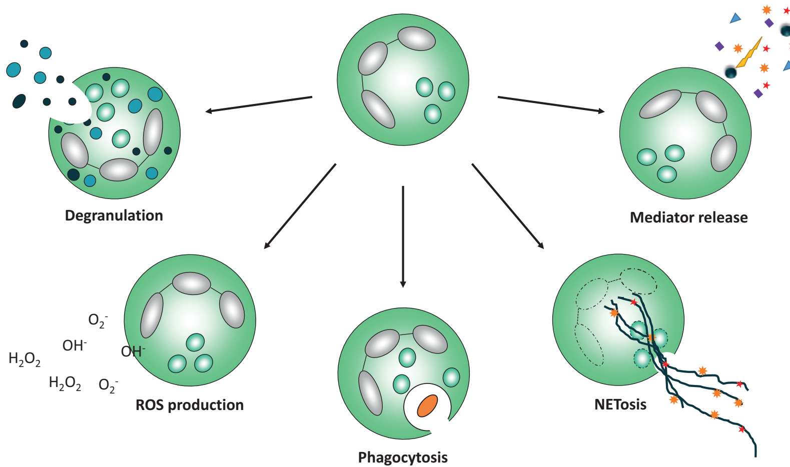

Figure 1: Schematic overview of neutrophil functions. Neutrophils can employ different strategies for pathogen elimination upon encountering a pathogen or other activating stimuli.

Neutrophils and the adaptive immune system

For a long time, neutrophils were considered to be truly innate immune cells, whose primary role was solely killing pathogens. Their primary task is indeed eliminating pathogens, but neutrophils also influence other immune cells and thus can modulate cells from both the innate immune system and the adaptive immune system. Neutrophils release cytokines that promote the migration of various immune cells to the site of infection. Neutrophils can also modulate T-cell responses. They can do this

directly by activation of naïve CD8+ T cells via MHC class I antigen presentation[28]. But they also do this indirectly by influencing DC-driven T-cell responses. For example, NE present in neutrophil granules promotes the polarization of naïve T-cells into Th17 cells[4]. Furthermore, NETs can reduce the activation threshold of T-cell responses[29]. NETs also influence other immune cells including activating DC subsets[30] and activating memory B cells via TLR9 stimulation[31]. Furthermore, neutrophils have been reported to directly modulate B-cell activation and differentiation via secretion of the cytokine BAFF[32]. Although we know that neutrophils play a role in modulating different adaptive immune responses, the extent of their role in this process is yet unknown. Further research needs to be performed to elucidate the exact role of neutrophils in controlling adaptive immune responses. This potentially is important in autoimmune diseases, such as inflammatory joint diseases.

Neutrophils and inflammatory joint diseases

Chronic inflammatory joint diseases are a group of rheumatic diseases that mainly affects the joints. It is estimated that the prevalence of these diseases is between 5-20% depending on the subtypes included[33]. These diseases are characterized by chronic inflammation of the joints and this can lead to progressive joint damage and disability which causes pain and loss of function in patients suffering from these diseases. Furthermore, chronic inflammatory joint diseases provide a major health and economic burden, since patients with these conditions require long-term pharmacological interventions and frequent hospital care. Different subtypes of chronic inflammatory joint diseases are associated with different pathology. There are many subtypes of inflammatory joint disease, but two common ones that are also within the scope of this thesis are rheumatoid arthritis (RA) and spondyloarthritis (SPA). RA is characterized by persistent inflammation of the peripheral joints, often resulting in the destruction of cartilage and bone [34]. SpA is characterized by inflammation in the spine and the peripheral joints resulting in bone remodeling (i.e. both destruction and pathological new bone formation). Furthermore, SpA patients can suffer from inflammation of the gut (inflammatory bowel disease), skin (psoriasis), and eyes (uveitis)[35,36]. Currently, a lot is still unknown about the underlying pathophysiology of RA and SpA, and current treatment strategies are not always effective. Neutrophils are abundantly present in the inflamed joints of patients with RA and SpA. Furthermore, inflammation-induced tissue damage

is a problem in both of these diseases. Although neutrophils are very potent in eliminating pathogens and modulating the functions of other immune cells, many of these neutrophil mechanisms and the compounds they release are very cytotoxic. When these mechanisms are uncontrolled they can cause harm to healthy cells and surrounding tissue. This possibly occurs in RA and SpA as well. In both RA and SpA, the severity of the disease directly correlates to an increased neutrophil-to-lymphocyte ratio in blood[37]. Thus, it is clear that neutrophils are involved in the pathogenesis of inflammatory joint diseases. To what extent exactly, is a question that remains unanswered up until now.

Neutrophils and COVID-19

COVID-19 occurred very unexpectedly at the end of 2019 and turned into a pandemic in 2020. This made continuing research into neutrophils and inflammatory joint diseases difficult since no contact with the clinic was allowed. At the same time, the situation provided a very interesting opportunity for every immunologist to conduct relevant research amidst a global pandemic. Especially because neutrophils seemed to be involved in the pathogenesis of COVID-19. Moreover, while the majority of COVID-19 patients have mild or no symptoms, the disease can be very severe for many patients. The disease is caused by severe acute respiratory syndrome-associated coronavirus-2 (SARS-CoV-2), a single-stranded RNA virus[38]. Most patients suffer from symptoms in the upper respiratory tract, including cough and shortness of breath. Patients with severe COVID-19 infection can suffer from respiratory failure, septic shock, and organ failure[39]. The mechanisms involved in severe COVID-19 infection are poorly understood. However, it is known that during severe COVID-19 infection there is excessive infiltration of immune cells in the lungs, overproduction of pro-inflammatory cytokines, and prominent changes in blood coagulation[40]. Also, there is strong evidence that neutrophils play an important role in the pathophysiology of COVID-19. The disease is associated with an elevated neutrophil-to-lymphocyte ratio, neutrophils are in an activated state, and neutrophil-related cytokines, including, IL-1β, IL-6, and CXCL-8 are elevated during severe infection[6,41,42]. In addition, NETosis is elevated in COVID-19 patients and SARS-CoV-2 can directly induce NETosis in neutrophils[43,44]. Therefore, more knowledge of the role of neutrophils and neutrophil activation could be beneficial for finding novel treatments for (severe) COVID-19 infections.

Extracellular Vesicles

Cell communication is essential in all processes in the human body. Cell communication can occur via direct cell-cell contact, via the release of various soluble mediators, and via the release of EVs. EVs are small lipid-bilayer enclosed particles, ranging from 30 nm to 1 μm in size[45]. This lipid bilayer protects nucleic acids and proteins from the extracellular space, where they will be degraded. Therefore the layer allows a stable exchange of proteins and nucleic acids[46]. Also, EVs can carry lipids or membrane proteins to other cells within their lipid bilayer. Furthermore, EVs can communicate complex signals between cells, as they can deliver multiple different components into one vesicle[47]. The biogenesis of EVs can occur in two ways. EVs can be produced as intraluminal vesicles within endosomes of the parent cell and thereafter released, these EVs are called exosomes. Alternatively, EVs can be released from budding from the parent cell membrane, these EVs are called microvesicles[48]. However, it is impossible to determine the method of biogenesis for the different vesicles. Furthermore, EVs are termed based on the field of study, such as ectosomes[49] or oncosomes[50]. Due to a lack of discriminatory markers, all these vesicles are collectively termed EVs to avoid confusion[51].

Extracellular vesicles and neutrophils

Just like any other cell neutrophils can release EVs. Many studies have investigated neutrophil-derived EVs and their effects on other cell functions. However, their findings are diverse and contradictory[52]. Besides these shortcomings in neutrophil-derived EV studies, there is consensus on some facts. First of all, the size of neutrophil-derived EVs seems to be between 100 nm and 700 nm[52,53]. Secondly, neutrophil-derived EVs express amongst others CD66b and MPO on their surface[52], markers that are common for activated neutrophils. The number of EVs released by neutrophils differs greatly in various studies and might depend on the activation status of the neutrophil they are derived from[52]. Neutrophil-derived EVs can affect other cell functions. Studies have shown that they can affect the function of other cells including, neutrophils, macrophages, NK-cells, and T-cells[54–57]. However, much of their effect on cell function is still unclear and more research is therefore necessary.

Extracellular vesicles and inflammatory joint diseases

EVs are present in the synovial fluid (SF) of joints and an elevated concentration of EVs is present in the SF of patients with inflammatory joint diseases[58]. EVs in SF from inflamed

joints originate from different cell types, such as neutrophils, monocytes, synovial fibroblasts, erythrocytes, and T-cells[59–62]. Next to EVs derived directly from cells present in the synovial environment, EVs in SF can also derive from blood plasma and infiltrate the joint[63]. Studying EVs in SF is challenging because SF is very viscous. This makes the isolation of EVs by differential ultracentrifugation difficult since the viscosity of SF limits the sedimentation of EVs[63,64]. Although the exact role of EVs in inflamed joint diseases is unclear, there is evidence that EVs are involved in the pathogenesis of various inflammatory joint diseases and they have been reported to induce both pro-inflammatory and anti-inflammatory responses in inflamed joints[65,66]. Additional research is needed to study and determine the exact function of EVs in inflammatory joint diseases.

Thesis outline

The original aim of this research project was to analyze cellular communication via EVs between neutrophils and DCs, focused on the activation and polarization of T cells in the context of spondyloarthritis. Due to the COVID-19 pandemic the research project's aim was broadened to analyze the role of neutrophils in the context of COVID-19 as well.

In **chapter 2** we provided a review describing the role of extracellular vesicles derived from innate immune cells in the orchestration of adaptive immune responses.

In **chapter 3** we studied how neutrophils are activated. We challenge the dogma that neutrophils first need to be primed by a priming stimulus whereafter neutrophils can be activated by an activated stimulus. We show that efficient neutrophil activation requires two simultaneous activating stimuli and that this is independent of the combination of stimuli used.

In **chapter 4** we studied the effect of dexamethasone and other immunomodulatory drugs, which were vitamin D3 and retinoic acid on neutrophil activation in the context of COVID-19. Dexamethasone is a drug that is often used in the treatment of critically ill COVID-19 patients. We found that dexamethasone dampens the inflammatory neutrophil response. Vitamin D3 did not affect neutrophil function, but retinoic acid increased neutrophil function.

In **chapter 5** we analyzed the synovial fluid (SF) of SpA and RA patients and discovered that neutrophils were present in an inactivated state. We show that the

SF of these patients inhibits activation of blood-derived neutrophils from healthy donors. Subsequently, we identified hyaluronic acid in the SF as the main factor responsible for this inhibitory effect on neutrophils.

In **chapter 6** we studied the effect of EVs derived from resting and activated neutrophils on DC function. We show that EVs derived from resting and activated neutrophils do not affect DC maturation and DC-driven Th17 cell development. EVs derived from resting neutrophils induce IL-8 production by DCs and therefore have an indirect pro-inflammatory effect. Contrastingly, EVs derived from activated neutrophils have an anti-inflammatory effect on DC cytokine production.

In **chapter 7** we summarize our findings and put those in perspective to current literature.

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'I never thought to look in here!' she whispered excitedly. 'I got this out of the library weeks ago for a bit of light reading.'
'Light?'

Hermione and Ron in Harry Potter and the Philosopher's Stone.

CHAPTER 2

The role of extracellular vesicles when innate meets adaptive

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Abstract

Innate immune cells are recognized for their rapid and critical contribution to the body's first line of defense against invading pathogens and harmful agents. These actions can be further amplified by specific adaptive immune responses adapted to the activating stimulus. Recently, the awareness has grown that virtually all innate immune cells, i.e. mast cells, neutrophils, macrophages, eosinophils, basophils, and NK cells, are able to communicate with dendritic cells (DCs) and/or T and B cells, and thereby significantly contribute to the orchestration of adaptive immune responses. The means of communication that are thus far primarily associated with this function are cell-cell contacts and the release of a broad range of soluble mediators. Moreover, the possible contribution of innate immune cell-derived extracellular vesicles (EVs) to the modulation of adaptive immunity will be outlined in this review. EVs are submicron particles composed of a lipid bilayer, proteins and nucleic acids released by cells in a regulated fashion. EVs are involved in intercellular communication between multiple cell types, including those of the immune system. A good understanding of the mechanisms by which innate immune cell-derived EVs influence adaptive immune responses, or vice versa, may reveal novel insights in the regulation of the immune system and can open up new possibilities for EVs (or their components) in controlling immune responses, either as a therapy, target, or as an adjuvant in future immune modulating treatments.

Key words

Extracellular vesicle, exosome, microvesicle, microparticle, innate immune cells, adaptive immunity

Introduction

For a long time, it has been a general principle in immunology that the development of effector T cells (Th1, Th2, Th17 or regulatory T cells) is directly driven by antigen-primed dendritic cells (DCs)[1]. However, it is now more and more recognized that both at inflammatory sites and in secondary lymphoid structures, DCs can interact with innate immune cells (i.e. mast cells, neutrophils, macrophages, eosinophils, basophils, and natural killer (NK) cells) resulting in the modulation of DC migration and function. These interactions subsequently influence T cell proliferation and/or polarization into effector cell subsets [2–9]. Moreover, the concept that innate immune cells can directly communicate with $CD4^+$ and $CD8^+$ effector T cells, regulatory T cells, γδ-T cells, and B cells, and thereby influence each other's function, adds another layer of complexity in the regulation of innate and adaptive immune responses [10–16]. These accumulating new insights indicate that in contrast with the conventional idea that innate immune cells only participate in immune responses as terminal effector cells, they also significantly contribute to the initiation and shaping of adaptive immune responses.

Mechanisms that are involved in the cross-talk between innate and adaptive immunity obviously include cell-cell contacts and the release of a diverse array of soluble factors, including cytokines and chemokines. Another increasingly investigated and appreciated mechanism via which cells may exert their modulatory effect is through the release extracellular vesicles (EVs). EVs are submicron structures, typically of 50 to 200 nm in size, and released by cells in a regulated fashion [17]. EVs can either be formed and released by outward budding from the plasma membrane (then often termed microparticles or microvesicles), or formed as intraluminal vesicles in endosomal multivesicular bodies and released (as so-called exosomes) from cells upon fusion of the multivesicular body with the plasma membrane[17, 18]. Since the route of biogenesis cannot be determined once the different vesicle subsets are found in biological fluids or culture supernatants due to a lack of discriminatory markers, they are collectively termed EVs [19]. The content of EVs entails lipids, proteins, and nucleic acids, and therefore EVs can be seen as multi-component communication devices. The selective incorporation of this cargo depends on the activation state of the EV-releasing cell, and differs between different cell types [20]. Importantly, the combined presence of different functional molecules in EVs can result in different cellular responses compared to the individual components [21]. Additionally and in

contrast to soluble proteins, some membrane-associated proteins may be resistant to inhibition as was shown for neutrophil elastase activity for example [22], or can induce more potent responses, as was shown for EV-associated Hsp70 [23]. Taken together, the selective incorporation of cargo determines how and where EVs exert their function.

It is clear that EVs can substantially influence different physiological and pathological processes, including immune responses [24, 25], cancer [26], and cardiovascular diseases [27]. As it is now increasingly recognized that innate immune cells may modulate the adaptive immune response, we will focus in this review on the contribution of EV-mediated cross-talk between cells of the innate and adaptive immune system to the initiation or regulation of immune responses. More specifically, we will focus on macrophages, mast cells, neutrophils, eosinophils, basophils and NK cells as producers of EVs that modulate T and B cell functions (either by directly targeting these cells, or indirectly by targeting DCs). Since DCs are well-known for their role as master regulator of adaptive immunity and because the immune-modulating characteristics of DC-derived EVs, including their use in clinical trials aiming at inducing tumor-specific T cell responses have been reviewed extensively [25, 28–30], we did not include DC-derived EVs in the current review. Finally, multiple different protocols are used for the isolation of (subsets of) EVs, and EV research is frequently complicated by the possible presence of contaminants in EV samples, e.g. protein aggregates and lipoproteins. Efforts have been made to standardize EV-related experimental methods and documentation [31, 32], and to improve the interpretation of the reviewed data, we concisely indicated the EV isolation methods (i.e. pelleting speed, possibly followed by density gradient ultracentrifugation (DGC)) that were used to obtain the described EV content or their immune modulatory effects.

Mast cells

Mast cells are tissue-resident cells that are particularly found at sites that are in contact with external environments such as the skin, gut, and airways. Moreover, mast cells can be found in lymphoid tissues, where their presence can significantly increase during inflammatory responses [33–36]. Physiologically, mast cells appear to have a prominent role in orchestrating innate and adaptive immunity required for host defense against microbial infections (parasites, bacteria, virus), and animal venoms. Moreover, these cells are associated both with the induction of immune tolerance

but also with the initiation and progression of multiple immune disorders [33, 37, 38]. Although direct cellular contacts and soluble mediator release are two important mechanisms employed by mast cells to exert these functions [2, 11, 39], mast cells release EVs under resting and activated conditions that are indicated to play a role.

Several studies have demonstrated that mast cells can express MHC class II (MHC-II) and co-stimulatory molecules under specific inflammatory conditions (LPS, IFN-γ, IL-4) [40–44], or acquire MHC-II after contact with DCs [45], and thereby regulate effector memory $CD4^+$ T cell responses [42–45]. In line with these data, MHC-II was shown to be present on EVs (70,000g (70K) pellets) from murine bone marrow-derived mast cells (BMMC) and RBL-2H3 cells (rat basophilic leukemia used as a mast cell model) using electron microscopy in combination with immunolabeling, western blotting, and ELISA [46–48]. In BMMC cell cultures, MHC-II-containing EVs were released both by degranulating [46] and unstimulated BMMCs [49], two conditions that result in the release of two completely different EV subsets based on size, and protein and lipid composition [50]. In an artificial model using hemagglutinin peptide-loaded HLA-DR1-expressing RBL-2H3 cells, EVs from degranulated cells (70K pellets) could significantly induce the proliferation of hemagglutinin-specific Jurkat T cells, but only in the presence of immature DCs [47]. Merely weak T cell activation was observed in the absence of DCs, indicating that mast cell-derived EVs must be taken up by professional antigen presenting cells or that additional DC-derived costimulatory signals were required for T cell activation. A comparable requirement was shown for MHC-II-positive DC-derived EVs in order to induce substantial effects on T cell responses [25, 51]. So far, no additional data exists that further substantiate a role for mast cell-EVs in the transfer of MHC-II-peptide complexes and in direct antigen presentation to T cells. Others demonstrated that antigen (ovalbumin)-primed unstimulated BMMCs can transfer endocytosed and partially processed antigens via EVs (70K pellets) to immature bone marrow-derived DCs (BMDCs) in vitro, or to DCs in vivo after EV injection. Interestingly, this EV-associated ovalbumin was far more efficiently presented by DCs than soluble ovalbumin in the presence of LPS. The authors further showed that antigen-loading into EVs was dependent on heat shock proteins (Hsp60 and Hsc70), and that the low density lipoprotein receptor-related protein 1 (LRP1/CD91) expressed on DCs was involved in the uptake of hsp-positive EVs [52]. Moreover, these mast cell-derived EVs were shown to induce BMDC maturation (upregulated expression of MHC-II, CD80, CD86, and CD40), and IL-12p70 release by DCs. This effect

on DCs was likely responsible for additionally observed effects: enhanced B and T cell proliferation both in vitro, and in in vivo murine models upon intraperitoneal injection of antigen-primed unstimulated BMMC-derived EVs (increased IL-2 and IFN-γ release, no IL-4). Furthermore, efficient antigen-specific antibody responses (IgG1/IgG2a) could be elicited in mice after subcutaneous injection of these EVs in the absence of conventional adjuvants [48, 52].

Although clear immunomodulatory functions are described for different heat shock proteins [53, 54], their functional role in mast cell EVs other than loading of antigenic cargo have not been demonstrated. It should be noted that although we also identified Hsp60, Hsp70, and Hsp90 in murine spleen-derived and peritoneal mast cell EVs, we did not observe any effects on the maturation of BMDCs upon incubation with EVs isolated from unstimulated mast cells (100K pellets, purified by DGC, or only using 100K pellets comparable to the above described papers) ([50] and our own unpublished data). These contradicting findings indicate that either mast cells derived from different precursors release functionally different EVs, and/or that this difference in functionality is due to different culture conditions employed, such as the required treatment with IL-4 to generate EVs with DC activating properties [48, 52]. As such, it is likely that mast cells only release immune stimulatory EVs when exposed to specific inflammatory conditions, since it would be physiologically unfavorable when constitutively released EVs continuously activate surroundings DCs and induce B and T cell activation.

Another mechanism of antigen-transfer from mast cells to DCs may involve antigen-IgE complexes bound to mast cell EVs. The presence of the high-affinity IgE receptor (FcεRI) on EVs from both unstimulated and degranulated RBL-2H3 cells (EV isolation protocol unknown) and unstimulated BMMCs (120K pellets) was shown [55, 56], though we only detected the Fc receptor γ-chain in EVs isolated (100K pellets purified by density gradient ultracentrifugation) from degranulated murine peritoneal mast cells [50]. Such FcεRI-IgE complexes were shown to be able to transfer antigen from BMMCs to BMDCs in a direct cell contact-dependent manner following induction of mast cell degranulation, although a contribution of EVs was not investigated [57].

Mast cell EVs have also been shown to contain lipases and lipid mediators that may be involved in the modulation of adaptive immune responses. Using a human mast cell line (LAD2), Cheung et al. showed that EVs derived from IFN-α-activated mast

cells contain active cytosolic phospholipase A2 (PLA2G4D), which can be transferred to CD1a-expressing cells (either a CD1a-expressing leukemic cell line or monocyte-derived DCs), leading to the generation and presentation of neolipid antigens. This subsequently induced the activation of lipid-specific CD1a-reactive T cells of psoriasis patients leading to the production of IL-22 and IL-17A [58]. Although EVs were isolated using a 'exosome extraction reagent' which is not a generally accepted EV isolation method, PLA_2 was also shown in EVs isolated after ionomycin-mediated RBL-2H3 activation (100K pellet) [59], and also we found PLA_2 activity in EVs isolated from degranulated peritoneal mast cells (100K pellets purified by DGC, own unpublished data). Interestingly, PLA_2 can generate lysophospholipids, such as lysophosphatidylcholine (LPC), which could lead to membrane damage and IL-33 release. In turn, IL-33 can directly signal through ST2 on T cells to induce Th2 cell responses, as was shown in mice [60]. LPC themselves can also directly influence T cell chemotaxis and function [61, 62], NKT cell activation [63], and DC maturation [64]. Furthermore, PLA_2 can directly induce DC maturation [65]. Besides PLA_2, RBL-2H3-derived EVs (100K pellets) also contained PLD_2 activity, arachidonic acid, and its derivatives including prostaglandin E2 (PGE2) and PGD2 [59, 66]. These prostaglandins may directly modulate DCs and T and B cells leading to both pro- and anti-inflammatory effects [67]. Moreover, PLD_2 hydrolyzes PC to generate choline and phosphatidic acid (PA), which in turn can by hydrolyzed by PLA_2 into lyso-PA that can inhibit DC function, and effect DC migration, cytokine release (inhibition of TNF-α and IL-12, enhanced IL-10 release), and their capacity to induce Th1 cell differentiation [68, 69]. Together, these findings implicate that mast cell-derived EVs may directly or indirectly be involved in the generation of lipid mediators that can modulate adaptive immune responses.

Finally, it was shown that EVs from degranulated murine peritoneal mast cells (100K pellets purified by density gradient ultracentrifugation) contain biologically active mast cell-specific proteases [50]. Association of proteolytic enzymes to EVs may be a mechanism to effectively position combinations of enzymes at distant sites, where mast cell proteases could influence adaptive immunity by proteolytic processing or degradation of cytokines and chemokines, including the IL-1 cytokine family members IL-18 and IL-33, as well as IL-15 [70, 71]. Moreover, proteases may directly target T cells. In a murine model it was found that mast cell protease-6 was involved in suppression of Th2 cytokines by activating PAR2 on Th2 cells [72].

Evidence for the direct targeting of T and B cells by mast cell EVs is still limited. Using EV-bead flow cytometry it was suggested that unstimulated BMMCs release CD40L$^+$ EVs (ExoQuick isolation) that were undetectable in culture supernatants upon co-culture with murine splenic B cells suggesting that they were captured by B cells. This transfer of EVs was suggested to be involved in the generation of IL-10 competent B cells [73]. CD40L was also found on BMMC-derived EVs previously [48], and may possibly also influence isotype switching, somatic hypermutation, plasma and memory B cell formation, as well as DC maturation [74]. Lastly, another group showed that OX40L$^+$ EVs derived from unstimulated BMMCs (120K pellets) promote Th2 cell differentiation by targeting anti-CD3/CD28-stimulated T cells in the presence of IL-4 [75]. However, this latter study has several methodological shortcomings, limiting the value of this finding.

Neutrophils

Neutrophils are circulating cells that are generally rapidly recruited to peripheral sites upon initiation of inflammation, were their main known function is the phagocytosis and killing of invading pathogens and clearance of debris [76]. Moreover, it is increasingly recognized that different neutrophil phenotypes with distinct functions exists [77, 78], that neutrophils also infiltrate secondary lymphoid organs [79], and that neutrophils are more than just final effector cells. Neutrophils are now known to be involved in orchestrating adaptive immune responses by influencing various cell types, either by releasing multiple soluble mediators or via direct cellular contacts. For instance, neutrophils can modulate DC migration, maturation, and their CD4$^+$ T cell polarizing capacity [2, 80]. In addition, they can suppress or enhance B cell, CD4$^+$ T cell, and γδ T cell functions (independent of MHC), or even act as antigen-presenting cells under specific activating conditions and thereby drive antigen-specific T cell responses [10, 79]. Limited studies have been performed so far indicating that neutrophil-derived EVs may regulate some of these immune modulatory processes. Nevertheless, already quite some research has been conducted on the characterization of vesicles released from resting or differentially activated neutrophils, which revealed the presence of several molecules that have the capacity to modulate adaptive immunity.

Human neutrophil degranulation induced by N-Formyl-methionyl-leucyl-phenylalanine (fMLP) resulted in the release EVs (160K pellets) that have inhibitory

effects on monocyte-derived DCs [81]. These EVs altered the morphology of LPS-activated DCs by inhibiting the formation of dendrites and reduced the phagocytic activity and maturation (lower expression of HLA-DP/DQ/DR, CD40, CD80, CD83, CD86), resulting in an attenuated capacity to induce T cell proliferation. Furthermore, the release of the cytokines IL-8, IL-10, IL-12 and TNF-α by LPS-activated DCs was inhibited by these EVs, while the release of TGF-β1 was increased [81]. These effects on cytokine release were in line with previous observations showing that resting, and LPS- or zymosan A-activated macrophages decrease the release of pro-inflammatory cytokines (TNF-α, IL-6, IL-8) and increase TGF-β1 release upon targeting by human neutrophil derived EVs (160K pellets, or concentrated culture supernatants) [82–84]. Phosphatidylserine (PS) is one of the modulatory molecules present in the membranes of neutrophil-derived EVs [81, 85, 86], and was shown to be involved in mediating anti-inflammatory effects in line with the generally known immunosuppressive function of this phospholipid [87]. Indeed, blocking PS with Annexin V before adding the EVs to the activated DCs significantly reduced the above described effects [81]. However, a follow-up study from the same group showed that the presence of PS on the EV surface is required for the binding and/or uptake of EVs, but not sufficient to induce the release of TGF-β1 from macrophages [84]. Other EV-associated factors responsible for the release of TGF-β1 were not further indicated.

The increased release of TGF-β1 by DCs and macrophages upon incubation with neutrophil-derived EVs can have diverse effects on the adaptive immune system, but is primarily known for its suppressive effects on $CD4^+$ Th1 and Th2 cell differentiation, and induction of regulatory T cells. Moreover, it can promote Th9 and Th17 cell generation, limit $CD8^+$ T cell and B cell functions, influence DC migration, and induce anti-inflammatory pathways in DCs leading to dampening of T cell responses [88]. These findings suggest that neutrophil-derived EVs have an indirect, primarily anti-inflammatory effect on the adaptive immune system by influencing mediator release by targeted DCs and/or macrophages. However, another study showed direct effects of EVs (100K pellets) derived from UV-B light-induced apoptotic human neutrophils on $CD4^+$ T cells [89]. These EVs were found to bind anti-CD3/CD28-activated $CD25^-CD127^+$ (naïve and resting central memory) and $CD25^+CD127^+$ (recently activated effector) T cells and reduced their release of TNF-α. In contrast, these EVs only suppressed the proliferation of $CD25^-CD127^+$ cells, likely by reducing both IL-2 secretion and CD25 upregulation upon stimulation [89]. The neutrophil

granule proteins MPO and arginase-1 were detected in the EV preparations, and although their described immunosuppressive properties (described below), they were not found to mediate the suppressive effects on T cells [89]. In addition, EVs from neutrophils are enriched in CD11b (possibly enhanced in EVs from apoptotic neutrophils) [85, 86, 89, 90], which may be involved in their binding to activated T cells [91]. Collectively, these data support the immune suppressing roles of neutrophil-derived EVs mediated by DCs and macrophages, and indicate that EVs released by apoptotic neutrophils may contribute to the prevention of uncontrolled activation and expansion of resting T cells, thereby supporting the termination of immune reactions.

Besides these studies that explored immune modulating functions of neutrophil-derived EVs, others have focused on the characterization of their release, size, and content, and identified several EV-associated molecules that can potentially influence DC and T cell functions. In this regard, it was shown that annexin A1 is present on the surface of EVs released from fMLP-stimulated neutrophils (100K pellets) [92–94]. Annexin A1 is immune modulating mediator with a broad range of actions including immune suppressive (inhibition of T cell proliferation and Th2 cell development, induction of regulatory T cells) and activating (favoring Th1- and Th17-mediated responses) capacities [95]. Arginase-1 and lactoferrin are other anti-inflammatory proteins identified in EVs derived from apoptotic neutrophils as described above [89], and EVs (15.7K pellets) from resting or S. aureus-activated neutrophils, respectively [90]. Arginase-1 depletes the nonessential amino acid L-arginine which can result in limited T cell proliferation and impaired T cell function [10]. Lactoferrin can increase DC recruitment to sites of infection, and was also shown to inhibit DC migration to draining lymph nodes. Moreover, contradicting results were found on the role of lactoferrin in the induction of DC maturation [2, 76]. Furthermore, several proteases have been identified in neutrophil-derived EVs (15.7-200K pellets) that may influence adaptive immunity, such as myeloperoxidase (MPO), cathepsin G, proteinase 3, and elastase [85, 93, 90, 96, 97]. Interestingly, EV-associated MPO was found more effective in influencing epithelial cell function when compared to recombinant soluble MPO, possibly due to targeting MPO activity or an increased MPO stability [97]. Although these proteases are also found in so-called neutrophil extracellular traps (NETs), which potentially contaminate pelleted EV fractions, we confirmed the association of elastase to EVs (10K and 100K pellets) after their purification by DGC (own unpublished data). Anti-inflammatory effects have been

described for MPO, which can suppress various aspects of DC function in vitro and in vivo, including their migration, antigen uptake, maturation, and subsequent induction of T cell responses [98]. Elastase, cathepsin G, and proteinase 3 have the capacity to inactivate several cytokines (including IL-2, IL-6, TNF-a, IL-15 and IL-33) and to shed IL-2 and IL-6 receptors from the plasma membrane by proteolytic cleavage, and may thereby inhibit diverse T cell functions [71, 99, 100]. Also, elastase has been described to induce TGF-β secretion by DCs, and to decrease their capacity to induce T cell proliferation [101], which may in part explain the above described TGF-β-inducing effects in DCs and macrophages of neutrophil EVs.

Next to these immune suppressive functions, it was found that elastase, cathepsin G, and proteinase 3 can activate IL-36 which can induce DC maturation, enhance Th1 or Th17 cell induction, and inhibit regulatory T cell development [102]. Moreover, elastase is able to potentiate the activation of γδ T cells via the activation of protease activated receptor 1 (PAR1) [103], and to critically influence the generation of human Th17 cells from naive $CD4^+$ T cells by cleaving IL-8 into a truncated form, which is subsequently required for Th17 cell development [80]. Another molecule that was identified in EVs (100K pellets) released from fMLP-activated human neutrophils was LTB_4 (together with LTB_4-synthesizing enzymes), which was shown to stimulate the directional migration of neutrophils themselves [93, 104]. Although possible consequences of EV-associated leukotriene was not further investigated on other cells, it may also enhance DC migration to sites of infection and/or the draining lymph nodes [105], and act as a chemoattractant for $CD4^+$ and $CD8^+$ T cells [106].

Macrophages

Macrophages are important phagocytic cells distributed in essentially all tissues, and are implicated in multiple different responses that are essential for host defense against invading pathogens, tissue development, and homeostasis (including tissue integrity and healing). Upon infection, macrophages can rapidly recruit various innate and adaptive immune cells. In addition, they can be involved in mediating $CD4^+$ and $CD8^+$ T cell responses and in the activation of B cells and invariant NKT cells [107–109]. Although cytokines play a prominent role in these effects, there is increasing evidence indicating that macrophage-derived EVs also contribute to this control of adaptive immunity.

Several groups demonstrated that macrophage-derived EVs directly or indirectly modulate DC and T cell responses. For example, mycobacterial-infected murine macrophages (J774 cell line) were found to release EVs (100K pellets, followed by DGC purification) that contain mycobacterial antigens and have the ability to induce BMDC maturation (increased expression of CD83, CD86, and MHC-II) and IL-12p40 expression [110]. Moreover, these EVs induced the proliferation and activation of $CD4^+$ and $CD8^+$ T cells in vitro, and in vivo after intranasal injection in mice, which resulted in the induction of a population of effector memory T cells. The presence of DCs was required for the optimal induction of T cell responses [110], comparable to the above described actions of mast cell- and DC-derived EVs. In contrast, another study showed that $MHC\text{-}II^+/CD86^+$ EVs (10K pellets) derived from ATP-activated mycobacterial infected bone marrow-derived macrophages (BMDM) can efficiently present endogenously processed antigens to BB7 T hybridoma cells and P25 TCR-transgenic naive T cells in vitro without need for additional DCs [111]. However, this seeming discrepancy can be the result of different EV populations studied due to different isolation protocols used, or to the different requirement for co-stimulation in the read-out T cell models.

Two other studies showed that DCs and macrophages themselves are major target cells of macrophages-derived EVs in mice. After intranasal injection of EVs (100K pellets, followed by DGC purification) derived from mycobacteria-infected human THP-1 cells targeting of both DCs and macrophages in the lungs was demonstrated [114], while after subcutaneous injection of EVs (100K pellets, no differential centrifugation) derived from a murine macrophage cell line (RAW264.7) targeting of DCs and macrophages was demonstrated in the draining lymph nodes [112, 113]. This latter study showed that EVs derived from macrophages with an M1 phenotype induced the release of Th1 cell promoting cytokines (IL-12, INF-γ) by both RAW264.7 macrophages and a DC cell line JAWSII, and induced a stronger antigen-specific cytotoxic T cell response in vivo when administered together with a peptide vaccine. Instead, EVs released by M2 like macrophages enhanced IL-4 and IL-10 release by macrophages and DCs [113]. Finally, EVs (100K pellets, followed by DGC purification) derived from mycobacterial-infected murine macrophages (RAW264.7 cells) induced the release of multiple cytokines and chemokines (e.g. TNF-α, CCL3, and CCL5) from uninfected macrophages that significantly enhanced the transmigration of $TCR\text{-}\beta^+$ T cells in in vitro assays, and may also influence the migration of DCs [114].

Besides the analysis of functional consequences of macrophage-derived EVs on DCs or T cells, several studies characterized EV content and/or their effects on uninfected macrophages which revealed immune modulatory components. As indicated, macrophage-derived EVs can contain microbial components and may as such transfer both antigens and activating signals to DCs, thereby promoting the development of adaptive immune responses comparable to what was described above for mast cell EVs. Glycopeptidolipids (GPLs, a major cell wall constituent of mycobacterium Avium), lipoarabinomannan (LAM), and 19-kDa lipoprotein were found in EVs (100K pellets, followed by DGC purification) derived from mycobacteria-infected macrophages (murine J774 cells and human THP-1 cells) and were transferred to, and activated uninfected macrophages in a TLR-dependent manner [112, 115]. Moreover, intranasal injection of these EVs in mice induced significant IL-12p40 and TNF-α release in the lung one day after injection, which may support the Th1 cell inducing effects of M1 macrophage EVs described above. Furthermore, LPS was found in EVs from Salmonella-infected THP-1 cells, which induced TLR-4-dependent BMDMs activation [112]. Also GP63, a surface protease of the Leishmania mexicana parasite, was shown to be present on EVs (100K pellets, followed by DGC purification) derived from infected J774 macrophages, yet functional implications were not further investigated [116].

It is yet poorly understood which components present in macrophage EVs other than microbial molecules can regulate the modulatory effects on DC and T cell responses. Yet, several molecules have been identified that are potentially involved. Increased amounts of Hsp70 for example were shown to be present in EVs (100K pellets, followed by DGC purification) derived from mycobacteria-infected RAW264.7 cell macrophages, which may contribute to the pro-inflammatory properties of these EVs [117]. However, Hsp70 is also associated with a tolerance promoting potential [54]. The ultimate actions of Hsp70 may depend on additional EV-associated signals, the expression of Hsp70 (membrane-exposed or luminal) or the exact form of Hsp70 present in EVs (e.g. protein modifications). Interestingly for IL-1β it was shown that it is present in its unprocessed and mature, bioactive form in EVs (isolated by annexin-bead pulldown) derived from ATP-activated human THP-1 cells [118], and murine BMDM-derived EVs (100K pellets) upon induction of inflammasome signaling upon activation with LPS/nigericin (a potent activator of the NRLP3 inflammasome) [119]. IL-1β can among others be involved in the modulation of DC function and migration, and in the expansion and differentiation T and B cells [120–122]. However, the presence of

IL-1β in EVs was not supported by Qu et al, using EVs derived from murine BMDMs [123]. Also, TNF-α and several chemokines (e.g. CCL2, CCL3, CCL4, and CCL5) were found in EVs (100K pellets) derived from LPS/nigericin-activated BMDMs and LPS-stimulated RAW264.7 cells [119, 124]. Although the mechanisms by which these EV-associated molecules can exactly function is still unclear, they may be involved in the enhanced activation and/or migration of DCs and T cells. Furthermore, the EVs from LPS-stimulated RAW264.7 cells contained miRNAs that can potentially target proteins involved in TLR and chemokine signaling, and as such may have the capacity to modulate DC-driven responses [124]. Another group showed that EVs (70K pellets) released from macrophages (P388D1 cell line) can contain complement C3 fragments on the outer membranes, which may be involved in enhancing T cell proliferation in the presence of antigen presenting cells [125]. Also proteins of the leukotriene pathway have been identified in EVs (100K pellets) released by human monocyte-derived macrophages, and these EVs had the capacity to convert LTA_4 to LTB_4 and LTC_4 [126]. Functional consequences of this EV-mediated leukotriene synthesis were not further addressed, but may encompass enhanced migration, activation, and antigen presentation by DCs, reduced IL-12 release by DC (thus enhanced Th2 cell development), and migration of, and cytokine production by $CD4^+$ and $CD8^+$ T cells [106]. One other immunomodulatory molecule that was recently demonstrated to be released within EVs (both 10K and 100K pellets) from murine pulmonary macrophages stimulated with bacteria or bacterial components is IL-36γ. The release of these EVs was again further enhanced by the additional activation with ATP [127]. How this encapsulated IL-36γ will be released from EVs and how it might influence adaptive immunity remains elusive, but DCs, B cells, and T cells can express IL-36R, which activation can induce DC maturation, enhance Th1 or Th17 cell generation, and inhibit regulatory T cell development [128].

Eosinophils, basophils, and NK cells

Eosinophils represent a small number of circulating granulocytes that primarily infiltrate sites of helminth infection or allergic inflammation, where their release of multiple (pre-formed) effector molecules mainly attributes to both physiologic and pathogenic effects, respectively. Moreover, eosinophils are found in several tissues under steady-state conditions where they maintain tissue, metabolic and immune homeostasis [14]. Eosinophils can modulate DC functions, and T and B cell responses [2, 12], but until now the contribution of eosinophil-derived EVs

in these processes is not addressed. One possible mechanism may involve the EV-mediated (100K pellets) transfer of the cationic proteins major basic protein (MBP) and eosinophil peroxidase (EPO). Eosinophils were shown to increase the release of EVs containing these proteins upon IFN-γ stimulation [129, 130]. Both MBP and EPO may enhance DC maturation (upregulated expression of CD80, CD83, and CD86 [131, 132]. Moreover, EPO can favor DC-driven Th2 cell development by increasing CCR7 and OX40L expression and the release of IL-6 and TNF-α by murine BMDCs, while significantly inhibiting IL-12p40 production [3, 132].

Basophils are found at low frequencies in the circulation and are primarily associated with the resolution of parasitic infections and with detrimental roles in allergic diseases, and autoimmune diseases as well [13]. Despite some clear evidence for their ability to influence DC function and DC-driven Th2 cell responses [2], there is currently no data available on basophils either as producers or targets of EVs.

NK cells are granular innate immune cells of the lymphoid lineage that are found in the circulation and in (secondary lymphoid) tissues, were they are primarily involved in the killing of virus-infected cells and tumor cells. NK cells can influence several immune responses and immune-related disorders by releasing several cytokines and chemokines that for example influence DC function, or Th1 cell development [7, 16, 133]. So far, there is no clear evidence that NK cell-derived EVs have any modulatory effect on DC, T cell, or B cell functions. However, although several groups described the capacity of NK cell-derived EVs to be involved in tumor cell lysis [134–136], one study also investigated cytotoxic effects on immune cells. Lugini et al showed that EVs (100K pellets) from both unstimulated and activated NK cells were able to lyse PHA-activated, but not resting PBMCs [134]. The cytotoxic molecules perforin and FasL were detected in NK-derived EVs (100K pellets, with and without DGC purification), and particularly perforin was found to be responsible for the lytic effects [134–136]. Since PHA is a selective T cell mitogen, these findings implicate a selective effect on activated T cells, and suggest a possible role for these EVs in in dampening T cell-mediated inflammation.

Concluding remarks

Based on the summarized literature in this review it is becoming clear that the EVs released by different innate immune cells can significantly influence the course of adaptive immune responses, or may at least have the capacity to do so based on their

cargo. A point of caution is the fact that some of the functions and cargo attributed to EVs is not truly EV-associated but based on co-isolated or aggregated material during the EV isolation process, especially since in many of the referred studies EVs were isolated by centrifugation only protocols.

In general it can be concluded that the majority of the effects induced by EVs derived from innate immune cells are mediated via targeting of DCs and subsequent antigen delivery or influencing DC maturation or migration. However, some papers described also direct effects on T cells by innate immune derived EVs. Moreover, while mast cell- and macrophage-derived EVs appeared to be primarily pro-inflammatory, EVs released from either activated or apoptotic neutrophils are thus far mainly associated with immune suppressive functions. Nevertheless, since the nature of the activating stimulus can significantly affect the content and biological functions of released EVs, their capacity to either enhance or inhibit adaptive immunity may significantly alter when isolated after culturing innate immune cells under suppressive or inflammatory conditions that are thus far unexplored. Due to lack of research, immune modulatory functions of eosinophil, NK cell, and basophil EVs still remain largely elusive. Besides, it is striking that evidence for a possible role of T cell- and B cell-derived EVs in influencing innate immune cell functions is understudied, with the exception of two studies from one group describing a role of anti-CD3/CD28-activated $CD4^+$ T cell EVs (20K or 100K pellets, unknown) in inducing mast cell degranulation and cytokine release [137]. Moreover, it was demonstrated that T cell-derived EVs (20K pellets) elicited mast cell activation resulting in the upregulation of several clusters of genes, especially cytokine and chemokine related ones, including IL-24 [138]. Based on these findings it is well possible that T cell EVs also target other innate immune cells, especially at inflammatory sites were activated T cells and multiple different innate immune cell types are present simultaneously.

In conclusion, multiple studies performed to date indicate a significant role for EVs in the communication between innate and adaptive immunity, and especially the contribution of mast cell-, neutrophil-, and macrophage-derived EVs to this cross-talk is investigated thus far. A good understanding of the capacity of EVs released by innate and adaptive immune cells to control the two arms of the immune system may reveal new insights on the complex regulatory circuits operating in the immune system. Moreover, this can uncover new possibilities for the use of EVs (or their components) in controlling immune responses, either as a therapy or as a target in future immune modulating treatments.

Table 1. Overview of molecules associated to innate immune cell-derived EVs that where either shown to be involved in the modulation of adaptive immune responses, or that are identified in innate immune cell-derived EVs and have potential immune modulatory capacity.

Cell type	EV-associated molecule(s)	(Potential) target cell	(potential) target molecule	(potential) effects	references
Mast cells					
EV content shown to be involved in immune modulation					
	MHC-II	DCs, T cell		T cell activation	46-49
	Endocytosed antigens	DCs		Antigen transfer	52
	Hsp60, Hsc70	DCs		Cargo selection and uptake by DCs immune activation or suppression	50, 52
	PLA_2	DCs	phospholipids	Generation of neolipid antigens and lysophospholipids DC activation	58, 59
	CD40	B cells		EV binding	48, 73
EV content with potential immune modulatory functions					
	FcεRI	DCs		Transfer of IgE-antigen complexes	50, 55, 56
	PLD_2		Phosphatidyl-choline (PC)	Phosphatidic acid (PA) generation, may lead to lyso-PA which inhibits DC function and affects Th1 cell generation	59, 66
	PGD2, PGE2	DCs, T cells, B cells		Variable, immune activation or suppression	59, 66
	Proteases	T cells	Cytokines	Cytokine processing and inactivation (e.g. IL-15, IL-18, IL-33) Th2 cell induction	50

Table 1. Continued

Cell type	EV-associated molecule(s)	(Potential) target cell	(potential) target molecule	(potential) effects	references
Neutrophils					
EV content shown to be involved in immune modulation					
	Phosphatidyl-serine (PS)	DC		EV binding/uptake Induction of TGF-β1 release	81, 85, 86
EV content with potential immune modulatory functions					
	CD11b	T cell		EV binding to activated T cells	85, 86, 89, 90
	Elastase	DCs, T cells	Cytokines, membrane receptors	Cytokine inactivation (e.g. IL-2, TNF-α), activation (IL-36), and processing (IL-8) Receptor shedding (CD25, CD126) Induction of TGF-β secretion by DCs, suppressing DC stimulatory capacity Th17 cell induction Activating γδ T cells	85, 96
	MPO	DCs		Suppression of migration, antigen uptake, maturation, and cytokine release	85, 90, 93, 96, 97
	Proteinase 3		Cytokines, membrane receptors	Cytokine inactivation (e.g. IL-2, TNF-α) and activation (IL-36) Receptor shedding (CD25)	85
	Cathepsin G		Cytokines, membrane receptors	Cytokine inactivation (e.g. IL-2, TNF-α, IL15, IL-33) and activation (IL-36) Receptor shedding (CD25, CD126)	93
	LTB_4	DCs, T cells		Increased migration	93, 104
	Annexin A1	DCs, T cells		Induction of Th1, Th17 or regulatory T cells, limiting Th2 cell development Suppression of T cell proliferation	92-94
	Lactoferrin	DCs		Modulation of migration and maturation	90
	Arginase-1		L-arginine	Suppression of T cell proliferation and function	89

Table 1. Continued

Cell type	EV-associated molecule(s)	(Potential) target cell	(potential) target molecule	(potential) effects	references
Macrophages					
EV content shown to be involved in immune modulation					
	MHC-II-peptide complexes	DCs, T cells		MHC-II-peptide complexes transfer to DCs T cell activation	110, 111
EV content with potential immune modulatory functions					
	Microbial components	DCs		Transfer of antigens and activating signals	110, 112, 115, 116
	Hsp70	DCs		Variable, immune activation or suppression	117
	IL-1β	DCs, T cells, B cells		Increased DC migration and IL-12 release Increased T cell expansion, inducing IFN-γ- and IL-17-producing $CD4^+$ T cells and granzyme B^+ $CD8^+$ T cells Induction of regulatory B cells	118, 119
	TNF-α and CCL 2-5	DCs, T cells		Enhancing activation and/or migration	119, 124
	C3 fragments	DCs, B cells		Enhancing T cell proliferation	125
	Leukotriene-related proteins		LTA_4	Increased migration, activation by DCs, reducing IL-12 release Increased migration and cytokine production by T cells	126
	IL-36γ	DCs, T cells, B cells		Increased DC maturation Enhanced Th1 or Th17 cell generation, limiting regulatory T cell development	127
Eosinophils					
EV content with potential immune modulatory functions					
	MBP	DCs		Enhancing DC maturation	129, 130
	EPO	DCs		Increased maturation, altered cytokine release Favouring Th2 cell development	129, 130
NK cells					
EV content with potential immune modulatory functions					
	Perforin and FasL	Activated T cells		Cell lysis	134

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'I think I'll just go down and have some pudding and wait for it all to turn up… it always does in the end…'

Luna Lovegood in Harry Potter and the Order of the Phoenix

CHAPTER 3

Efficient Neutrophil Activation Requires Two Simultaneous Activating Stimuli

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Abstract

Neutrophils are abundantly present in the synovium and synovial fluid of patients suffering from arthritis. Neutrophils can be activated by a multitude of stimuli and the current dogma states that this is a two-step process, consisting of a priming step followed by an activation step. Considering that neutrophil activation occurs in an inflammatory environment, where multiple stimuli are present, we argue that a two-step process is highly unlikely. Here, we indeed demonstrate that neutrophils require simultaneous ligation of two different receptors for efficient activation. We isolated human peripheral blood neutrophils and cultured them with various combinations of stimuli (GM-CSF, fMLF, TNF, and LPS). Next, we evaluated essential neutrophil functions, including degranulation and ROS production using flow cytometry, mediator release using ELISA, NETosis by a live cell imaging method, phagocytosis by imaging flow cytometry, and extracellular vesicle (EV) release quantified by high-resolution flow cytometry. Exposure of neutrophils to any combination of stimuli, but not to single stimuli, resulted in significant degranulation, and mediator and EV release. Furthermore, ROS production increased substantially by dual stimulation, yet appeared to be more dependent on the type of stimulation than on dual stimulation. Phagocytosis was induced to its maximum capacity by a single stimulus, while NETosis was not induced by any of the used physiological stimuli. Our data indicate that neu-trophil activation is tightly regulated and requires activation by two simultaneous stimuli, which is largely independent of the combination of stimuli.

Keywords

degranulation; mediator release; ROS production; NETosis; phagocytosis; extracellular vesicle release

Introduction

Neutrophils are usually the first responders to an infection, and their primary role is killing invading pathogens [1]. In addition, neutrophils influence other immune cells and thereby can modulate both innate and adaptive immune responses [2,3]. Furthermore, neutrophils are present in high amounts in synovium and synovial fluid of arthritis patients, where they can have damaging effects in inflamed joints of these patients [4]. In peripheral blood, neutrophils are present in a resting state but in response to invading microbes, neutrophils migrate and become activated at the site of infection. Neutrophils employ a variety of mechanisms to eliminate pathogens and modulate the function of surrounding cells [5,6]. These mechanisms include degranulation, phagocytosis, reactive oxygen species (ROS) production, and the release of soluble mediators, neutrophil extracellular traps (NETs), and extracellular vesicles (EVs) [1,7–9]. Because of the cytotoxic nature of the majority of neutrophil-derived compounds, uncontrolled neutrophil activation can lead to tissue damage, for example in autoimmune diseases [6]. Indeed, neutrophils from rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients exhibit excessive ROS production and NETs contribute to development and disease activity [10]. Therefore, tight regulation of neutrophil migration and activation is important for optimal health.

The current dogma is that neutrophil activation is a two-step process: first neutrophils require pre-activation, also known as priming, which allows them to respond to an activating stimulus to become fully activated [11, 12]. Such a process would be plausible if priming occurs by factors involved in migration (e.g., chemokine receptors or adhesion molecules), while activating factors comprise pathogen associated molecular patterns (PAMPs) and damage associate patterns (DAMPs). However, in the literature there is no clear distinction made between priming and activating stimuli. For example, LPS and fMLF are reported to act both as priming and activating stimuli [12–19], which is physiologically unrealistic, as both molecules are PAMPs. In contrast, GM-CSF and TNF are often described as priming stimuli [12,20,21], but these two cytokines are usually abundantly present at inflammatory sites [22]. Consequently, as neutrophils encounter pathogens that carry or induce many different inflammatory stimuli at the same time at the infection site, the true existence of a (spatio)temporal, two-step activation process may often be unlikely. Therefore, we hypothesized that the current designation of priming and activating

stimulating agents is a rather artificial concept that is often not physiologically relevant. In this study, we used four different stimuli (GM-CSF, fMLF, TNF and LPS) to show that indeed dual stimulation is required to induce optimal neutrophil degranulation, cytokine release, ROS production and EV release. Consequently, we propose to dismiss the distinction between priming and activating stimuli and that efficient neutrophil activation is primarily a result of ligation of at least two activating receptors simultaneously, rather than a two-step priming and activating process.

Results

Dual Stimulation of Neutrophils Is Necessary for Efficient Degranulation

Full neutrophil activation is induced, according to literature, by subsequent exposure of a priming and activating stimulus. As certain stimuli are used both for priming and activating the stimulus, we questioned this system. Therefore, in order to investigate the effect of different stimulating agents on neutrophil activation, we measured degranulation, a well-known and essential antimicrobial mechanism of neutrophils. Neutrophils contain four different types of granules: azurophilic granules, specific granules, gelatinase granules, and secretory vesicles [23, 24]. We analyzed CD63, CD66b, and CD16 membrane expression to determine the fusion of different granules with the plasma membrane upon activation. CD63 is present in azurophilic granules [23] and CD66b is present in specific gelatinase granules [23,24]. CD16 is expressed by resting neutrophils, but is also present in secretory vesicles [25], and is cleaved from the surface by the sheddase ADAM17 upon activation [26]. We defined fully degranulated neutrophils as CD16-CD63+ (Figure 1A) and the gating strategy is shown in Figure S1. These CD16-CD63+ neutrophils consistently displayed high CD66b expression (Figure 1B). We determined the CD16, CD63 and CD66b expression at 2 h after start of culture, since this culture period resulted in the detection of a clear activated phenotype (Figure S2A) with limited cell death (Figure S2B).

Neutrophils stimulated with either fMLF or TNF alone at increasing concentrations did induce, to different levels, degranulation in part of the neutrophils (Figure 1C). Additionally, the highest concentrations of GM-CSF significantly induced full degranulation, although this was with a very limited number cells (mean <1.2%).

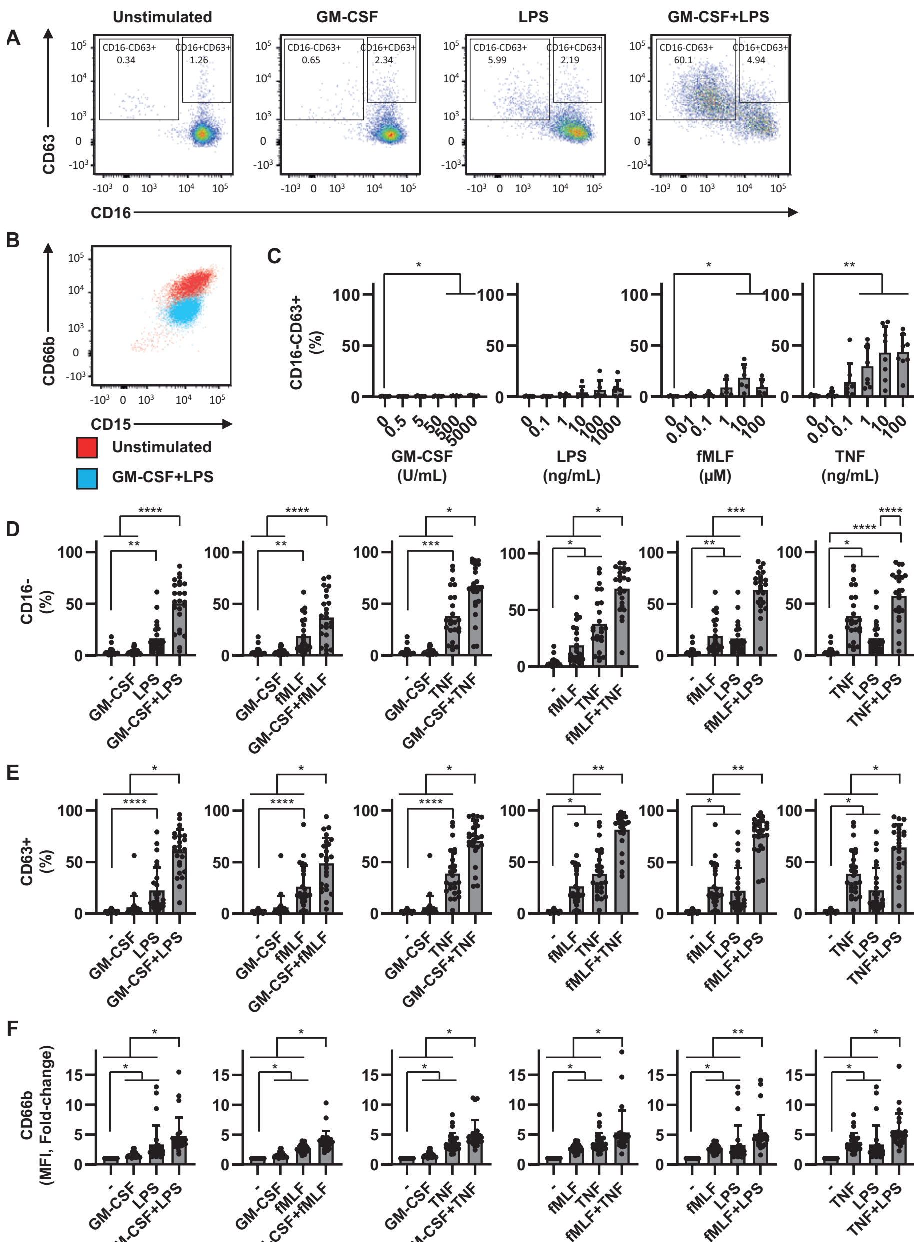

Figure 1. Dual stimulation of neutrophils is required for efficient degranulation. Neutrophils were cultured for 2 h in the absence or presence of different stimuli used at fixed concentrations (GM-CSF: 50 U/mL, LPS: 10 ng/mL, fMLF: 1 µM, and/or TNF: 1 ng/mL), (**A**,**B**,**D**–**F**) or increasing concentrations (**C**). (**A**) Flow cytometry plot demonstrating gating strategy to determine

Figure 1. Continued

neutrophil degranulation: CD16-CD63+, or azurophilic degranulation: CD63+ (CD16-CD63+ and CD16+CD63+). (**B**) Flow cytometry plot demonstrating that neutrophils expressing CD16-CD63+ are consistently high in degranulation of specific and gelatinase granules (CD66b). (**C**) Effects of increasing concentrations of the different stimuli on neutrophil degranulation displayed as percentages of CD16-CD63+ neutrophils. (**D**) Effect of single and double stimulation on degranulation as measured by percentages of CD16- neutrophils. (**E**) Effect of single and double stimulation on degranulation as measured by percentages of CD63+ neutrophils. (**F**) Effect of single and double stimulation on specific and gelatinase granule degranulation as measured by changes in mean fluorescent intensity (MFI) of CD66b, expressed in fold change compared to unstimulated. Data are representative of 6 (**C**) and 25 (**D**,**E**,**F**) independent experiments, and are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. The p-values were calculated using a paired t-test (**C**) and Friedman test with Dunn's correction.

Importantly, large differences were observed between individual donors, especially after incubation with fMLF and TNF (Figure 1C). For subsequent experiments, we selected suboptimal dosages that did not induce full degranulation in the majority of donors. Exposure of neutrophils to a combination of two stimuli consisting of either GM-CSF (50 U/mL), LPS (10 ng/mL), fMLF (1 μM), or TNF (1 ng/mL), resulted in a strong synergistic decrease of CD16 membrane expression (Figure 1D), and increase of CD63 and CD66b membrane expression (Figure 1E,F) compared to a single stimulus or no stimulus. This effect was observed with any combination of stimuli. Interestingly, the combination of two previously termed priming stimuli (GM-CSF and TNF) induced similar neutrophil degranulation as a combination of two activating stimuli (fMLF and LPS) or a more standard combination of a priming and an activating stimulus (GM-CSF and LPS). Although cell death was consistently higher upon double stimulation compared to single stimulation, this was always below 5% after 2 h of stimulation (shown for GM-CSF and LPS in Figure S2B).

Another important feature of neutrophil activation is the loss of L-selectin (CD62L), which is important for neutrophil migration [27]. In contrast to degranulation, CD62L membrane expression is completely downregulated by neutrophils activated with any of the single and double stimuli used (Figure S3A,B).

To enter the inflammatory site, neutrophils have to be exposed to chemotactic agents, like IL-8. To mimic this, we incubated neutrophils in the presence of IL-8 and/or LPS. We observe no 'priming' effect of IL-8, since degranulation was not induced in neutrophils stimulated with IL-8 and LPS compared to neutrophil stimulated with LPS alone (Figure S4).

Together, these data demonstrate that activation of neutrophils with more than one stimulus leads to a synergistic enhancement of neutrophil degranulation. Importantly, the choice and combination of previously termed priming and activating stimuli appears irrelevant to induce neutrophil degranulation and CD16 cleavage.

Dual Stimulation of Neutrophils Is Required for Optimal Mediator Release

Upon neutrophil activation, many different mediators are released into the extracellular environment, such as pre-stored antimicrobial peptides, or de novo synthesized cytokines, and chemokines [28,29]. We analyzed the release of neutrophil elastase (NE), a well-known pre-stored mediator in azurophilic (CD63 positive) granules. In line with the CD63 expression data described above, the release of NE was significantly increased by dual stimulation compared to single stimulation (Figure 2A), and significantly correlated (R = 0.76) with full degranulation (Figure 2B).

In contrast to NE, IL-8 is synthesized de novo upon activation and is regulated differently from expression and release of NE [28]. Consequently, IL-8 release may depend differently on single or dual stimulation. Single stimuli hardly induced any IL-8 secretion, while dual stimulation, independent of the combination of activating or priming stimuli, significantly enhanced IL-8 release (Figure 2C). Similarly for degranulation, we observed high inter-individual differences in IL-8 release, and although both degranulation and IL-8 were positively correlated (Figure 2D), this association was not straightforward. High IL-8 release always corresponded with high degranulation, while fully degranulated neutrophils did not always release high amounts of IL-8. We could not relate this discrepancy to individual differences, not to different stimulatory factors.

Collectively, similarly to degranulation, for optimal mediator release, a dual stimulation is essential, independent of the combination of stimuli used.

ROS Production Is Less Dependent on Dual Stimulation

Next, we investigated whether efficient ROS production is dependent on one or more activating stimuli or on a specific stimulus. Intracellular ROS production was determined by flow cytometry using the ROS indicator dihydrorhodamine-123 (123-DHR) (Figure 3A). ROS production was generally unaffected in neutrophils stimulated with either GM-CSF or LPS at increasing concentrations, although LPS induces ROS production in some donors. In contrast, neutrophils stimulated with fMLF or TNF clearly increased in ROS production at higher concentrations.

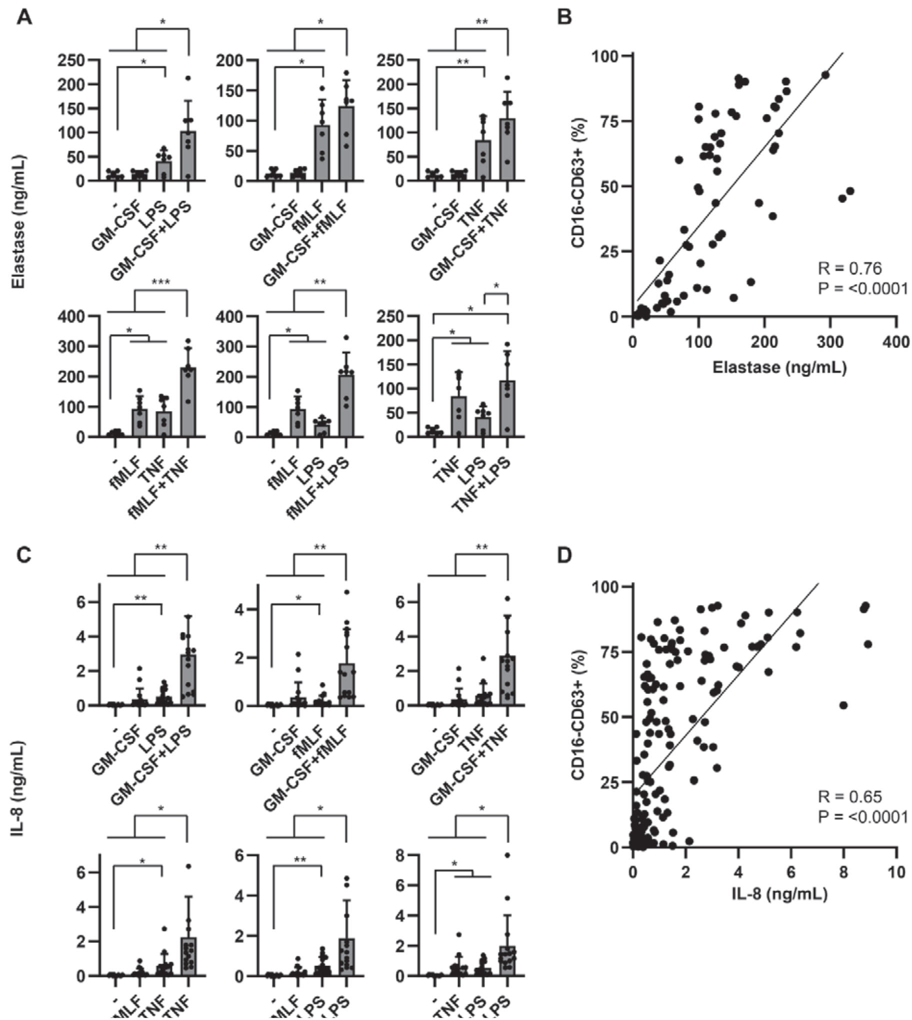

Figure 2. Dual stimulation of neutrophils is required for optimal mediator release. Neutrophils were cultured for either 2 or 24 h in the absence of presence of different stimuli (GM-CSF (50 U/mL), fMLF (1 μM), TNF (1 ng/mL) and/or LPS (10 ng/mL). (**A**) NE was measured in culture supernatants collected 2 h after activation (n = 7). (**B**) Correlation between NE release and degranulation. Neutrophil degranulation was assessed by flow cytometry as shown in Figure 1. Data consist of 7 independent experiments, with multiple conditions in each experiment. (**C**) IL-8 was measured in culture supernatants collected 24 h after activation (n = 11). (**D**) Correlation between IL-8 release and degranulation. Neutrophil degranulation was assessed by flow cytometry as shown in Figure 1. Data consist of 11 independent experiments, with multiple conditions in each experiment. (**A**,**C**) Data are presented as means ± SD, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA with Tukey's correction. (**B**,**D**) Linear regression was applied to determine R.

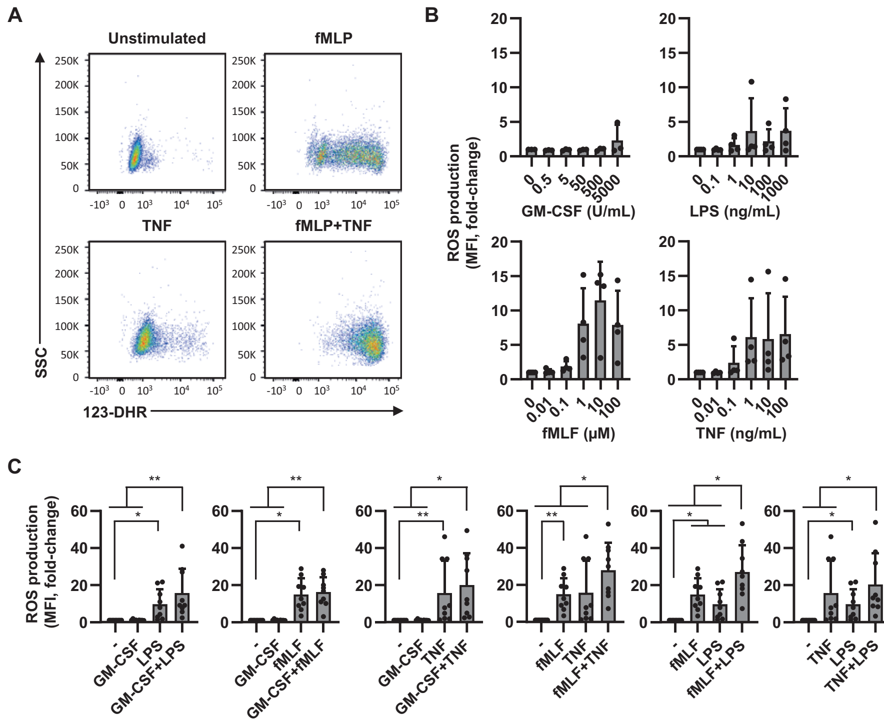

Figure 3. Effects of neutrophil activation by single or double stimuli on ROS production. Neutrophils were cultured in the presence of 123-DHR and in the absence or presence of GM-CSF (50 U/mL), fMLF (1 µM), TNF (1 ng/mL), and/or LPS (10 ng/mL). After 1 h of culture ROS production was analyzed using flow cytometry. (**A**) Representative dot plots showing the influence of neutrophil stimulation on ROS production. (**B**) Effects of increasing concentrations of the different stimuli on ROS production (n = 4). (**C**) Effect of single and double stimulation on ROS production. Total ROS production is expressed as fold change on MFI of 123-DHR compared to unstimulated. Data are presented as mean ± SD. * $p < 0.05$, and ** $p < 0.01$. The p-values were calculated using a paired t-test (**B**) and Friedman test with Dunn's correction (**C**).

Again, just as for neutrophil degranulation, we observed large differences in ROS production between individual donors after incubation with either LPS, fMLF, or TNF (Figure 3B,C). Overall, fMLF showed to be the most prominent inducer of ROS production. Neutrophils activated with two stimuli containing fMLF in combination with either LPS or TNF, but not GM-CSF, significantly further increased ROS production compared to single stimulated and unstimulated neutrophils. With other combinations of stimuli we observed a trend in increased in ROS production compared to single stimulated cells, but this was not significant nor seemed to be

synergistic (Figure 3C). These data indicate that although dual stimulation induces strong ROS production, fMLF is an important stimulator of ROS production, especially when combined with LPS or TNF. ROS production is therefore more dependent on the type of stimulation than on dual stimulation.

Individual Stimuli or Combinations Do Not Induce NETosis

NETosis is a specific process employed by neutrophils in order to entrap and kill pathogens through the release of nuclear content to form a meshwork of chromatin, citrullinated histones and antimicrobial molecules [30,31]. Since neutrophil activation with GM-CSF and LPS induced profound degranulation, mediator release and ROS production, we first only assessed NETosis after stimulation with GM-CSF, LPS, or the combination of both. Unexpectedly, NETosis was not observed at any time point, whereas the non-physiological stimulus PMA induced clear NETosis (Figure 4A–C). After 4 h of stimulation with PMA, a clear presence of NETs was observed, which was less after 8 and 16 h of stimulation, probably due to the disappearance of DNA from neutrophils that underwent NETosis. In parallel, increasing numbers of dead cells were detected in response to PMA stimulation (data not shown). Although NETosis was not observed with GM-SCF/LPS stimulation, we also analyzed NETosis upon stimulation with fMLF or TNF, or any of the possible combinations of two stimuli. None of these stimuli could induce NETosis within 16 h of stimulation (data not shown). These results show that NETosis is not induced by single or combined stimulation with GM-CSF, LPS, fMLF, and/or TNF at concentrations that induce strong degranulation, mediator release, and ROS production.

Phagocytosis Is Independent of Dual Stimulation

Phagocytosis is another mechanism employed by neutrophils to engulf and eliminate pathogens. We investigated the effect of differential activation with single and double stimuli on phagocytosis using 1µm-sized FITC-labeled melamine beads. The proportion of neutrophils that engulfed FITC-labeled beads was quantified by flow cytometry (Figure 5A). Neutrophils stimulated with either single or double stimulation, using GM-CSF and LPS, demonstrated a significant increase in phagocytosis compared to unstimulated neutrophils, which was further increased by the addition of another stimulus (Figure 5B). To verify whether FITC-positive neutrophils detected by flow cytometry were positive as a result of bead uptake, and

not due to aspecific binding to the extracellular plasma membrane, we performed ImageStream analyses. These analyses demonstrated no uptake when cells were kept at 4 °C, whereas the beads were efficiently taken up at 37 °C (Figure 5C). Moreover, with ImageStream analysis, we confirmed that FITC-positive cells were due to bead internalization, implying that the increased detection of FITC-positive neutrophils shown in Figure 5B was caused by phagocytosis. Collectively, these data indicate that phagocytosis is, in general, readily induced by a single stimulation and further increased by an additional stimulus.

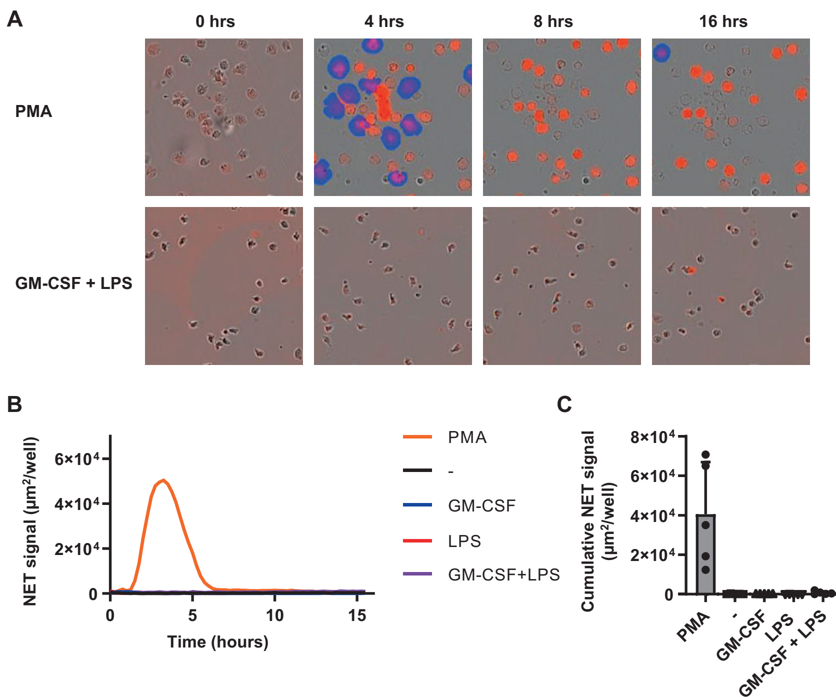

Figure 4. Physiological stimuli do not induce NETosis. Neutrophils were cultured for 16 h in the presence of a cell impermeable fluorescent DNA-binding dye in the absence or presence of GM-CSF (50 U/mL) and/or LPS (10 ng/mL), or PMA (100 μg/mL). Fluorescence was measured every 15 min to determine NET formation and cell death. (**A**) Overlays of phase contrast and fluorescence images showing accessible DNA in red, and extracellular DNA (>400 μm^2) in blue (NETs). Images are representative of 5 independent experiments. (**B**) NETosis was determined as fluorescence signal area in μm^2 per well. Only areas larger than 400 μm^2 were used for calculations (n = 5). (**C**) NETosis expressed as fluorescence area after 4 h of stimulation (n = 5). Data are presented as mean ± SD.

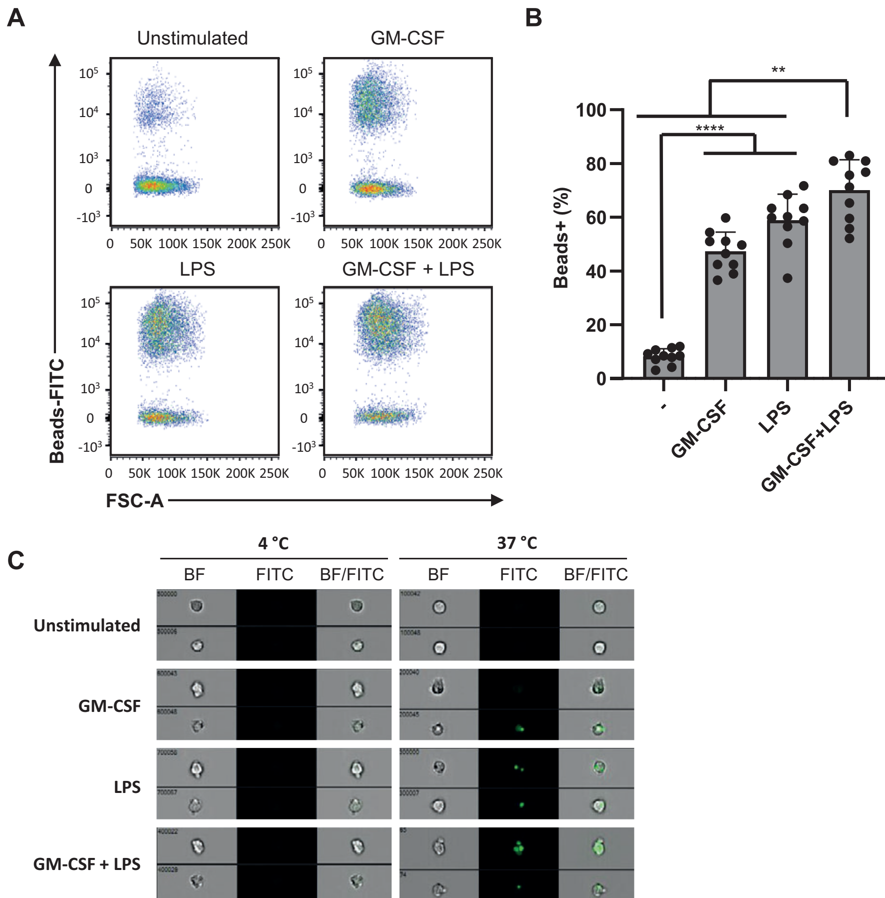

Figure 5. Phagocytosis is independent of dual stimulation. Neutrophils were stimulated for 2 h in the presence of FITC-labeled melamine beads and in the absence or presence of GM-CSF (50 U/mL), fMLF (1 μM), TNF (1 ng/mL), or LPS (10 ng/mL). (**A**) Phagocytosis was analyzed using flow cytometry. (**B**) Phagocytosis, determined by flow cytometry as percentages of FITC positive neutrophils (n = 10). (**C**) Imagestream microscopy images of neutrophils stimulated at 37 °C or 4 °C with GM-CSF (50 U/mL) and/or LPS (10 ng/mL) in the presence of FITC-labeled melamine beads. Location of phagocytosed beads is shown in green (left), and overlay of phase contrast neutrophil (right). Data are presented as mean ± SD. Asterisks indicate significant differences: * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$, one-way ANOVA with Tukey's correction.

Dual Stimulation of Neutrophils Increases the Release of EVs

EVs, small lipid bilayer-enclosed vesicles released by cells, are an important mode of communication between cells and are released in a controlled manner by many cell types, including neutrophils. Here we investigated whether EV release was influenced by dual

stimulation. EV populations were isolated from culture supernatants of unstimulated, single, or dual stimulated neutrophils using GM-CSF and LPS and pelleted at 10,000 g (10 kg) or 100,000 g (100 kg), followed by sucrose density gradient separation. First, we determined with western blot analysis the presence of common EV proteins CD9 and CD63. For 10 kg EVs CD9 and CD63 signals were observed in the expected EV densities (1.18–1.22 g/mL and 1.10–1.16 g/mL) for both unstimulated and dual-stimulated neutrophils (Figure 6A). Furthermore, a CD63 signal, but not a CD9 signal, was observed in the 1.24 g/mL–1.27 g/mL density fraction of dual-stimulated neutrophils, which could be caused by massive degranulation (Figure 6A). For 100 kg EVs CD9 and CD63 signals were mainly observed in EV densities 1.10–1.16 g/mL for dual stimulated neutrophils and these signals were much lower for unstimulated neutrophils (Figures 6A and S5A). Importantly, dual stimulation strongly increased the signal of CD9 and CD63 of 100k EVs in 1.10–1.16 density fraction. To analyze the amount of EV release by neutrophils, EVs pelleted at 10 k or 100 kg were labeled with a lipophilic dye (PKH67) followed by sucrose density gradient separation and subsequently analyzed using fluorescence triggered single EV flow cytometric analysis [32]. The scatter plots of time-based measurements of individual fluorescently labeled EVs in fractions 1.18–1.22 g/mL and 1.10–1.16 g/mL show clear differences in scatter profiles between 10 k and 100 k EVs in all conditions from unstimulated, single- and dual-stimulated neutrophils, indicating differences in EV subsets. This corroborates the differences in CD9 and CD63 distribution between these fractions as observed in the western blot. Furthermore, a strong increase in the amount of EVs in the 1.10–1.16 g/mL density fraction was observed after dual stimulation which match the Western blot data (Figure 6B). Quantitative analysis shows that activation of neutrophils by dual stimulation significantly increased the release of EVs and especially of the 100 kg EVs in density fraction 1.10–1.16 g/mL (Figure 6C). In line with this observation, the number of 100 k EVs released by neutrophils moderately correlated with neutrophil degranulation (Figure S5B). Although the amount of cell death was always low, a moderate correlation between 10 kg EVs present in 1.10–1.16 g/mL density fraction and dead cells was observed. There was no correlation observed between 10 k EVs present in 1.18–1.22 g/mL and 100 kg EV number and percentage of dead cells (data not shown), indicating that most 100 kg EVs detected are released by viable cells and were neither released as a result of apoptosis nor other types of cell death. Taken together, these data indicate that dual stimulated neutrophils do release more EVs than unstimulated or single-stimulated neutrophils which is most prominent for 100 kg EVs in density fraction 1.10–1.16 g/mL.

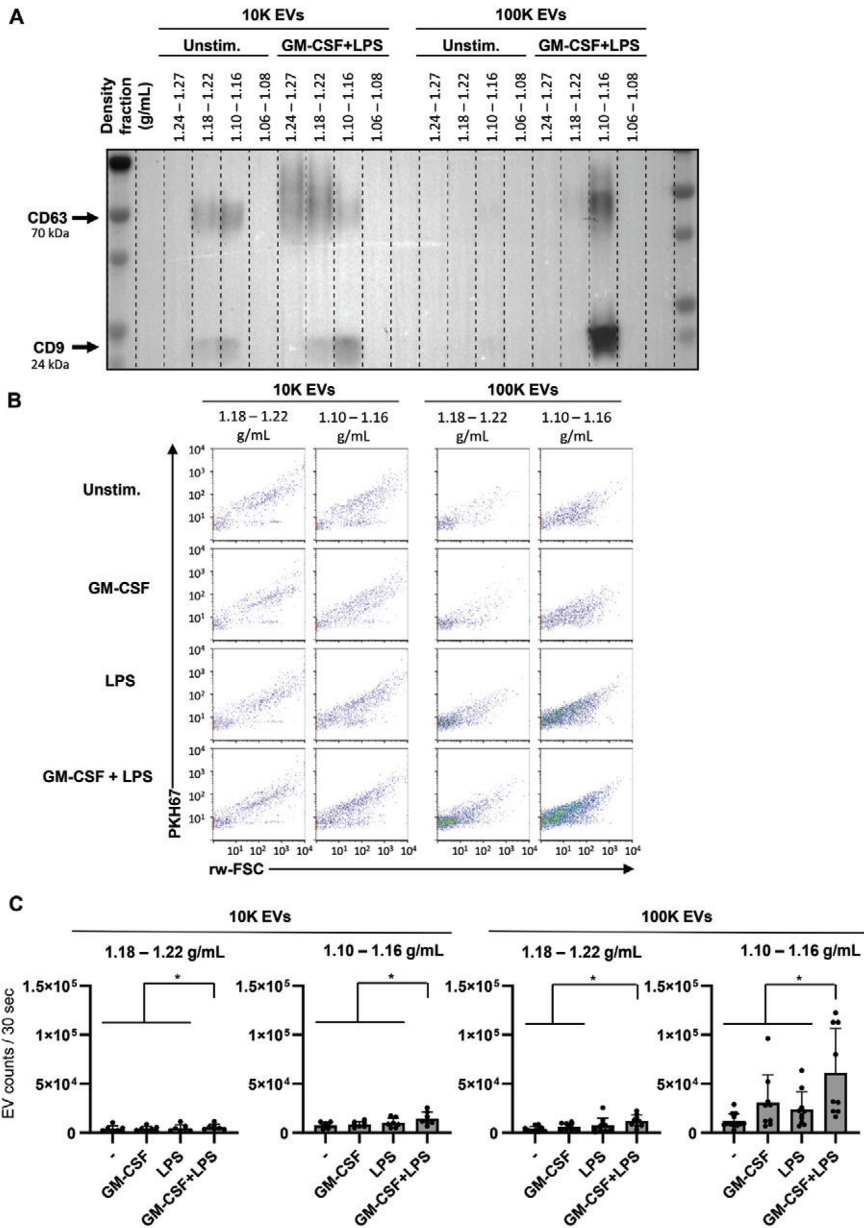

Figure 6. Dual stimulation of neutrophils increases EV release. Neutrophils were cultured for 2 h in the absence of presence of GM-CSF (50 U/mL) and/or LPS (10 ng/mL). After 2 h, culture supernatants were collected for the analysis of EV release. (**A**) Protein analysis (Western blotting) of EVs pelleted at 10 kg and 100 kg and floated into a sucrose gradient from neutrophils from a representative donor. Analysis is shown for CD9 and CD63 (tetraspanins; general EV-markers).

Figure 6. Continued

(**B**) High-resolution flow cytometric analysis of purified PKH67-labeled EVs pelleted at 10 k and 100 k and floated in a sucrose density gradient from neutrophils stimulated with GM-CSF and/or LPS. (**C**) Quantification of EV release in the EV density fractions (1.10–1.16 g/mL and 1.18–1.22 g/mL) as determined by high-resolution flow cytometry. Indicated are the numbers of detected events within the fixed time frame of 30 s, with multiple conditions in each experiment. Data are presented as mean ± SD. Asterisks indicate significant differences: * $p < 0.05$, ($n = 7–11$), one-way ANOVA and mixed-effects analysis with Tukey's correction.

Discussion

In the current study we provide evidence that peripheral blood neutrophils from healthy donors require two simultaneous stimuli for optimal activation resulting in degranulation, mediator release and EV release. In contrast, ROS production is more dependent on the type of stimulation than on dual stimulation, while phagocytosis appears already close to its maximum capacity when only one type of stimulus is present. Surprisingly, we found that the neutrophil function NETosis was not induced by any of the used physiological stimuli or combinations thereof, but could only be induced by the artificial stimulus PMA. Moreover, our data suggest that the current use of priming and activating agents is often not necessarily physiologically relevant. Instead, we demonstrated that neutrophils require activation by two different stimuli which is independent of the combination of stimuli. Any combination of GM-CSF, TNF (both generally considered priming stimuli), LPS, or fMLF (both generally considered activating stimuli) are very well capable of inducing various aspects of neutrophil activation. This contradicts the current dogma stating that neutrophil activation is a two-step process of priming followed by activation. Certain single stimuli; however, already induce neutrophil activation to some extent. At higher concentrations (generally ≥1 ng/mL) TNF enhances degranulation and increases ROS production compared to unstimulated neutrophils. Importantly, our results indicate that degranulation or IL-8 release is strongly increased by the combination with another stimulus.

Quite unexpectedly, we observed a large variation in neutrophil degranulation and ROS production between donors, both after applying a single (except for GM-CSF) and a double stimulus. Neutrophils from some donors did not react at all to a single stimulation, while neutrophils from other donors showed some degree of activation. Although at present we cannot explain these inter-individual differences, the high

donor-to-donor variation may reflect the effectiveness of neutrophils to react to certain microbes. On the other hand, it may mirror the propensity of individuals to disorders associated with neutrophil activation-induced damage. It has been previously described that immunosenescence could play a role, because neutrophils from aged donors (above 60 years of age) can respond differently [33]. However, this cannot account for the observed donor-to-donor variation, since all of our donors were below 60 years of age. Furthermore, we observed no correlation between age and neutrophil degranulation or ROS production.

These observations question the distinction between priming and activating stimuli. TNF is a stimulus that is classically linked to priming whereas it can induce activation of neutrophils itself to some extent. In contrast, LPS is mostly designated as an activating stimulus but not, or only moderately induces any neutrophil activation when presented as a single stimulus.

For this study we isolated neutrophils from peripheral blood using Lymphoprep density centrifugation directly after drawing blood. Many isolation methods have been indicated to increase neutrophil activation [34]. In contrast, our isolation methods seem to have a limited effect, as unstimulated neutrophils truly resemble unstimulated neutrophils. Furthermore, it has been suggested that chemo attraction may influence neutrophil activation. However, we did not observe any effect on degranulation of the potent chemoattractant IL-8, alone or in combination with LPS. The same is true that other stimuli may have a different effect on neutrophil activation. In this study, we only use four different stimuli for neutrophil activation. These are four widely used stimuli in terms of neutrophil activation.

In contrast to degranulation, mediator release, ROS production, EV release and to some extent phagocytosis, which were significantly enhanced by ligation of two types of receptors, no NETosis was observed with any combination of stimuli tested. The formation of NETs, consisting of nuclear chromatin with contents of granules and antimicrobial molecules, has been identified as an important mechanism of neutrophils to trap and disarm invading microbes. Moreover, NETosis has been associated with various chronic inflammatory and infectious diseases including SLE [10,35], RA [36], and COVID-19 [37]. We only observed NETosis when neutrophils were stimulated by PMA, a very robust but artificial stimulus, indicating that the assay that we set up to measure NET formation is valid. Some earlier studies

have observed NETosis in response to TNF and LPS [13,38], whereas others could not detect NETosis with these stimuli [49]. A reason for these differences could be the difference in LPS that was used, or the culture condition. Previous research has shown that neutrophils can selectively release NETs when stimulated with LPS from different bacterial resources under serum-free conditions [40]. Another factor that may play a role is the use of FCS as it has recently been reported that serum and serum albumin inhibit LPS-induced in vitro formation of NETs [41]. No serum or only 0.5% serum was used in NETosis assays, where a response was observed to LPS [13,38], but this setup resulted in rapid cell death in our assay. Since we could detect PMA-induced NETosis in medium containing 10% FCS, we find this a less likely explanation for the absence of NETosis. GM-CSF and TNF are cytokines that are abundant in disorders associated with NETs and frequently linked to disease pathogenesis [42,43]. Circulating TNF levels were elevated in SLE patients versus controls and TNF was proposed as biomarker for SLE disease activity [44]. Therefore, it is surprising that these cytokines do not induce NETosis in our study. This may indicate that either the induction of NETosis is induced by a different set of stimuli, via a different mechanism, or at other concentrations of the stimuli than those used in this study. Another more valid explanation could be that induction of NETosis requires a more complex activation system such as multiple stimuli that are present in microbial stimulation. The latter mechanism would be physiologically reasonable as the formation of NETs has been associated with detrimental tissue damage [6,45,46] and therefore requires tight regulation with various safety valves.

Here we show that the release of EVs by neutrophils also is enhanced by a dual stimulation. Nevertheless, single stimulation by GM-CSF or LPS also induced EV release. We recently showed that LPS-induced neutrophils increase in 100 k EV release, and that this is correlated with degree of degranulation [47], a finding that is supported by data presented in this study. However, neutrophils stimulated with GM-CSF showed little to no increase in degranulation was induced, while there was an increased release of small EVs. This indicates that increased EV release can occur independently of primary or secondary granule release. The increase of EVS was most pronounced in EVs pelleted at 100 kg. Although we did not observe clear differences in the mean buoyant density between these two EV types, the light scattering signals do show differences between the 10 kg and 100 kg EVs, which could indicate differences in size and/or composition. Since molecular cargo selection of EVs is not

a random process, but rather regulated via distinct and regulated processes that are dependent on the type and state of the cell, the type of stimuli that were received, and the subcellular origin of EVs [48,49]. For mast cells for example, we previously observed clear differences in the buoyant density, light scattering, and CD9 and CD63 content of EVs released from unstimulated and activated cells [50]. Moreover, it has been shown that neutrophil-derived EVs indeed have a distinct protein composition, and have antimicrobial capacities [51,52]. It remains to be elucidated whether these composition differences also exist between EVs of single or double stimulated neutrophils.

The observation that a single stimulus is insufficient to fully activate a cell, and to induce a full executive program is not novel. For instance, abundant IL-12 production by dendritic cells is only induced by a double stimulus, e.g., a PAMP in combination with IFN-γ [53]. IL-12 is an important activator of Th1 cells that via the production of IFN-γ activate macrophages, which may cause massive tissue damage when over activated. Furthermore, for the production and secretion of high levels of IL-23 and IL-1β, important cytokines for Th17 cell development, a PAMP in combination with Fc-receptor ligation on dendritic cells is necessary [54]. Additionally, IL-17-producing Th17 cells have been shown to be potentially detrimental and they are associated with various chronic inflammatory disorders. Nevertheless, they are crucial in the fight against bacterial and fungal infections [55]. These examples underline that important processes that are potentially detrimental to the host when overactivated, are often regulated at multiple levels. This could be a physiological explanation as to why neutrophils require more than one stimulus in order to start releasing a plethora of harmful contents.

In this study, we show that full neutrophil activation is tightly regulated and requires the ligation of at least two different types of receptors. Moreover, there is no need for a spatio-temporal separation of these stimulatory actions, as neither would be the case where a pathogenic microbe enters the body resulting in rapid activation of neutrophils. Infection or inflammation results in the rapid release of inflammatory associated molecules such as TNF and GM-CSF. These cytokines are also highly active in arthritis patients. As neutrophils are exposed these and other stimuli concomitantly, full activation of neutrophils will happen rapidly, resulting in the killing and elimination of invading microbes by one or more of the effective mechanisms that neutrophils possess or leading to excessive damage as observed in arthritis.

Materials and Methods

Neutrophil Isolation

Blood was collected from healthy volunteer donors after informed consent into sodium heparin tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Blood was diluted with Hanks balanced salt solution (HBSS, Sigma-Aldrich Inc., St. Louis, MO, USA) and granulocytes and erythrocytes were separated from peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation on Lymphoprep (d = 1.077 ± 0.001 g/mL; Axis-Shield, Oslo, Norway). Erythrocytes were lysed in ice-cold erythrocyte lysis buffer (containing 0.155 M NH_4Cl (Sigma-Aldrich, Inc., USA), 1 mM $KHCO_3$ and 80 μM EDTA (both Merck KGaA, Darmstadt, Germany), dissolved in sterile water, pH 7.3) for 10 min on ice. Subsequently, neutrophils were cleared from remaining erythrocytes with a second lysis step for 5 min on ice, and washed twice in PBS. Neutrophils were then resuspended in IMDM (Gibco; Thermo Fischer Scientific Inc, Waltham, MA, USA) supplemented with 10% heat inactivated (HI) fetal bovine serum (FBS; Hyclone; Thermo Fischer Scientific Inc, Waltham, MA, USA) and gentamycin (86 μg/mL; Duchefa Biochemie B.V., Haarlem, The Netherlands) and used immediately. Neutrophil purity was analyzed by flow cytometry and was always >97%.

Neutrophil Culture, Stimulation and Flow Cytometric Analysis

Neutrophils were seeded at a density of 0.4×10^6 cells/mL in 250 μL in a flat bottom 96-well plate (Costar, Corning Inc. Corning, NY, USA) in IMDM medium containing 10% HI-FBS and gentamycin. subsequently, neutrophils were cultured for 1, 2, or 24 h at 37 °C in the absence or presence of the following reagents: granulocyte-macrophage colony-stimulating factor (GM-CSF) (Schering-Plough B.V., Brussels, Belgium, catalog no. PSR 99M0408), N-formyl-methionyl-leucyl-phenylalanine (fMLF) (Sigma, catalog no. F3506), lipopolysaccharide (LPS) (Sigma-Aldrich, catalog no. L3024, from Escherichia Coli. O111:B4), and tumor necrosis factor (TNF) (Miltenyi Biotec, catalog no. 130-094-022). After 2 or 24 h culture, supernatants were collected for the analysis of neutrophil elastase and IL-8, respectively. For flow cytometric analysis of ROS production, neutrophils were cultured and stimulated for 1 h at 37 °C in the presence of 25 μM 123-dihydrorhodamine (123-DHR; Marker Gene Technologies, Eugene, OR, USA). For the flow cytometric analysis of CD63, CD66b, CD16, and CD62L, neutrophils were stimulated for 2 h. After stimulation,

cells were harvested and washed twice in cold PBA (PBS-0.5% w/v BSA-0.05% w/v azide), followed by antibody labeling in PBA. Degranulation (CD63 and CD66b), CD16 and CD62L expression, and cell viability (PI) were determined using flow cytometry after 2 h of culture. The following antibodies were used: αCD15-FITC (1:100; HI98), αCD16-PECy7 (1:1000; 3G8), αCD62L-APCCy7 (1:25; Greg-56), αCD63-APC (1:100; H5C6), αCD66b-PE (1:100; G10F5), (all Biolegend, San Diego, Calif). DAPI dihydrochloride (DAPI; 20 μM) or propidium iodide (PI; 500 ng/mL) (both from Sigma-Aldrich) were used to determine cell viability. A total of 10,000 cells were acquired in the live gate on a FACSCanto (BD Biosciences, San Jose, Calif.) and further analyzed using FlowJo software (BD Biosciences, San Jose, CA, USA).

IL-8 ELISA

IL-8 concentrations were determined in culture supernatants collected after 24 h of culture using an IL-8 ELISA (Invitrogen Life Technologies, Breda, The Netherlands). In brief, flat-bottom EIA/RIA 96-well plates (Costar, Corning Inc.) were coated at 4 °C overnight with aniti-IL-8 antibody (1:1000, 893A6G8, Invitrogen) diluted in carbonate buffer (0.5M, pH 9.6). Plates were washed with PT (PBS-0.1% v/v TWEEN 20) and blocked with PTB (PT-1% w/v bovine serum albumin) at 37 °C for 1 h. Next, wells were incubated with culture supernatants (commonly diluted 10 times in PT) or recombinant IL-8 for 1 h, washed thrice with PT, and incubated for 1 h with IL-8 detection antibody (1:1000, 790A28G2, Invitrogen) diluted in PTB. Subsequently, wells were washed thrice and incubated for 45 min with strep-poly HRP (1:10000, M2032, Sanquin, The Netherlands) diluted in PT containing 2% protifar (Nutricia, Netherlands). Finally, plates were washed five times and developed with 3,3′,5,5′-tetramethylbenzidine (TMB, Merck, Darmstadt, Germany). The reaction was stopped by adding an equal amount of 1M H2SO4. Absorbance was measured at 450 nm with reference at 655 nm by using a VersaMax microplate reader (Molecular devices, California, CA, USA).

Neutrophil Elastase ELISA

Neutrophil elastase (NE) concentration was analyzed in culture supernatants collected after 2 h of cell culture as described in Souwer et al [3]. Briefly, flat-bottom EIA/RIA 96-well plates were coated overnight with polyclonal rabbit IgG directed

against NE (1.5 ng/mL, Sanquin) at 4 °C. Plates were washed with PT thrice before adding culture supernatants, usually diluted 250 times in PTG (PT-0.2% v/w gelatin), and incubated for 1 h. Next, plates were washed with PT and incubated for 1 h with biotinylated rabbit anti-human elastase diluted in PTG (1 ng/mL, Sanquin blood Supply). Subsequently, after a washing step, plates were incubated for 30 min with streptavidin-peroxidase diluted 1:1000 in PTG (Amersham Life Science, Buckinghamshire, UK). Finally, the ELISA was completed using TMB as a substrate and measured as described above.

NETosis Assay

NET formation (NETosis) was analyzed using an Incucyte S3 Live-Cell Analysis System (Essen BioScience, Newark, UK) and a previously described IncuCyte® NETosis assay [56]. Briefly, neutrophils were seeded at a density of 1.0×10^5 cells/mL in 200 µL in a 96-well IncuCyte® Imagelock plate (Essen BioScience) in IMDM medium containing 10% HI-FBS and gentamycin, and incubated with the above indicated stimuli, or with 100 ng/mL PMA (a well-known NET-inducing agent) for 16 h. The cell-impermeant nucleic acid binding dye YOYO™-3 Iodide (invitrogen Carlsbad, California, CA, USA) was present at 400 nM in the medium to stain free available DNA. Neutrophils were imaged every 15 min using phase contrast and red fluorescent exposure channels present in the IncuCyte, using a 20× dry objective lens. Data were analyzed using the IncuCyte Basic Software (Essen BioScience). Neutrophils were identified by phase contrast, objects smaller than 100 μm^2 were excluded as cells. Neutrophils that underwent apoptosis were identified by red staining of the cell, neutrophils undergoing NETosis were identified by red staining in the cell and red staining visibly crossing the cell membrane, this was marked by red fluorescent objects larger than 400 μm^2 in area. For the red fluorescent channel, edge sensitivity was set to 0 and hole fill was set to 100 μm^2. The TopHat method and the edge split tool were used for background correction and for accurate quantification of individual cells. NETosis was quantified by the total signal of red fluorescent area of objects >400 μm^2 per well.

Phagocytosis Assay

Neutrophils were seeded at a density of 1×10^6 cells/mL in 1 mL in a flat bottom 24-well plate in IMDM medium containing 10% HI-FBS and gentamycin, and stimulated with the above indicated stimuli for 2 h at 37 °C or at 4 °C in the presence

of 0.0003% w/v FITC-marked microparticles based on melamine resin (FITC-beads; Sigma-Aldrich). After stimulation, neutrophils were harvested, washed twice in cold PBA buffer, and labeled in PBA buffer using the following antibodies: αCD16-PECy7 (1:1000; 3G8), αCD63-APC (1:100; H5C6), αCD66b-PE (1:100; G10F5) (all Biolegend). Analysis was done using flow cytometry and using advanced imaging flow cytometry. For this latter analysis, a total of 100,000 cells were acquired with an Amnis ImageStream (Luminex, TX, USA) and further analyzed using IDEAS software (Amnis, Washington, DC, USA).

EV Isolation and Fluorescent Labeling

To prepare EV-depleted FBS, 30% FBS in IMDM was ultracentrifuged for 16 h at 100,000× g in an SW32 rotor (Beckman Coulter, Fullerton, CA). For isolation of EVs, 6×10^6 neutrophils were cultured per stimulation condition for 2 h in 24-well plates (Costar, Corning), 1×10^6 neutrophils per well in 1 mL IMDM medium containing 1% EV-depleted FBS. After 2 h, culture supernatants were collected gently to minimize co-isolation of neutrophils, and sequentially subjected to differential centrifugation and floatation into sucrose gradients as described previously [32]. Briefly, supernatants were centrifuged twice at 200× g for 10 min, and twice at 500× g for 10 min. Subsequently, EVs were pelleted by ultracentrifugation of the 500× g supernatant for 30 min at 10,000× g (10 kg EVs) using an SW40 rotor (Beckman Coulter), and by ultracentrifugation of the collected 10,000× g supernatant for 65 min at 100,000× g (100 kg EVs) using an SW40 rotor in a Beckman-Coulter ultracentrifuge at 4 °C. EV-containing pellets were resuspended in 20 µL PBS containing 0.2% EV-depleted bovine serum albumin (BSA) and labeled with 7.5 mM PKH67 (Sigma, St. Louis, MO, USA) in 180 µL diluent C. The reaction was stopped by adding 100 µL of IMDM with 10% EV-depleted FBS. PKH67-labeled pellets were mixed with 1.5 mL 2.5 M sucrose and a linear sucrose gradient (2.0–0.4 M sucrose in PBS) was added on top in a SW40 rotor. Gradients were centrifuged at 192,000× g (average) for 16–17 h at 4 °C, after which 1 mL gradient fractions were collected by pipetting from the top of the tube. Densities of all fractions were determined by refractometry.

Western Blotting

Gradient sucrose fractions from EV isolation 1–3 (1.27 g/mL–1.24 g/mL), 4–6 (1.22 g/mL–1.18 g/mL), 7–10 (1.16 g/mL–1.10 g/mL), and 11–12 (1.08 g/mL–1.06 g/

mL) were pooled for protein identification by Western blotting and diluted in PBS in SW40 tubes (Beckman Coulter, Fullerton, CA, USA). EVs were pelleted at 125,755 g for 70 min at 4 degrees. Pellets were resuspended in 30 μL non-reducing SDS-PAGE sample buffer (0.25M Tris/HCl, pH 6.8, 40% v/v glycerol, 8% w/v SDS, 0.01% w/v bromophenol blue), heated at 100 degrees, run on pre-cast gel (Criterion TGX Gels, BioRad, CA, USA) and transferred onto a 0.2 μm polyvinyldidene difluoride membrane. After blocking for 1 h in blocking buffer (0.2% Fish skin gelatin +0.1% Tween-20), blots were incubated overnight at 4 °C with primary antibodies against CD9 (clone HI9, Biolegend, San Diego, CA, USA; dilution 1:1000) and CD63 (clone H5C6, BD Bioscience, dilution 1:500,) in blocking buffer. Washed three times in PBS-tween and incubated for 1 h with HRP-coupled secondary antibody (Jackson ImmunoResearch, PA, USA; dilution 1:10000). Blots were washed again three times in PBS-Tween, followed by two washes in plain PBS and incubation with ECL solution (ThermoScientific, SuperSignal West Dura Extended Duration Substrat, cat. 34075). Blots were analyzed using BioRad Chemidoc imager (BioRad, CA, USA) and Rad Image Lab V5.1 software (BioRAD).

3

Neutrophil EV Quantification by High-Resolution Flow Cytometry

High-resolution flow cytometric analysis of PKH67-labeled EVs was performed using an optimized jet-in-air-based BD influx flow cytometer that was dedicated and optimized for detection of submicron-sized particles (BD Biosciences, San Jose, CA, USA). Detailed descriptions of both the hardware adaptions and methods used were previously described in detail [32,57]. Forward scatter was detected with a collection angle of 15–25° (reduced wide-angle forward scatter (rw-FSC)). Briefly, fluorescence threshold triggering was applied to distinguish PKH67-labeled EVs from non-fluorescent events. PKH67 was excited with a 488 nm laser (Sapphire, Coherent 200 mW) and fluorescence was collected through a 530/40 bandpass filter. Prior to EV-measurements fluorescent polystyrene 100 and 200 nm yellow-green (505/515) FluoSphere beads (Invitrogen, Carlsbad, CA, USA) were measured to calibrate the fluorescence and rw-FSC settings in order to minimize day to day variations. The instrument was aligned until predefined MFI and scatter intensities were reached with the smallest possible coefficient of variation for rw-FSC, SSC and fluorescence. After optimal alignment, PMT settings required no or minimal day to day adjustment. For EVs a fluorescence threshold was set allowing an event rate of 10–20 events/second while acquiring a clean PBS sample. For quantitative analysis of PKH67-labeled EVs

sucrose gradient fraction between 1.06 g/mL and 1.20 g/mL were diluted at least 20× in PBS prior to analysis to keep the event rate <10,000/s to avoid coincident particle detection and occurrence of swarm [58]. Upon loading on the influx, the sample was boosted into the flow cytometer until events appeared, after which the system was allowed to stabilize for 30 s. Measurements were performed by a fixed 30 s time. Scatter and EVs count data were analyzed using FlowJo software (BD Biosciences).

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was done in Graphpad Prism version 8.3.0 for Windows by using statistical tests, depending on the experimental data. For multiple comparisons p-values were calculated on selected pairs either using a one-way ANOVA or mixed-effects analysis with Tukey post-test correction or using Friedman tests with Dunn post-test correction. For single comparisons p-values were calculated using paired t-tests. p-values < 0.05 were considered as statistically significant.

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Graphical abstract was created with BioRender.com

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Figures

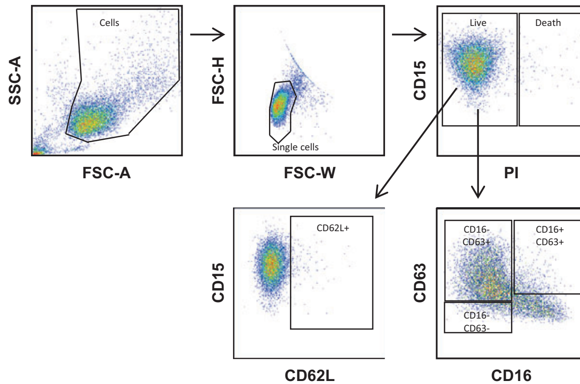

Figure S1. Flow cytometry gating strategy for neutrophil degranulation. Neutrophils were first gated on forward scatter(FSC-A) and side scatter (SSC-A) plot. Then single cells were gated and then live and death cells were gated. With the live cells gates were set for activation markers.

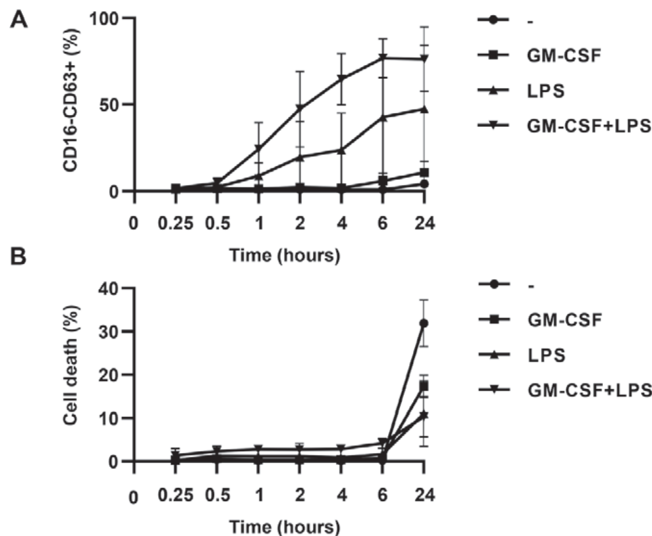

Figure S2. Neutrophils were cultured in the absence or presence of GM-CSF (50 U/mL), LPS (10 ng/mL), or their combination. After different culture durations flow cytometry was used to asses activation status. (a) Full degranulation over time, measured by percentages of CD16-CD63+ neutrophils. (b) Cell death over time (n = 3–5 per time point). Data are presented as mean ± SD.

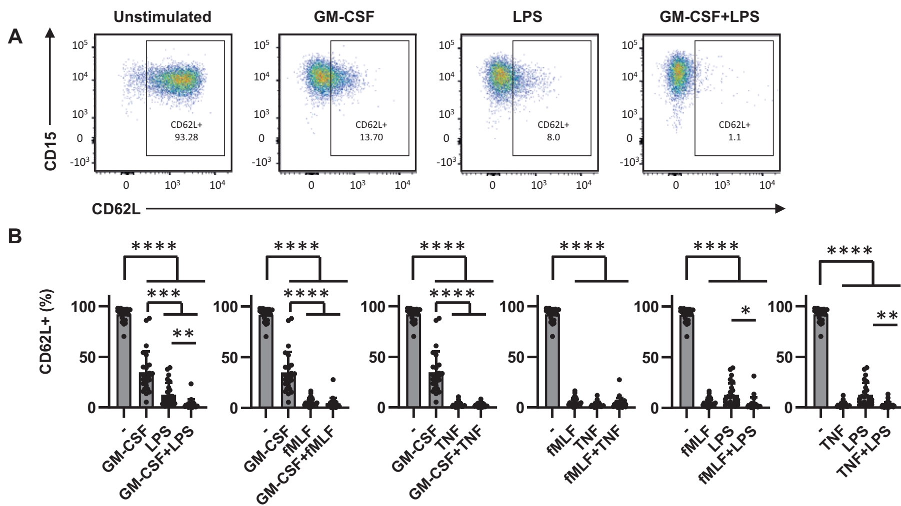

Figure S3. (A) Flow cytometry plot demonstrating gating strategy to determine CD62L membrane expression. **(B)** Neutrophils were cultured for 2 h in the absence or presence of different stimuli (GM-CSF (50 U/mL), LPS (10 ng/mL), fMLF (1 μM), and/or TNF (1 ng/mL)). CD62L membrane expression, expressed by percentages of CD62L+ neutrophils n = 20. Data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, One-way ANOVA.

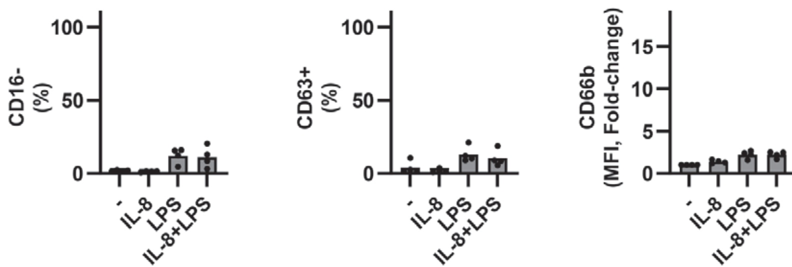

Figure S4. Neutrophils were cultured for 2 h in the absence or presence of LPS (10 ng/mL) and/or IL-8 (100 ng/mL)). CD16- and CD63+ membrane expression, expressed by percentages of CD16- and CD63+ neutrophils. CD66b membrane expression, as measured by changes in mean fluorescent intensity (MFI) of CD66b, expressed in fold-change compared to unstimulated. n = 4. Data are presented as mean ± SD.

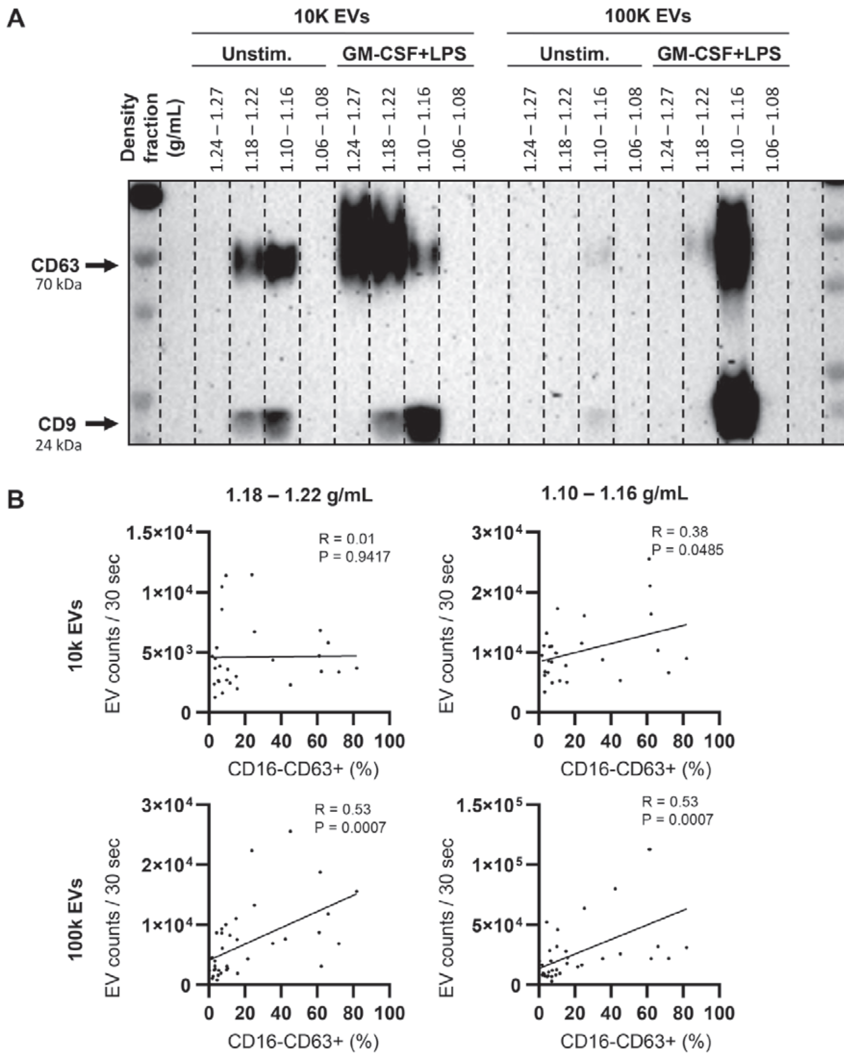

Figure S5. (a) Prolonged exposure of the western blot of Figure 6A of EVs pelleted at 10kg and 100 kg and floated in a sucrose density gradient from neutrophils from a representative donor. Analysis is shown for CD9 and CD63 (tetraspanins; general EV-markers). (b) Linear relationships were determined between EV release shown in (Figure 6B) and full degranulation. Linear regression was applied to determine R.

“Escaping goblins to be caught by wolves!” he said, and it became a proverb though we now say “out of the frying-pan into the fire” in the same sort of uncomfortable situations.

J.R.R. Tolkien, The Hobbit

CHAPTER 4

Dexamethasone, but Not Vitamin D or A, Dampens the Inflammatory Neutrophil Response to Protect At-risk COVID-19 Patients

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Abstract

Dexamethasone (DEX) was the first drug shown to save lives of critically ill COVID-19 patients suffering from respiratory distress. A hyperactivated state of neutrophils was found in COVID-19 patients compared to non-COVID pneumonia cases. Given the beneficial effects of DEX in COVID-19 patients, we investigated the effects of DEX and of other immunomodulatory drugs vitamin D3 (VD3) and retinoic acid (RA) on neutrophil function. DEX, but not VD3 or RA, significantly inhibited all tested aspects of neutrophil function, e.g. degranulation, intracellular ROS production, CXCL8 release and NETosis. Interestingly, RA displayed the opposite effect by significantly increasing both CXCL8 and NET release by neutrophils. Taken together, these data suggest that the lower COVID-19 mortality in DEX-treated patients may in part be due to the dampening effect of DEX on the inflammatory neutrophil response, which could prevent neutrophil plugs with NETS in the lungs and other inflamed organs of patients.

Keywords

Neutrophils; COVID-19; Dexamethasone; Vitamin D3; Retinoic Acid

Introduction

COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was officially declared a global pandemic in March 2020 by the World Health Organization[1], with currently over 353 million confirmed cases and 5.6 million associated deaths[2]. While the majority of COVID-19 patients is asymptomatic or shows mild symptoms, one-fifth of patients will develop severe illness, symptoms including acute respiratory distress syndrome, sepsis and multiorgan failure[3]. An elevated neutrophil-to-lymphocyte ratio (NLR) has been identified as an early risk factor for severe COVID-19[4]. Severe COVID-19 is characterized by a cytokine storm, to which pro-inflammatory monocytes and neutrophils contribute[5]. Neutrophils in the lungs are both enriched and in a hyperactivated state, with upregulated IL-1β and CXCL8 expression, in COVID-19 patients compared to non-COVID pneumonia cases[5]. Neutrophil plugs with NETs were notably present in the lungs and other inflamed organs such as the heart, kidney and brain of deceased COVID-19 patients, affirming an elevated activation status of neutrophils[6,7]. Therefore, targeting the excessive neutrophil inflammatory response could be a crucial step in lowering the probability of progression to severe respiratory distress and eventually organ failure in COVID-19 patients.

Dexamethasone (DEX), an inexpensive and commonly applied corticosteroid, was the first drug shown to save lives of people suffering from severe COVID-19 in a large randomized, controlled trial[8,9]. The effect of DEX was most pronounced in patients on ventilators amongst whom deaths were reduced by one-third[10]. In contrast, no effect was observed in people without respiratory distress. Therefore, treatment guidelines recommend administration of DEX only in hospitalized patients who require supplemental oxygen. DEX is regarded as a potent general immunosuppressive drug[8], which reduces CXCL8 and TNF expression in neutrophils[11,12]. How DEX affects other aspects of neutrophil function is less well-known. In addition to DEX, vitamin D3 (VD3) supplementation has been proposed as a beneficial strategy to reduce the impact of COVID[13]. Furthermore, it has been suggested that retinoic acid (RA) metabolism is defective during the COVID-19 cytokine storm, which causes excessive cytokine release[14,15]. Hence, RA supplementation could also be considered for treatment. However, little is known about the effects of these immunosuppressive drugs on neutrophil function.

Therefore, we investigated the effects of DEX, VD3 and RA on function of human neutrophils by determining degranulation, CXCL8 release and intracellular ROS production upon stimulation with TLR7/8 ligand Resiquimod (R848)[16] and TNF. Furthermore, we assessed the effects of these drugs on PMA-induced NETosis. We

found that DEX dampens all aspects of neutrophil function assessed in this study. In contrast, VD3 did not affect function. Interestingly, RA did not alter degranulation and ROS production, but increased CXCL8 release and NETosis. Taken together, these data support a potential neutrophil dampening role for DEX, thereby providing a rationale for the use of DEX in treatment of critically ill COVID-19 patients.

Material & Methods

Neutrophil isolation

Blood was collected from healthy volunteer donors after informed consent. The blood collection protocol was approved by the institutional review board of the Amsterdam Medical Centre (METC 2015_074). Neutrophils were isolated using a density gradient followed by erythrocyte lysis, as previously described[17]. Neutrophils were then resuspended in IMDM (Gibco; Thermo Fischer Scientific Inc, Waltham, Mass) supplemented with 10% heat inactivated (HI) fetal bovine serum (FBS; Hyclone; Thermo Fischer Scientific Inc, Waltham, Mass) and gentamycin (86 µg/mL; Duchefa Biochemie B.V., Haarlem, The Netherlands) and used immediately. Neutrophil purity was analyzed by flow cytometry and was always > 97%.

Neutrophil culture, stimulation and flow cytometric analysis

Neutrophils were seeded at a density of 0.5 x 10^6 cells/mL in 200 µL in a flat bottom 96-well plate (Costar, Corning Inc. Corning, NY) in IMDM medium containing 10% HI-FBS and gentamycin. Subsequently, neutrophils were pretreated for 30 minutes with DEX (40 nM; #D2915 from Merck), VD3 (2.5 µM; #17936 from Sigma-Aldrich), RA (10 µM; #R2625 from Sigma-Aldrich), or controls medium, ethanol or DMSO, respectively. Then R848 (1 µg/mL; Invivogen, USA) and TNF (1 ng/mL; Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) were added and neutrophils were cultured for 2 hours (degranulation), or 24 hours at 37 °C. 24-hour culture supernatants were collected for the analysis of neutrophil CXCL8 release, by ELISA (Invitrogen Life Technologies, Breda, The Netherlands), as described previously[17]. For assessment of ROS production, neutrophils were stimulated for 1 hour with R848 (500 ng/mL) and TNF (250 pg/mL) in the presence of 250 nM 123-dihydrorhodamine (123-DHR; Marker Gene Technologies, OR, USA), after 15 minutes pretreatment with drugs or controls. For flow cytometric analysis of CD16, CD63, and CD66b cells were washed after stimulation, stained and analyzed as previously described[17].

NETosis assay

NETosis was analyzed using an Incucyte S3 Live-Cell Analysis System (Essen BioScience, Newark, UK) and a previously described IncuCyte® NETosis assay[18]. Briefly, neutrophils were seeded at a density of 1.0 x 10^5 cells/mL in 200 µL in a 96-well IncuCyte® Imagelock plate (Essen BioScience) in IMDM medium containing 10% HI-FBS and gentamycin, and incubated for 15 minutes in the presence of DEX (40 nM), VD3 (2.5 µM) or RA (10 µM) or controls medium, ethanol or DMSO, respectively. 1.5 ng/mL PMA was added after 15 minutes and neutrophils were incubated for 12 hours in presence of the cell impermeant nucleic acid binding dye YOYO™-3 Iodide (Invitrogen). Neutrophils were imaged every 15 minutes using phase contrast and red fluorescent exposure channels, using a 20x dry objective lens. Data were analyzed using the IncuCyte Basic Software (Essen BioScience), with the same parameters as previously described[17].

4

Statistical analysis

Data are expressed as mean ± SD or as mean. Statistical analysis was done in GraphPad Prism version 9.1.0 for Windows by using statistical tests, depending on the experimental data. The Shapiro-Wilk test was performed to test normality of data. For multiple comparisons, p-values were calculated on selected pairs (drug versus vehicle control) using a one-way ANOVA with Holm-Sidak's post hoc correction on raw data. For single comparisons, p-values were calculated using two-tailed paired t-tests. P-values < 0.05 were considered statistically significant.

Results and Discussion

Neutrophil degranulation is dampened by DEX, but not VD3 or RA

A hyperinflammatory response of neutrophils is associated with severe COVID-19[19]. Since drugs that dampen neutrophil activation may be useful in fighting SARS-CoV-2 infection, we studied whether DEX, VD3 or RA affect neutrophil degranulation by analyzing CD16 (FCγRIII), CD63 and CD66b membrane expression. Fusion of azurophilic granules with the plasma membrane increases CD63 expression, while CD66b indicates specific and gelatinase granules[20,21], and CD16 is cleaved from the surface upon the release of secretory vesicles[20–23]. Neutrophils were stimulated with R848 and TNF, a mimic for viral activation, in the presence of DEX, VD3, RA or

relevant controls (medium, ethanol or DMSO, respectively) and we titrated the drugs to determine the used concentration in all experiments (**Supplementary Fig. 1**). Data were obtained by flow cytometry and were analyzed with the gating strategy shown in **Fig. 1A**. Exposure of stimulated neutrophils to DEX, resulted in significant inhibition of CD16 cleavage from the membrane, while this was not affected by VD3 and RA compared to vehicle controls (**Fig. 1B**). Furthermore, DEX significantly decreased CD63 expression (**Fig. 1C**), while none of the immunomodulatory drugs affected CD66b membrane expression (**Fig. 1D**). Ethanol alone reduced CD16 cleavage and CD63 expression (**Supplementary Fig. 1, Fig. 1B, C).** These data indicate that DEX predominantly dampens degranulation of azurophilic granules (CD63) and secretory vesicles (CD16), rather than specific and gelatinase granules. Taken together, our study is the first to demonstrate that neutrophil degranulation is restricted by DEX, while VD3 an RA have no effect on degranulation. Administration of DEX to hospitalized COVID-19 patients may reduce hyperinflammatory neutrophil degranulation.

CXCL8 release is dampened by DEX and strengthened by RA

During infection neutrophils release many different mediators, including CXCL8 which is an important chemoattractant for neutrophils[22,24,25]. We analyzed the release of CXCL8 after 24 hour-stimulation with R848 and TNF. In line with previous reports[11], CXCL8 release by neutrophils in presence of DEX was decreased by approximately 50%, with an average of 1.02 ± 0.52 ng/mL (mean ± SD) CXCL8 release by DEX-treated neutrophils versus 2.24 ± 1.14 ng/mL by medium control neutrophils (**Fig. 2**). Surprisingly, RA significantly increased CXCL8 release by stimulated neutrophils by 1.5-fold, whereas VD3 did not influence CXCL8 release (**Fig. 2).** This RA-induced effect on neutrophil function was not found for degranulation. CXCL8 is synthesized de novo upon activation and is thus regulated differently than degranulation, where granules are already pre-stored in the neutrophils and rapidly released within two hours. This could underlie the variable effects of RA on different aspects of neutrophil function. The opposite effects of RA and DEX on CXCL8 release by neutrophils could be due to opposite effects on NFκB activity. NFκB transcription factors are the main regulators of CXCL8 transcription[26,27]. Corticosteroids, including DEX, inhibit CXCL8 transcription via repression of NFκB activity[27]. Reduced expression of NFκB transcription factors by DEX was confirmed in human neutrophils[28]. Elevated CXCL8 secretion upon RA treatment is possibly due to increased NFκB activity, which was shown in human keratinocytes[26]. However, to our knowledge, increased CXCL8 release by RA was not previously shown in neutrophils.

Collectively, our data show that similar to degranulation, CXCL8 release is dampened by DEX and VD3 had no effect. Interestingly, RA increased CXCL8 secretion, whereas no effect of RA was observed on neutrophil degranulation.

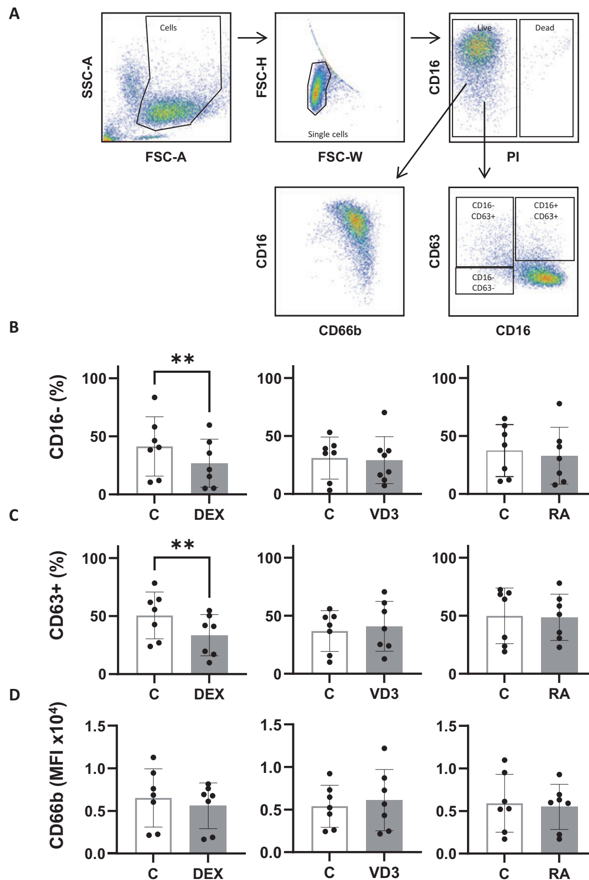

Figure 1: Neutrophil degranulation is dampened by DEX, but not VD3 or RA.

Neutrophils were pretreated with DEX, VD3, RA or their respective controls medium, ethanol or DMSO (all controls abbreviated as C in figures), and cultured for 2 hours in the presence of R848 and TNF. (**A**) Flow cytometry plot demonstrating gating strategy to determine neutrophil degranulation. Neutrophils were gated on forward scatter (FSC-A) and side scatter (SSC-A), followed by a single cell and live gate from which the expression of CD16, CD63 and CD66b was assessed (**B**) Secretory vesicle degranulation as measured by percentage of CD16- neutrophils is depicted. (**C**) Azurophilic degranulation as measured by percentage of CD63+ neutrophils is shown. (**D**) Degranulation of specific and gelatinase granules is depicted as mean fluorescent intensity (MFI) of CD66b. Data are representative of 7 independent experiments and are presented as mean ± SD. ** $p < 0.01$.

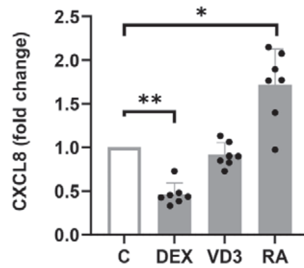

Figure 2: CXCL8 release by neutrophils is affected by DEX and RA
Neutrophils were stimulated by R848 and TNF and in the presence of DEX, VD3, RA or relevant controls. CXCL8 was measured in 24-hour culture supernatants (n=7). Data are shown presented as mean ± SD relative to controls. *p < 0.05, ** p < 0.01.

ROS production is reduced by DEX

In addition to degranulation and CXCL8 secretion, neutrophil ROS production is important in the clearance of unwanted pathogens[29]. However, it has been suggested that excessive ROS production by neutrophils during COVID-19 exacerbates the host immunopathological response resulting in tissue damage[30]. Intracellular ROS production was determined by flow cytometry using the ROS indicator 123-DHR in R848/TNF-stimulated neutrophils in the absence or presence of DEX, VD3 or RA (**Fig. 3A**). Similar to neutrophil degranulation and CXCL8 release, intracellular ROS production was significantly reduced in neutrophils exposed to DEX. Accordingly, neutrophils from human volunteers injected with DEX were shown to exhibit lower extracellular ROS generation[31]. In contrast, neutrophils stimulated in the presence of RA or VD3 showed no difference in ROS production compared to neutrophils stimulated with relevant controls, neither when assessing the percentage of intracellular ROS+ cells or the mean fluorescence intensity (MFI) of neutrophils (**Fig. 3B and C**). RA was previously shown to increase N-formyl-methionyl-leucyl-fenylalanine (fMLF)-stimulated production of intracellular ROS[32], but we did not find an effect on intracellular ROS production, which could be stimulus-dependent. We used a double stimulus rather than a single stimulus for optimal neutrophil activation, which is more physiologically relevant than single stimuli given that cells encounter a plethora of pro-inflammatory cytokines and microbial or viral components[17]. Our data indicate that DEX restricts ROS production in neutrophils, again demonstrating anti-inflammatory potential of DEX on neutrophil functions.

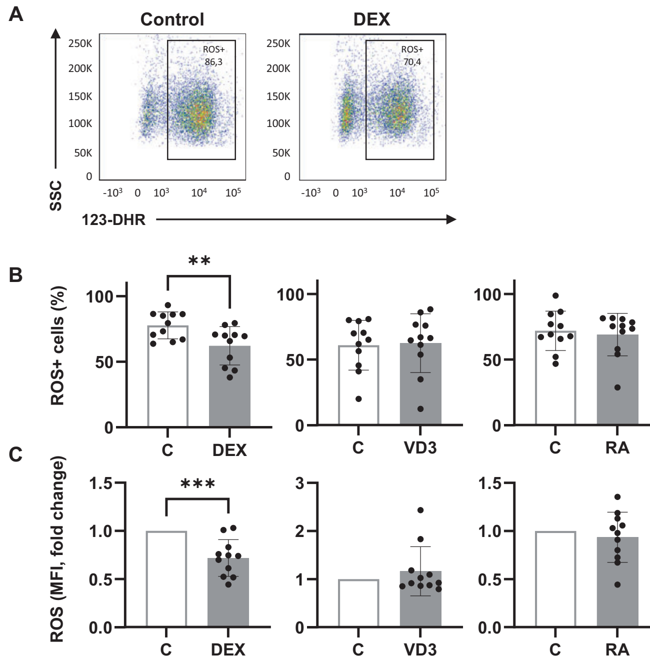

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Figure 3: ROS production in neutrophils is reduced by DEX, but not VD3 or RA
Neutrophils were incubated for 1 hour in the presence of 123-DHR and activated by R848 and TNF, in the presence of DEX, VD3, RA or relevant controls. ROS production was analyzed using flow cytometry. (**A**) Representative flow cytometry image of neutrophils incubated with DEX or relevant control. (**B-C**) Effects of DEX, VD3 and RA on ROS production (n=11). (**B**) Intracellular ROS generation is expressed as percentage of ROS+ cells. (**C**) Total ROS production is expressed as MFI of 123-DHR relative to controls, with MFI 6166 ± 3636 (mean ± SD) medium control, 4252 ± 3103 ethanol control and 5998 ± 5398 DMSO control. Data are presented as mean ± SD. ** $p < 0.01$ and *** $p < 0.005$.

DEX reduces and RA increases NETosis

NETosis is a mechanism used by neutrophils to entrap and kill pathogens through the release of nuclear and granular content that forms a network[33]. Although NETosis is important as an antimicrobial function, it requires tight regulation, since excessive NETosis can lead to severe tissue damage and exacerbation of inflammation[34,35]. Neutrophil plugs with NETs were found in deceased COVID-19 patients and NET quantity correlates to disease severity[6,7,36]. To examine whether DEX, VD3 or RA could dampen NETosis in neutrophils, neutrophils were incubated with 1.5 ng/mL PMA in the absence or presence of DEX, VD3 or RA. With time-lapse immunofluorescence

microscopy we analyzed NETosis (**Fig. 4A**). Maximal NETosis was observed after 4 hours of PMA-stimulation, which was significantly reduced by DEX. VD3 did not have any effect on NETosis, while NETosis was increased by RA (**Fig. 4 B-C**). Reduced NET release in presence of DEX was reported upon stimulation of neutrophils with Staphylococcus aureus, but not with PMA[37]. However, we used a 20-fold lower dose of PMA, possibly allowing DEX to interfere with NETosis. RA was previously shown to enhance both PMA- and fMLF-induced NETosis[32]. Similar to CXCL8 release, we observed opposite effects of RA and DEX on NETosis. It has been shown that inhibition of the NFκB pathway reduces NETosis[38]. Hence, the differential effects of RA and DEX on NFκB activity could underlie their observed effects on NETosis. Moreover, peptidyl arginine deiminase 4 (PAD4) plays a critical role in the formation of NETs[39] and it has been shown that corticosteroid treatment of rheumatoid arthritis patients decreases synovial expression of PAD4[40], indicating that DEX may affect PAD4 expression in neutrophils. In contrast, treatment of acute promyelocytic leukemia cells with RA, to differentiate them into granulocytic cells, increases PAD4 expression[41]. Taken together, distinct effects of DEX and RA on NFκB and PAD4 activity could underlie the opposing effects of these drugs on NETosis.

In this study, we confirmed the well-established anti-inflammatory effect of DEX on CXCL8 release[11,12] and importantly, we show that this dampening effect of DEX extends to other aspects of neutrophil function, including intracellular ROS production, degranulation and NETosis. We observed no effects of VD3 on neutrophil function when compared to the vehicle control (ethanol), while neutrophils do express mRNA of the VD3 receptor[42]. The effects of VD3 on neutrophils are rarely studied and results are contradictory, e.g. elevated versus decreased CXCL8 release by VD3 treatment[43]. A limitation of our study is that we did not use (pseudo)-SARS-CoV-2 as stimulus for neutrophils. Although neutrophils may not be infected by SARS-CoV-2[44], its components, e.g. nucleocapsid, spike proteins or ssRNA, may activate neutrophils. Purified nucleocapsid and spike proteins from SARS-CoV-2 were shown to induce NETosis, while they did not increase intracellular ROS production[45]. The effect of these proteins on the release of other neutrophil derived factors, e.g. granules and CXCL8, remains to be established. Here, we used R848 in combination with TNF to activate neutrophils. Our earlier work showed that two different stimuli are needed for optimal neutrophil activation[17]. R848 is a synthetic ligand that activates TLR7 and TLR8, the latter expressed by neutrophils, which recognize ssRNA[46]. TNF is an important modulator of immune responses, including the response to viruses.

Therefore, neutrophil stimulation with R848 and TNF may represent an attractive model to study candidate drugs for dampening neutrophil activation in COVID-19.

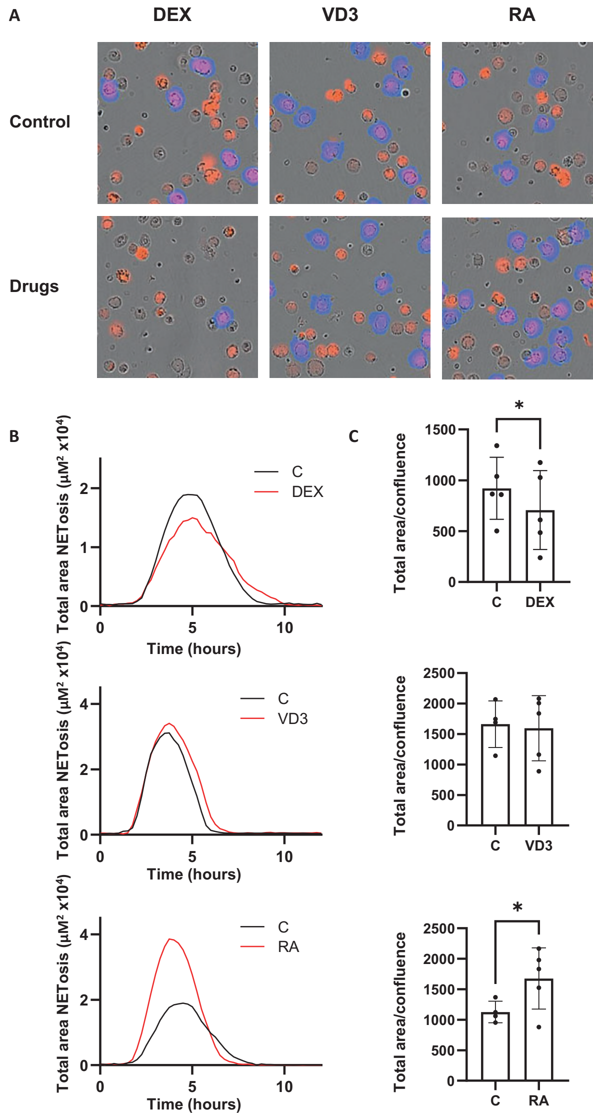

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Figure 4: DEX reduces, while RA increases NETosis
Neutrophils were stimulated for 12 hours with PMA (1.5 ng/mL) in the presence of DEX, VD3, RA or relevant controls as well as a cell impermeable fluorescent DNA-binding dye. Fluorescence

Figure 4. Continued

was measured by time-lapse immunofluorescence microscopy every 15 minutes to determine NETosis. (**A**) Overlays of phase contrast and fluorescence images showing accessible DNA in red, and extracellular DNA (>400 μm^2) in blue (NETs). Images are representative of 5 independent experiments. (**B**) NETosis was determined as fluorescence signal area in μm^2 per well shown in a 12 hour time course. Only areas larger than 400 μm^2 were used for calculations (n=5). Data are presented as mean. (**C**) Normalized NETosis expressed as total fluorescence area divided by cell confluence after 4 hours of stimulation is depicted (n=5). Data are presented as mean ± SD. * $p < 0.05$.

Taken together, our data support previous reports on a pro-inflammatory effect of RA on neutrophils and this may be of importance to treatment of neutrophil immunodeficiencies[47], while caution is warranted for potential use as a tolerogenic adjuvant in autoimmune disorders or other diseases associated with hyperactivation of neutrophils, such as COVID-19[19,43,48]. The anti-inflammatory effect of DEX on neutrophil function supports the use of DEX in hospitalized COVID-19 patients suffering from respiratory distress.

Acknowledgements

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Conflicts of Interest

The authors declare no potential conflicts of interest.

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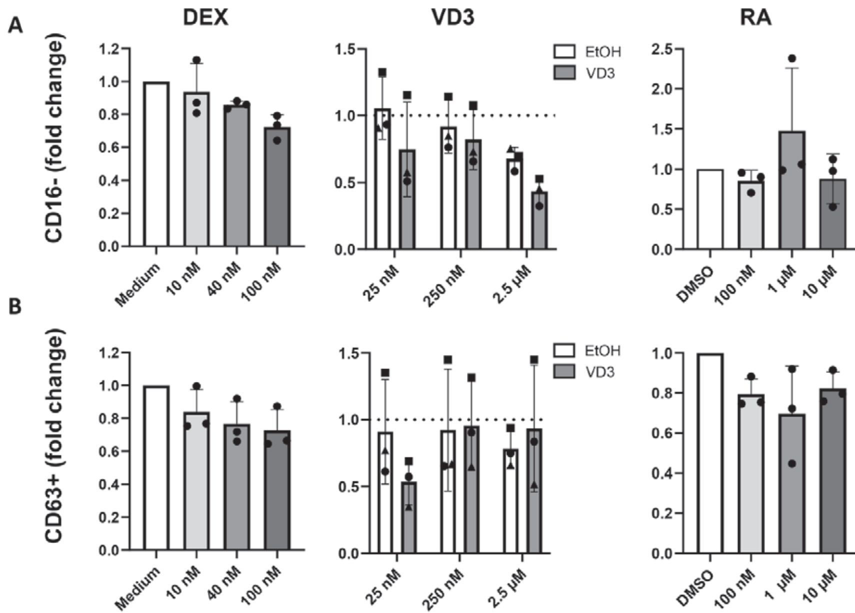
Supplementary Figures



Supplementary Figure 1: DEX dose-dependently inhibits neutrophil degranulation Neutrophils were pretreated with DEX, VD3, RA or their respective controls medium, ethanol or DMSO and cultured for 2 hours in the presence of R848 and TNF. (A) Secretory vesicle degranulation as measured by percentage of CD16- neutrophils is depicted, normalized to controls, mean ± SD. In the middle panel, data is normalized to medium control (35.2 ± 21.0 % CD16- neutrophils), indicated by the line at 1 and each donor is represented by a different symbol. Grey bars indicate VD3, while respective ethanol dilutions are shown in white bars. (B) Azurophilic degranulation as measured by percentage of CD63+ neutrophils is shown, normalized to controls, mean ± SD, with medium control 27.0 ± 14.3 % CD63+ neutrophils). Three independent experiments were performed.

There is nothing like looking, if you want to find something. You certainily usually find something, if you look, but it is not always quite the something you were after.

J.R.R. Tolkien, The Hobbit

CHAPTER 5

Hyaluronic acid in synovial fluid prevents neutrophil activation in spondyloarthritis

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Abstract

Spondyloarthritis (SpA) patients suffer from joint inflammation resulting in tissue damage, characterized by the presence of numerous neutrophils in the synovium and synovial fluid (SF). As it is yet unclear to what extent neutrophils contribute to the pathogenesis of SpA, we set out to study SF neutrophils in more detail. We analyzed the functionality of SF neutrophils of 20 SpA patients and 7 disease controls, determining ROS production and degranulation in response to various stimuli. In addition, the effect of SF on neutrophil function was determined. Surprisingly, our data show that SF neutrophils in SpA patients have an inactive phenotype, despite the presence of many neutrophil-activating stimuli such as GM-CSF and TNF in SF. This was not due to exhaustion as SF neutrophils readily responded to stimulation. Therefore, this finding suggests that one or more inhibitors of neutrophil activation may be present in SF. Indeed, when blood neutrophils from healthy donors were activated in the presence of increasing concentrations of SF from SpA patients, degranulation and ROS production were dose-dependently inhibited. This effect was independent of diagnosis, gender, age, and medication in the patients from which the SF was isolated. Treatment of SF with the enzyme hyaluronidase strongly reduced the inhibitory effect of SF on neutrophil activation, indicating that hyaluronic acid that is present in SF may be an important factor in preventing SF neutrophil activation. This finding provides novel insights into the role of soluble factors in SF regulating neutrophil function and may lead to the development of novel therapeutics targeting neutrophil activation via hyaluronic acid or associated pathways.

Keywords

Neutrophils; Neutrophil activation; Spondyloarthritis; Synovial fluid; Hyaluronic acid

Introduction

Chronic inflammatory joint diseases are highly prevalent with estimates ranging from 5-20% depending on the subtypes included[1]. Besides the most common type of arthritis, i.e. rheumatoid arthritis (RA), spondyloarthritis (SpA) is the second most prevalent inflammatory joint disease that affects roughly 0.2 – 1.6% of the world population[2]. SpA patients suffer from pain and stiffness in the spine and/or peripheral joints due to inflammation and structural damage. They may also exhibit inflammation of the bowel mucosa, eyes (i.e. anterior uveitis, and the skin (i.e. psoriasis)[3–6]. Current effective treatments are non-steroidal anti-inflammatory drugs (NSAIDs) aimed at inhibiting cyclooxygenase, conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) like methotrexate or sulfasalazine (for peripheral joints), and biological or targeted synthetic DMARDs (bDMARDs) that block pro-inflammatory cytokines such as tumor necrosis factor (TNF), or IL-17A, or intracellular signal transduction pathways like JAK/STAT signaling[7–9].

In the inflamed joints of SpA patients, neutrophils are present in high numbers both in the synovial tissue and in the synovial fluid (SF)[10]. Neutrophils are the most common leukocyte subset in blood and one of the first responders of the host defense system during an infection. The main function of neutrophils is to capture and destroy invading pathogens like bacteria and fungi. To do so they are equipped with various effector functions such as degranulation, mediator release, reactive oxygen species (ROS) production, phagocytosis, and neutrophil extracellular trap (NET) formation[11,12]. However, neutrophils are also associated with various immune-mediated inflammatory diseases, including inflammatory bowel disease (IBD)[13–15]. In IBD, a disease closely related to SpA, disease severity correlates with the number of invasive neutrophils[13]. Furthermore, it has been reported that the mucosa of IBD patients has higher levels of neutrophil elastase compared to healthy controls, indicating the local presence of active neutrophils[16]. The abundant presence of neutrophils in psoriatic skin lesions serves as a typical histopathologic hallmark of psoriasis[17]. The neutrophil-to-lymphocyte ratio (NLR), the activity of neutrophils, and the number of NETotic cells were significantly higher in psoriasis patients compared to healthy controls[18]. In RA and SpA, the severity of the disease correlates with an increased neutrophil-to-lymphocyte ratio[14]. In addition, neutrophils present in SF of RA patients produce more reactive oxygen species (ROS) and the formation of neutrophil extracellular traps (NETosis) is increased[19]. The observation that IL-17 levels in SF of SpA patients are increased

compared to levels in SF of OA patients, suggests that neutrophils may be involved in Th17 activation, the main producers of IL-17[20,21].

Although there is evidence for the presence and involvement of neutrophils in various forms of arthritis, the exact role of neutrophils in the disease process is still elusive. Therefore, we set out to study SF neutrophils derived from SpA patients in more detail. First, we characterized surface markers involved in neutrophil degranulation. Here we demonstrated that, surprisingly, neutrophils present in SF of SpA patients are neither activated nor exhausted, as they can respond in vitro to stimuli by degranulation and ROS production. We show that soluble factors present in SpA SF can inhibit neutrophil activation and identified hyaluronic acid (HA) as a potential factor preventing excessive neutrophil activation. In the current study, we provide more insight into the behavior of neutrophils in inflamed joints of SpA patients. Furthermore, our data provide arguments for using intra-articular HA supplementation as (additional) therapy in patients with chronic inflammatory joint diseases in order to ensure the inactivity of neutrophils.

Results

Patient characteristics

SF samples were collected from 27 patients with an actively inflamed knee joint of whom 20 were classified as SpA, 5 as RA, and 2 as OA. SF was processed and analyzed immediately after collection. The majority of patients were male (18/27). Age was highly variable (31-85 years) as was the type of treatment, ranging from none to one or more (combinations of) NSAIDs, csDMARDs, and/or bDMARDs. SF derived from these patients contained highly variable amounts of cells (0.2-47.2x10^6 cells/ml) and percentages of neutrophils (2.2-96.4%). Demographics of individuals are listed in **table 1** and **supplemental table 1.**

SF-derived neutrophils of severely inflamed joints are inactive

To determine the activation state of SF-derived neutrophils of SpA patients, we analyzed the neutrophil degranulation status. Neutrophils contain four different types of granules: azurophilic granules, specific granules, gelatinase granules, and secretory vesicles[22,23]. We here assessed neutrophil degranulation by determining the membrane expression of CD16 (FCγRIII); expressed on resting neutrophils and cleaved from the surface by ADAM17 (present in secretory vesicles), CD63 (present in azurophilic granules),

Table 1. Description of the patient cohort and description of SF data from SpA patients. ND = not determined.

Patient#	Diagnosis	Gender M/F	Age years	Treatment	Type of bDMARd (ia)	Total cells in SF (cells/mL)	Amount of neutrophils in SF (%)
1	SpA	F	50	NSAID		ND	ND
2	SpA	M	59	NSAID, bDMARD	anti-IL17	$1.6x10^6$	77,9
3	SpA	M	34	NSAID, csDMARD		$20,2x10^6$	74,7
4	SpA	M	52	bDMARD	anti-TNF	$4,5x10^6$	82,3
5	SpA	M	31	None		$7,8x10^6$	42,1
6	SpA	F	58	bDMARD	anti-TNF	ND	25,1
7	SpA	M	75	NSAID, bDMARD	anti-TNF	ND	86,9
8	SpA	M	35	NSAID		ND	27,0
9	SpA	M	75	NSAID		$10,1x10^6$	96,4
10	SpA	F	48	NSAID, bDMARD	anti-IL17	$1.5x10^6$	2,2
11	SpA	M	35	NSAID, csDMARD, bDMARD	anti-TNF	$47,2x10^6$	39,4
12	SpA	M	55	bDMARD	anti-TNF	$8,0x10^6$	48,7
13	SpA	F	58	csDMARD, bDMARD	anti-TNF	$2,6x10^6$	44,4
14	SpA	M	36	NSAID, csDMARD, bDMARD	anti-TNF	$38,1x10^6$	66,8
15	SpA	F	58	NSAID, csDMARD, bDMARD	anti-IL17	$2,8x10^6$	16,1
16	SpA	M	36	NSAID, csDMARD, bDMARD	anti-TNF	$22,3x10^6$	33,6
17	SpA	M	64	csDMARD		$0,2x10^6$	40,9
18	SpA	F	52	csDMARD, bDMARD	anti-TNF	$4,5x10^6$	8,0
19	SpA	M	56	csDMARD		$4,4x10^6$	80,9
20	SpA	F	36	NSAID		$6,6x10^6$	62,8

and CD66b (present in both specific and gelatinase granules) by flow cytometry [22–26]. Thus, activated neutrophils display an upregulated expression of CD63 and CD66b while CD16 is downregulated compared to unstimulated neutrophils.

We compared the activation status of SF neutrophils from SpA patients (n=20) with that of blood-derived neutrophils from healthy donors (n=25), that were either resting or fully activated with GM-CSF (50 U/mL) and LPS (10 ng/mL) [24]. Surprisingly, we found that neutrophils isolated from SF greatly resembled unstimulated blood-derived neutrophils (**Fig. 1A**). Fully activated neutrophils, as defined by CD63high and CD16low, were only observed after GM-CSF+LPS stimulation of blood-derived neutrophils but not of directly measured SpA SF-derived neutrophils (**Fig. 1A and B**). Also, when analyzing single CD16 and CD63 expression, SF-derived neutrophils showed more similarity to unstimulated neutrophils than stimulated neutrophils. Furthermore, SF-derived neutrophils had a low expression of CD66b which was comparable to unstimulated blood-derived neutrophils. This phenomenon was observed in all 20 SpA SF samples. These samples were derived from SpA patients with high variability in age, gender, medication use, and amount of neutrophils in SF (**Table 1**), suggesting this phenomenon is independent of these factors. However, to draw conclusions for any relation to age, gender, medication use, and amount of neutrophils a larger sample set would be necessary.

To determine whether the observed activation status of neutrophils is disease-specific we also determined the degranulation status of SF-derived neutrophils from RA patients (n=5) and OA (n=2). Similar to neutrophils derived from SF from SpA patients, we observed that neutrophils derived from RA and OA SF greatly resembled unstimulated blood-derived neutrophils as well (**Suppl. Fig 1**). Although the number of included RA and OA SF samples was much smaller, these findings suggest that inactive neutrophils are present in SF of inflamed joints, which may be irrespective of the disease. Taken together, neutrophils found in SF of patients with SpA greatly resemble unstimulated blood neutrophils and have an inactive phenotype. This observation seemed independent of diagnosis, age, gender, medication, or neutrophil count.

SF neutrophils from SpA patients are not exhausted and can be activated outside of the SF environment

The observation that SF neutrophils from SpA patients display a non-activated phenotype could be due to the fact that these cells were either not activated yet or were exhausted due to the previous activation. To investigate this neutrophils were isolated from SF of SpA patients and stimulated in vitro with GM-CSF and LPS.

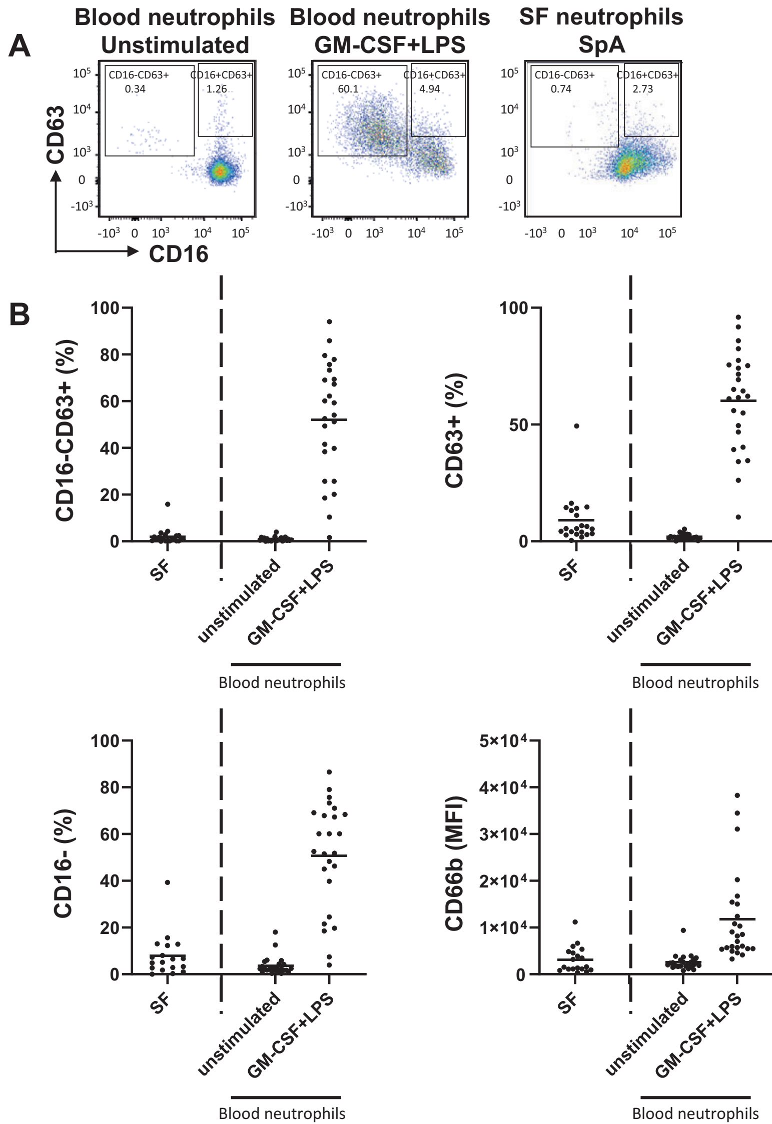

Figure 1. Neutrophils derived from SF of SpA patients do not degranulate.
Neutrophils from SF of SpA patients (n=20) were analyzed by flow cytometry and compared to neutrophils derived from blood of healthy donors (n=25). (A) Representative flow cytometry plot of unstimulated healthy donor-derived blood neutrophils, stimulated blood neutrophils, and SF-derived neutrophils of a SpA patient demonstrating CD16 and CD63 membrane expression. (B) Full neutrophil degranulation as measured by percentage of CD16-CD63+ neutrophils, secretory vesicle degranulation as measured by percentage of CD16- neutrophils, azurophillic degranulation as measured by percentage of CD63+ neutrophils, and specific and gelatinase degranulation as measured as mean fluorescent intensity (MFI) of CD66b. Data are presented as mean and individual points.

Stimulation of SF neutrophils induced the expression of CD63 and shedding of CD16 (**Fig. 2A and B)**. When analyzing CD16 and CD63 expression individually, we also observed a significant decrease in CD16 membrane expression and a significant increase in CD63 membrane expression. Furthermore, we observed a significant increase in CD66b membrane expression compared to unstimulated neutrophils (**Fig. 2B**). Besides degranulation, another effector function of activated neutrophils is the release of ROS. In line with the observed increase in degranulation, stimulation of SF neutrophils resulted in significantly increased ROS production compared to unstimulated SF neutrophils (**Fig. 2C and D**). For both degranulation as ROS production, no correlation was found between sample characteristics (e.g. neutrophil count) and percentage of degranulation or ROS production. Together, these data demonstrate that neutrophils in SF have an inactive phenotype, but are not exhausted as they can degranulate and produce ROS when activated in the absence of SF.

Blood neutrophil activation can be inhibited by SF from SpA patients

To investigate whether certain factor(s) in SF inhibit neutrophil activation, we stimulated blood-derived neutrophils of healthy donors in the presence or absence of SF of SpA patients (30% v/v). The presence of SF strongly inhibited the activation of blood-derived neutrophils (**Fig. 3A and B**). This was observed both for degranulation as demonstrated by the expression of CD63 and CD16 (**Fig. 3A**) and for ROS production (**Fig. 3B**). No correlation was found between sample characteristics (e.g. neutrophil count) and percentage of degranulation or ROS production. Furthermore, titration of SF demonstrated a dose-dependent effect of SF as 30% SF inhibited neutrophil degranulation and ROS production significantly, whereas for lower concentrations (1-3%) no significant effects were observed. (**Suppl. Fig. 2A and 2B**). Of note, unstimulated neutrophils were hardly affected by the incubation of SF (**Supl. Fig. 2C and 2D**). Collectively, these data indicate that SF from SpA patients may contain one or more factors that are limiting neutrophil activation.

Hyaluronic acid in SF of SpA patients inhibits neutrophil activation

Our results indicate that SF from SpA patients contains factors that restrict neutrophil activation. One of the major components of SF is hyaluronic acid (HA) which is present at a concentration of approximately 1.5-3.1 mg/mL[27]. It has been reported

that HA can inhibit inflammation in the adjuvant arthritis model[28] and can block neutrophil infiltration and acute lung injury[29], making HA a likely candidate that prevents neutrophil activation in SF. First, we analyzed the expression of CD44, .

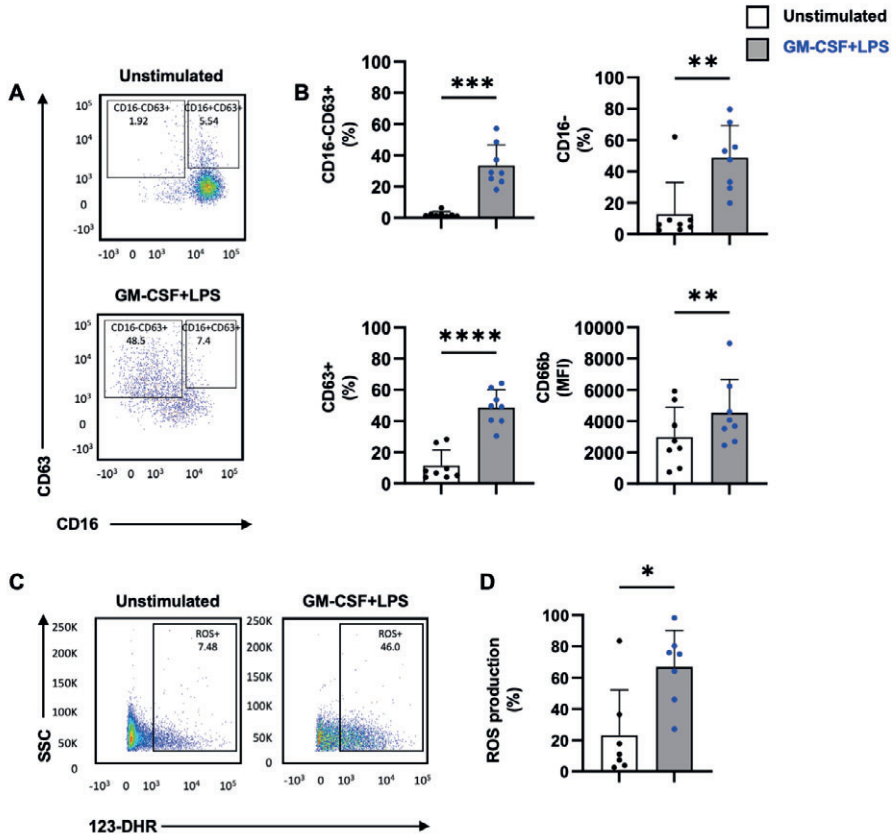

Figure 2. Neutrophils derived from SF of SpA patients are able to degranulate and produce ROS. (**A**) SF cells of SpA patients (n=8, pat# 4, 5, 7, 8, 10, 11, 12, 15) were cultured for 2 hours in the absence or presence of GM-CSF (50 U/mL) and LPS (10 ng/mL), Representative flow cytometry plot of synovial fluid neutrophils demonstrating CD16 and CD63 membrane expression for unstimulated and GM-CSF+LPS stimulated cells. (**B**) Percentage of CD16-CD63+, CD16-, CD63+, and MFI of CD66b membrane expression as a measure for degranulating neutrophils. (**C**) SF cells of SpA patients (n=7, pat# 10, 11, 12, 15, 17, 19, 20) were incubated for 1 hour in the presence of 123-DHR. Representative flow cytometry plot of SF neutrophils demonstrating ROS production for unstimulated and GM-CSF+LPS stimulated cells. (**D**) Intracellular ROS generation expressed as percentage of ROS+ cells. Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. The P-values were calculated using a paired t-test.

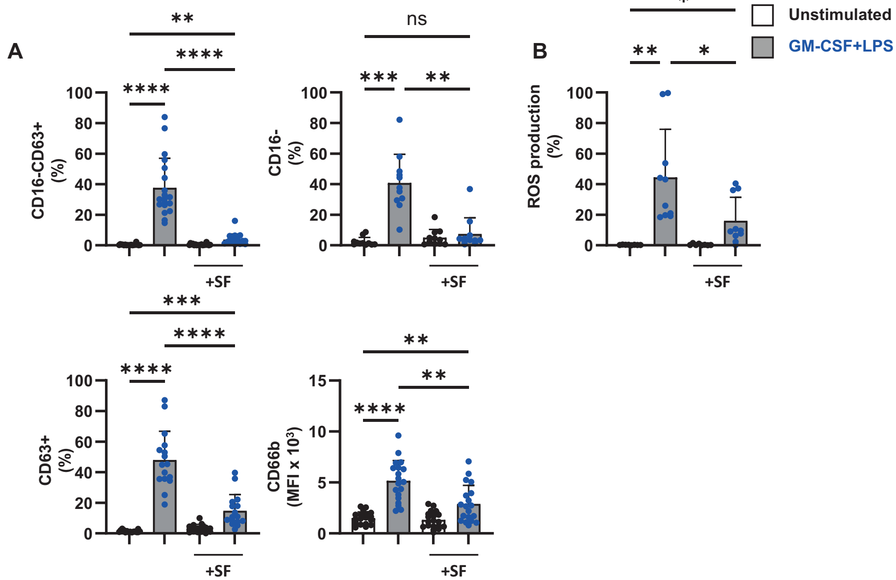

Figure 3. SF of SpA patients inhibits activation of healthy blood-derived neutrophils.(**A**) Blood-derived neutrophils from healthy donors (n=11-15) were cultured for 2 hours in the absence or presence of GM-CSF (50 U/mL) and LPS (10 ng/mL) and with or without 30% SF of SpA patients (n=9-12, pat# 7, 8, 10, 12-20). Percentage of CD16-CD63+, CD16-, CD63+, and MFI of CD66b membrane expression as a measure for degranulating neutrophils. (**B**) Blood-derived neutrophils from healthy donors (n=8) were cultured for 1 hour in the presence of 123-DHR and GM- CSF (50 U/mL) and LPS (10 ng/mL) and with or without 30% SF of SpA patients (n=6, pat# 13, 14, 16-19). Percentage of ROS production. Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. The P-values were calculated using a one-way ANOVA.

which is a common receptor for HA[30]. SF-derived and blood-derived neutrophils both express CD44 (**Fig 4A and 4B**). Next, we determined whether HA at a similar length and concentration range as present in SF of SpA patients was able to inhibit neutrophil degranulation. Indeed, HA at the concentration of 1.6 mg/mL and 3.2 mg/mL was able to significantly inhibit neutrophil degranulation (**Fig 4C**). Hyaluronidase (hyase) is an enzyme that catalyzes the degradation of HA[31]. We next analyzed whether incubation of SF with hyase could revert the inhibiting effect of HA on neutrophil activation. Hyase alone did not interfere with neutrophil degranulation or ROS production (**Suppl. Fig 3A and 3B**). However, treatment of SF with hyase resulted in a significant but not complete loss of the ability of SF to inhibit neutrophil degranulation (CD63+/CD16-) and ROS production (**Fig 4D and 4E**). Taken together our data shows that HA in SF has the capacity to prevent neutrophil activation

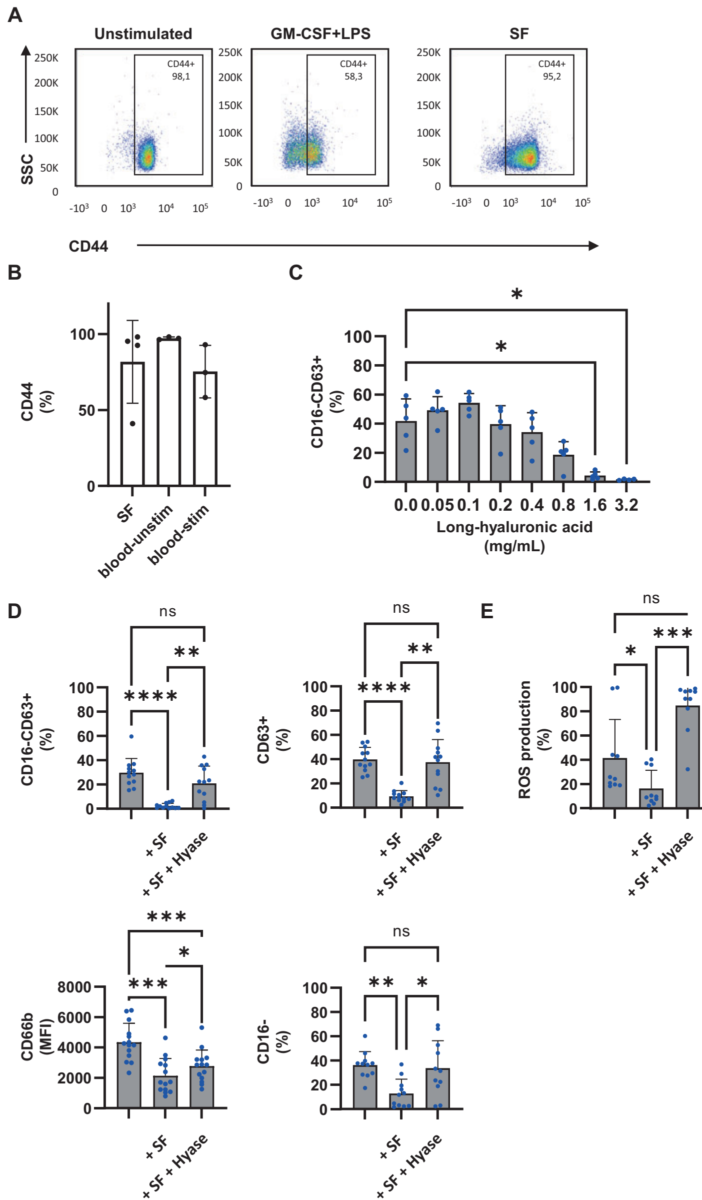

Figure 4. HA in SF inhibits neutrophil activation. (**A-B**) Blood-derived and SF-derived neutrophils were cultured for 2 hours in the absence or presence of GM-CSF (50 U/mL) and LPS (10 ng/mL). (**A**) Representative flow cytometry plot of unstimulated blood neutrophils, stimulated blood neutrophils, and synovial fluid neutrophils demonstrating CD44 membrane expression. (**B**) Percentage of CD44 membrane expression (n=3-4, pat# 16, 18, 19, 20).. (**C**) Blood-derived neutrophils were cultured for 2 hours in the presence of GM-CSF (50 U/mL) and LPS (10 ng/

Figure 4. Continued

mL) and with various concentrations of HA. Percentage of CD16-CD63+ as a measure of full degranulation shown. (**D**) Blood-derived neutrophils from healthy donors (n=11-19) were cultured for 2 hours in the presence of GM-CSF (50 U/mL) and LPS (10 ng/mL), without SF or with 30% SF or with 30% SF treated with hyase. SF was obtained from SpA patients (n=9-12, pat# 7, 8, 10, 12-20). Percentage of CD16-CD63+, CD16-, CD63+, and MFI of CD66b membrane expression as a measure for degranulating neutrophils. (**E**) Blood-derived neutrophils from healthy donors (n=10) were cultured for 1 hour in the presence of 123-DHR and GM- CSF (50 U/mL) and LPS (10 ng/mL), without SF or with 30% SF of with 30% SF treated with hyase. SF was obtained from SpA patients (n=6, pat# 13, 14, 16-19). Percentage of ROS production. Data are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001. The P-values were calculated using a one-way ANOVA.

Discussion

In this study, we show that SF-derived neutrophils of SpA patients are in an inactive state, based on low levels of degranulation and lack of ROS production ex vivo. Similar observations were made for SF-derived neutrophils of RA and OA patients, albeit with a limited sample size. Of note, SF-derived neutrophils are not exhausted as these neutrophils retained their capacity to be activated, degranulate and produce ROS outside of the SF environment. Moreover, activation of blood-derived neutrophils from healthy donors was dose-dependently inhibited by SF of SpA patients. Further analysis indicated that HA which is abundantly present in SF largely contributes to this impediment of neutrophil activation.

It was rather surprising that SF-derived neutrophils are in an inactive state since SF of patients with arthritis contains many neutrophil-activating factors, including TNF and GM-CSF. TNF is present in SF of both treated and untreated SpA or RA patients at a concentration between 94.2 and 378.2 pg/mL or 139.4 and 533.0 pg/mL, respectively[32]. In an earlier study, even higher concentrations of TNF were found in SF of different types of arthritis with average TNF levels of 0.97 (±0.6) ng/mL[33]. GM-CSF is present in SF of patients with various forms of arthritis, including RA and SpA, at concentrations ranging from 5.31 (± 3.9) pg/mL to 29.5 (±10.9) pg/mL[34]. Also, the neutrophil-activating factors IL-1β, IFNγ, and TGF-β have been found abundantly in SF [20,35]. Taken together, these studies indicate that multiple activating stimuli are present in SF of inflamed joints, which would normally lead to neutrophil activation, especially since dual stimulation of neutrophils with GM-CSF and TNF is efficient in activating neutrophils at relatively low concentrations[24].

Therefore, it is clear that one or more dominant factors are present in SF that prevent neutrophil activation. Here, we show that HA can act as a strong neutrophil inhibiting factor and that hyaluronidase treatment of SF, resulting in the degradation of HA, results in loss of neutrophil inhibitory capacity. HA is known to increase the density of SF creating a viscous, jelly-like consistency that acts as a lubricant to reduce friction between articular cartilages[36]. To our knowledge, we are the first ones to show that HA in SF of SpA patients prevents different aspects of neutrophil activation. The inhibitory effect of HA on neutrophil activation is not entirely new, but HA can also have pro-inflammatory characteristics[37]. HA has a high molecular mass (HMM) form and a low molecular mass (LMM) form. In SF HMM HA is present in high concentrations (1.5-3.1 mg/mL)[27]. SF derived from arthritic joints contains lower concentrations of HA and reduced chain length compared to SF from healthy joints[38,39]. HA in its HMM form has been shown to have immunosuppressive effects[28,29]. In addition, previous studies have demonstrated a clear inhibitory effect of HA on neutrophil ROS production[40] and neutrophil-mediated cartilage degradation[41]. This is in line with our results, showing that HMM HA in high concentrations inhibits neutrophil degranulation and ROS production.

To our knowledge, the effect of SF from SpA patients on neutrophils has not been described before. Although the sample size of other forms of arthritis in the current study is rather small, the data from SF of RA and OA patients suggest that the inhibition of neutrophil activation is a common effect and not only observed in SpA. Other studies have also investigated the effect of SF on neutrophil activation. Two recent studies showed that neutrophils in SF of patients with juvenile idiopathic arthritis (JIA) display an active phenotype based on increased levels of various activation markers, including CD16 and CD66b[42,43]. The same study also tested the effect of 20% SF of JIA patients on healthy blood neutrophils and found no change in degranulation surface markers, including CD16 and CD66b[43]. In other studies that used 10-25% SF of RA patients induction of NETosis[19,44] and ROS production[19,44–46] was found in healthy blood neutrophils, while non-RA (i.e. OA, PsA, and gout) SF inhibited ROS production[46]. Furthermore, previous studies found conflicting results on the effect of SF on apoptosis. While one study has found evidence for enhanced apoptosis of healthy blood-derived neutrophils after the addition of more than 50% SF from RA, SpA, and OA patients after 24 and 48 hours[47], another study has found evidence for inhibited apoptosis of healthy

blood-derived neutrophils after the addition of 50% SF from RA patients after 12 and 18 hours[48]. In our study, we did not observe enhanced or inhibited neutrophil apoptosis. We used different time points and concentrations, looked at neutrophils after 1 and 2 hours and used a maximum of 30%.Consequentily, our results might have been different if we looked at other time points or used different concentrations of SF. However, our study has been performed with SF from patients with differences in diagnosis, treatment and potentially also disease severity, which may to some extent account for the somewhat discrepant findings.

In the current study, we showed that HA in SF derived from inflamed joints of SpA patients prevents neutrophil activation. It is known that inflamed joints contain less HA compared to uninflamed joints. Moreover, the capacity of HA to inhibit neutrophil activation decreases significantly when the HA concentration is lower than 1.6 mg/mL. HA supplementation, also known as viscosupplementation, is a therapy that is commonly used in OA patients and has been demonstrated to restore lubrication in joints and stimulate the growth of cartilage and bone tissue[49,50]. In our view, patients with other forms of arthritis may also benefit from HA supplementation because in addition to improving lubrication we demonstrate that it can also prevent (neutrophil-induced) inflammation. Importantly, the effects of intra-articular (IA)-HA injection have been described to be much longer-lasting than IA corticosteroid injection (approximately 6 months vs. 1-2 months, respectively) and have no significant adverse effects[49]. However, IA-HA injections are not commonly used in arthritis types other than OA. Of note, one study showed that IA-HA injection is beneficial in RA patients[51]. In the current study, we demonstrated that using HMM-HA inhibits neutrophil activation. Therefore, we propose that HA supplementation may hold great potential in some arthritis patients and have beneficial effects that reach beyond improving the viscosity of SF and enhancing lubrication, since it might also reduce inflammation. This may be especially of value for patients with chronic monoarthritis that is persistent even with adequate systemic treatment. This may ultimately result in dose reduction or decrease the number of DMARDs that patients require and improve quality of life.

In conclusion, the present study demonstrates that SF-derived neutrophils of SpA patients display an inactive phenotype, although they are not exhausted as these neutrophils can be activated to degranulate and produce ROS outside the SF micro-environment. Importantly, we demonstrate that SF can also inhibit the activation of

blood-derived neutrophils from healthy donors. Finally, we show that HA present in SF can act as a strong inhibitor of neutrophil activation and that hyaluronidase treatment of SF, resulting in the degradation of HA, results in the loss of neutrophil inhibitory capacity. Our study can therefore be considered as an argument to look at HA as a potential novel treatment option for chronic inflammatory joint diseases, although this remains to be formally tested in a randomized controlled trial.

Materials and Methods

Synovial fluid collection and preparation of synovial cells and cell-free synovial fluid

Synovial fluid (SF) from inflamed knee joints was collected during active arthritis from 27 patients after obtaining informed consent. Patients' characteristics are described in **table 1** and **supplementary table 1**. SF was centrifuged at 650g, for 20 min to pellet the cells. The SF was centrifuged at 3000g for 30 minutes at RT to pellet all remaining cells and debris, the cell-free SF was collected and stored at -80°C until further use. Meanwhile, the SF-derived cells collected after the first 650g step were resuspended in IMDM (Gibco; Thermo Fischer Scientific Inc, Waltham, Mass) supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS; Hyclone; Thermo Fischer Scientific Inc, Waltham, Mass) and gentamycin (86 μg/mL; Duchefa Biochemie B.V., Haarlem, The Netherlands) and passed through a 70 μm single-cell filter. Then cells were resuspended in IMDM with 10% HI FBS at a concentration of 2 x 10^6 cells/mL for flow cytometry analysis and culture experiments.

Neutrophil isolation from blood

Blood was collected from healthy volunteer donors after obtaining informed consent into sodium heparin tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Neutrophils were isolated using density gradient followed by erythrocyte lysis, as previously described[24]. Neutrophils were then resuspended in IMDM (Gibco; Thermo Fischer Scientific Inc, Waltham, Mass) supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS; Hyclone; Thermo Fischer Scientific Inc, Waltham, Mass) and gentamycin (86 μg/mL; Duchefa Biochemie B.V., Haarlem, The Netherlands) and used immediately. Neutrophil purity was analyzed by flow cytometry and was always > 97%.

Neutrophil culture

Neutrophils were seeded at a density of 0.4 x 10^6 cells/mL in 250 µL in a flat-bottom 96-well plate (Costar, Corning Inc. Corning, NY) in an IMDM medium containing 10% HI-FBS and gentamycin. Subsequently, neutrophils were cultured for 1 or 2 hours at 37 °C (CO2 incubator) in the absence or presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Schering-Plough B.V., Brussels, Belgium) and lipopolysaccharide (LPS) (Sigma-Aldrich) and the absence or presence of SF, hyaluronic acid (HA, Sigma-Aldrich, St Louis, MO, USA) and SF pretreated with hyaluronidase (hyase, Sigma-Aldrich; 40 U/mL). For flow cytometric analysis of ROS production, neutrophils were cultured and stimulated for 1 hour in the presence of 25 µM 123-dihydrorhodamine (123-DHR; Marker Gene Technologies, OR, USA). For the flow cytometric analysis of CD63, CD66b, and CD16 neutrophils were stimulated for 2 hours. After stimulation, cells were harvested and used for measurement of degranulation or ROS production.

Measurement of degranulation markers

Cells were washed twice at 4°C in PBA (PBS-0.5% w/v BSA-0.05% w/v azide), followed by antibody labeling in PBA. CD16, CD63, and CD66b expression and cell viability (PI) were determined using flow cytometric analysis. The following antibodies were used: αCD15-FITC (1:100; HI98), αCD16-PECy7 (1:1000; 3G8), αCD63-APC (1:100; H5C6), αCD66b-PE (1:100; G10F5), (all Biolegend, San Diego, Calif). propidium iodide (PI) (Sigma-Aldrich) was used to determine cell viability. A total of 10,000 cells were acquired in the live gate on a FACSCanto (BD Biosciences, San Jose, CA, USA) and further analyzed using FlowJo software (BD Biosciences).

Measurement of ROS production

Cells were washed twice at 4°C in PBA (PBS-0.5% w/v BSA-0.05% w/v azide) and measured using flow cytometry. A total of 10,000 cells were acquired in the live gate on a FACSCanto (BD Biosciences) and further analyzed using FlowJo software (BD Biosciences).

Statistical analysis

Data are expressed as mean ± SD or as mean and individual points. Statistical analysis was done in Graphpad Prism version 9.1.0 for Windows by using statistical tests,

depending on experimental data. The Shapiro-Wilk test was performed to test the normality of data. For single comparisons, p values were calculated using two-tailed paired t-tests. For multiple comparisons, p-values were calculated using a one-way ANOVA. P-values below 0.05 were considered statistically significant.

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Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary table 1: Description of the patient cohort and description of SF data from OA and RA patients ND = not determined.

Patient#	Diagnosis	Gender M/F	Age years	Treatment	Type of bDMARd (ia)	Total cells in SF (cells/mL)	Amount of neutrophils in SF (%)
21	RA	F	85	NSAID		$0,3x10^6$	25,0
22	RA	M	67	None		ND	ND
23	RA	M	36	None		$10,7x10^6$	58,8
24	OA	M	59	NSAID, csDMARD, bDMARD	anti-TNF	$3,2x10^6$	21,0
25	OA	M	60	csDMARD, bDMARD	anti-TNF	ND	ND
26	RA	F	50	Steroid		$9x10^6$	21,6
27	RA	M	37	None		ND	ND

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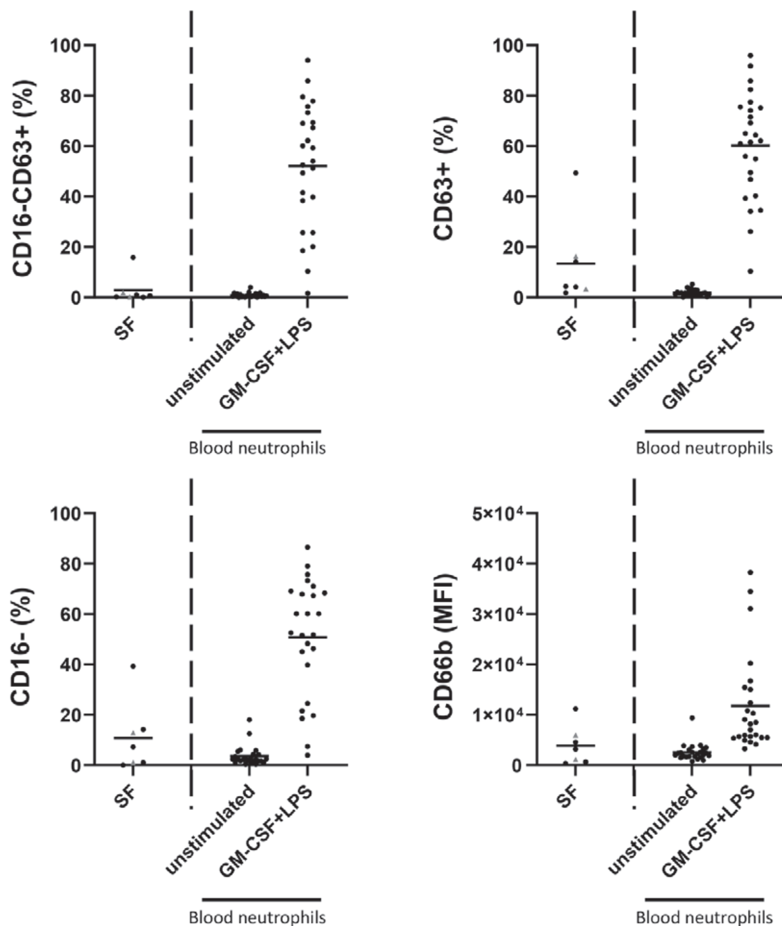
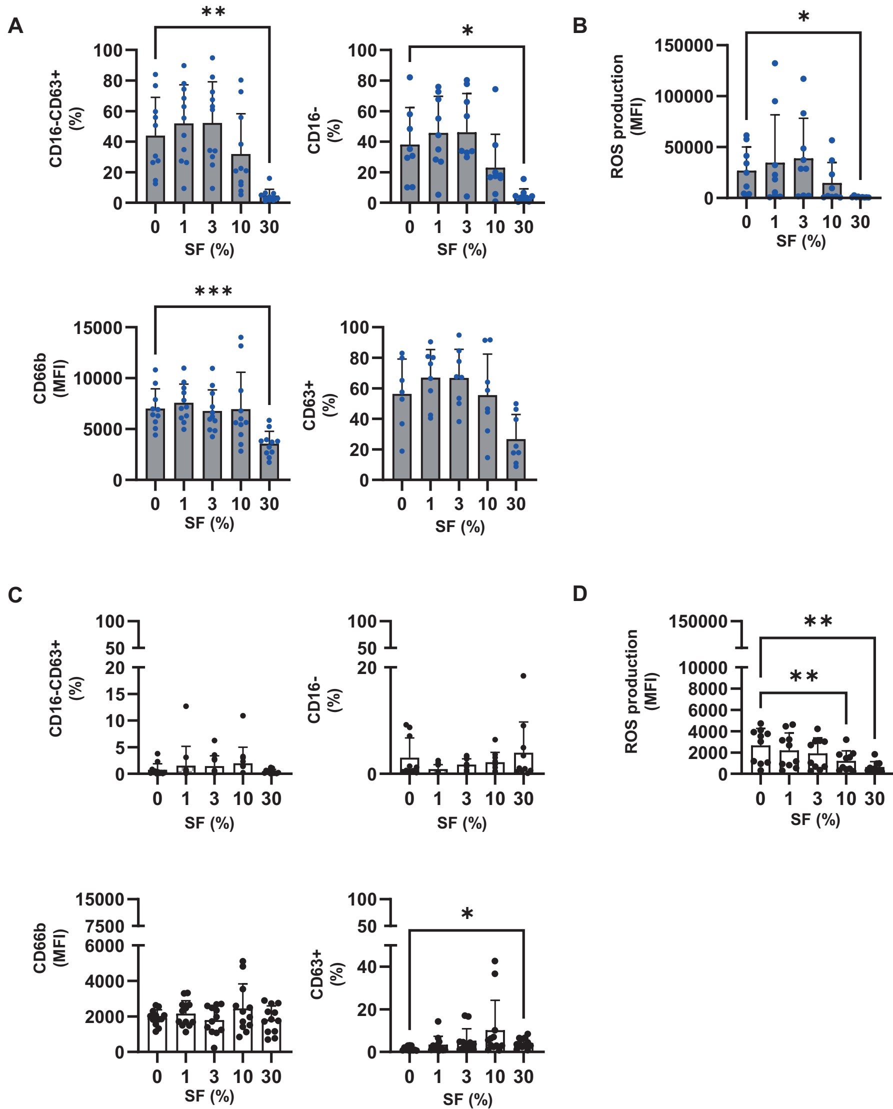


Supplemental figure 1: Neutrophils derived from SF of RA and OA patients do not degranulate. Neutrophils from SF from RA patients (black circles (n=5)) and OA patients (red triangles (n=2)) were analyzed by flow cytometry and compared to neutrophils derived from blood. Full neutrophil degranulation as measured by percentage of CD16-CD63+ neutrophils, secretory vesicle degranulation as measured by percentage of CD16- neutrophils, azurophillic degranulation as measured by percentage of CD63+ neutrophils, and specific and gelatinase degranulation as measured as mean fluorescent intensity (MFI) of CD66b. Data are presented as mean and individual points.



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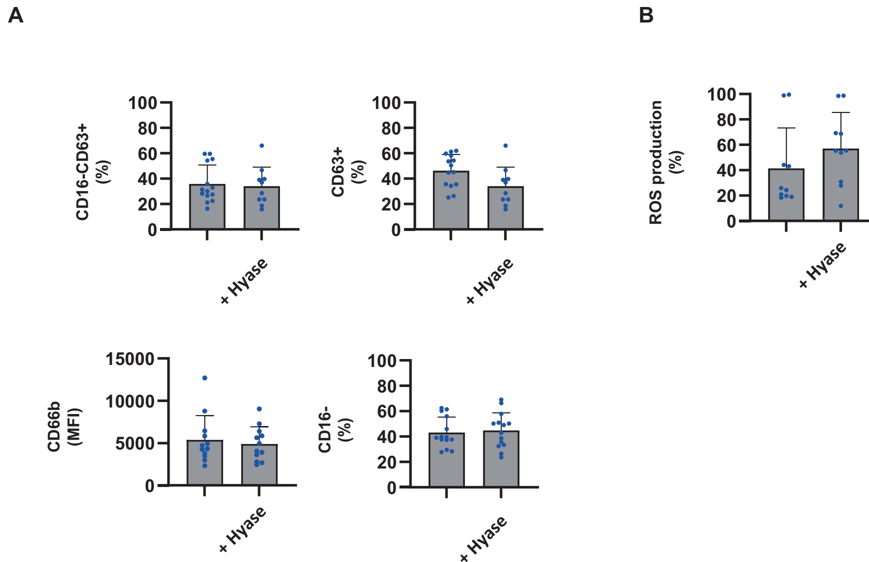
Supplemental figure 2: Titrations of SF on stimulated and unstimulated neutrophils. (**A**) Blood-derived neutrophils from healthy donors (n=11-14) were cultured for 2 hours in the presence of GM-CSF (50 U/mL) and LPS (10 ng/mL) and with various concentrations of SF of SpA patients (n=9-12, pat# 7, 8, 10, 12-20). Percentage of CD16-CD63+, CD16-, CD63+, and MFI of CD66b membrane expression as a measure for degranulating neutrophils. (B) Blood-derived neutrophils from healthy donors (n=9) were cultured for 1 hour in the presence of 123-DHR and GM- CSF (50 U/mL) and LPS (10 ng/mL) and with various concentrations of SF of SpA patients (n=6, pat# 13, 14, 16-19). Percentage of ROS production. (**C**) Blood-derived neutrophils from healthy donors (n=11) were cultured for 2 hours with various concentrations of SF of SpA patients (n=9, pat# 12-

Supplemental figure 2. Continued

20). Percentage of CD16-CD63+, CD16-, CD63+, and MFI of CD66b membrane expression as a measure for degranulating neutrophils. N=11. (D) Blood-derived neutrophils from healthy donors (n=10) were cultured for 1 hour in the presence of 123-DHR and with various concentrations of SF of SpA patients (n=6, pat# 13, 14, 16-19). Percentage of ROS production. Data are presented as mean ± SD. *P<0.05, **P<0.01, and ***P<0.001. The P-values were calculated using a one-way ANOVA.



Supplemental figure 3: Hyase has no effects on neutrophil activation. (**A**) Blood-derived neutrophils were cultured for 2 hours in the presence of GM-CSF (50 U/mL) and LPS (10 ng/mL), with or without hyase. Percentage of CD16-CD63+, CD16-, CD63+, and MFI of CD66b membrane expression as a measure for degranulating neutrophils. N=11-19 (**B**) Blood-derived neutrophils were cultured for 1 hour in the presence of 123-DHR and GM- CSF (50 U/mL) and LPS (10 ng/mL), with or without hyase. Percentage of ROS production. N=10. Data are presented as mean ± SD.

te veel mensen
te veel draaien
te veel mensen
draaien eromheen
te veel mensen
te veel zinnen
te veel woorden
voor een mens alleen

Mag het licht uit, de Dijk

CHAPTER 6

Extracellular vesicles from resting or activated neutrophils differentially affect DC function

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Abstract

Neutrophils are key players in the communication between the innate immune system and the adaptive immune system. Neutrophils influence various dendritic cell (DC) functions. Communication of neutrophils with other parts and cells of the immune system occurs via various mechanisms. One such mechanism is via the release of extracellular vesicles (EVs). The role of neutrophil-derived EVs in the regulation of adaptive immune responses is still poorly understood. Here, we compared EVs from resting and activated neutrophils and evaluated their effect on DC function. This study shows that EVs derived from activated neutrophils, in contrast to their cell of origin, do not affect DC maturation and DC-driven Th17 cell development, as measured by flow cytometry. Surprisingly, EVs derived from activated neutrophils displayed an anti-inflammatory effect on cytokine production by DCs, while EVs derived from resting neutrophils had a proinflammatory effect as demonstrated by increased IL-8 production by DCs. This study demonstrates that EVs derived from activated or resting neutrophils have divergent effects on DC function.

Keywords

Neutrophils, extracellular vesicles, ectosomes, exosomes, microvesicles, Dendritic cells, DC function

Introduction

Neutrophils are the most abundant cell type of the innate immune system and their rapid mobilization from peripheral blood into tissues upon microbial infection to kill pathogens is a first line of defense[1]. Using their effector functions like degranulation, mediator release, reactive oxygen species (ROS) production, and the release of neutrophil extracellular traps (NETs), infections can be cleared[1,2]. In the last decade, it has been recognized that neutrophils also play an important role in modulating adaptive immune responses[3,4]. Previously, we demonstrated that neutrophils are essential in promoting dendritic cell (DC)-driven Th17 cell development via the release of neutrophil elastase[5]. The cross-talk between neutrophils, DCs, and T-cells can occur via various mechanisms, including cell-cell contact,the release of soluble mediators or extracellular vesicles (EVs) [2]. EVs are small lipid bilayer-enclosed particles that are released by cells in a regulated fashion and are key players in intercellular communication[6]. Despite the increased investigation of neutrophil-derived EVs, their function in the regulation of adaptive immunes responses is poorly understood and sometimes their modulatory effects seem to be even contradictory[7]. This is illustrated by a recent study showing that neutrophils-derived EVs can have a pro-inflammatory, as well as an anti-inflammatory effect on neutrophils and endothelial cells, depending on the environmental conditions[8]. Moreover, previous research showed that EVs derived from activated neutrophils have an anti-inflammatory effect on DC function[9]. To decipher the potential role of neutrophil-derived EVs in T cell modulation, we here investigated whether EVs derived from resting and activated neutrophils could have differential effects on DC function and the induction of Th17 responses. Taking into consideration the role of EVs in keeping and restoring homeostasis, we investigated the effect of EVs derived from resting and activated neutrophils on DC function, activation, and cytokine production, as well as on the capacity to promote Th17 cell development. We show that EVs from activated neutrophils, in contrast to their cells of origin do not induce Th17 cell or DC maturation. However, EVs derived from activated neutrophils have an anti-inflammatory effect on DC cytokine production, while EVs derived from resting neutrophils have an indirect pro-inflammatory effect by inducing IL-8 production by DCs.

Material & Methods

Cell isolation

Blood was collected from healthy donors after informed consent into heparin tubes (Greiner Bio-One, Alphen a/d Rijn, The Netherlands). Blood was diluted with Hanks balanced salt solution (HBSS), Sigma-Aldrich Inc., St. Louis, MO, USA), and granulocytes and erythrocytes were separated from peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation on Lymphoprep (d = 1.077 ± 0.001 g/mL; Axis-Shield, Oslo, Norway). Subsequently, neutrophils were isolated from the granulocytes and erythrocytes pool, as described previously[2]. Neutrophil purity was analyzed by flow cytometry and was always >97%. PBMCs were separated into monocyte and peripheral blood lymphocytes (PBL) by density gradient centrifugation on percoll (GE Healthcare, Hoevelaken, The Netherlands). Monocyte-derived dendritic cells (moDCs) were generated from the monocyte layer, as described previously[5]. $CD4^+$ T cells were isolated from the PBL layer as previously described[5]. The purity of naïve T cells was always >98%.

Neutrophil EV preparation

After isolation, neutrophils were seeded at a density of 2.0 x 10^6 cells/mL in a flat bottom 24-well plate (Costar, Corning Inc. Corning, NY, USA) in IMDM medium (Thermo Scientific, Gibco, Waltham, MA, USA) supplemented with EV-depleted 10% heat-inactivated fetal bovine serum (HI-FBS) and gentamycin. EV-depleted HI-FBS was previously prepared by ultracentrifugation of 30% HI-FBS in IMDM for 16 h at 100,000xg in an SW32 rotor (Beckman Coulter, Fullerton, CA, USA). Per condition, 24 x 10^6 cells were used. Cells were either unstimulated (medium only) or stimulated with GM-CSF (Shering-Plough B.V., Brussels, Belgium; 50 U/mL) and fMLP (Sigma-Aldrich; 1μM). After 2 h, culture supernatants were gently collected and subjected to differential centrifugation, twice at 200 x g for 10 min at RT, and twice at 500 x g for 10 min. Thereafter, EVs were pelleted by ultracentrifugation of the 500 x g supernatant for 30 min at 10,000 x g at 4°C using an SW40 rotor (Beckman Coulter) to collect 10k EVs. Subsequently 100k EVs were collected by ultracentrifugation of the 10,000 x g supernatant at 100,000 x g for 65 min at 4°C using an SW40 rotor. EV-containing pellets were either resuspended in IMDM containing 5% HI-FBS and stored at -80°C for a maximum of one week before functional DC or T cell assays were performed, or resuspended in 20 μL PBS containing 0.2% EV-depleted bovine serum albumin (BSA) for EV-characterization.

Neutrophil EV characterization

Single EV-based flow cytometric analysis was performed on EV pellets labeled with PKH67 for generic fluorescent labeling and subjected to sucrose gradient floatation as previously described[10]. [2] In short, EV pellets that were resuspended in 20 µL PBS containing 0.2% EV-depleted BSA were labeled with 7.5 mM PKH67 (Sigma, St. Louis, MO, USA) in 180 µL diluent C. The reaction was stopped after 3 minutes by adding 100 µL of IMDM with 10% EV-depleted FBS. PKH67-labeled pellets were mixed with 1.5 mL 2.5 M sucrose and a linear sucrose gradient (2.0-0.4 M sucrose in PBS) was added on top. Gradients were centrifuged at 192,000 x g for 16 h at 4°C in an SW40 rotor. Hereafter 1 mL gradient fractions were collected by carefully pipetting from the top of the tube and subsequently measured by high-resolution flow cytometry on a BD influx flow cytometer that was optimized for the detection of submicron-sized particles (BD Biosciences)[10].

For western blotting EV pellets were diluted in PBS. The primary antibodies CD9 (clone HI9, Biolegend; dilution 1:1000) and CD63 (clone H5C6, BD Biosciences; dilution 1:500) were used for characterization as previously described[2].

Neutrophil elastase ELISA

Neutrophil elastase (NE) concentration was analyzed in culture supernatants collected after 2 hours of stimulation and in EV pellets and EV supernatants as described previously[5].

Hyphae generation

C. Albicans cultures (clinical isolates) were maintained at the Department of Experimental Microbiology, Academic Medical Center, Amsterdam. Yeast particles were resuspended in IMDM supplemented with 10% HI-FCS and plated at 10,000 particles per well on flat-bottom 96-well plates. Hyphae were formed by a 4-hour incubation at 37 °C, 5% CO_2, followed by heat-killing for 2 hours at 70 °C.

Th17 coculture

At day 6 of moDCs generation, immature DCs were harvested and extensively washed prior to use in culture. Th17 cocultures were cultured in IMDM + 5% HI humane serum (HS) in a flat-bottom 96-well plate containing 50,000 autologous

DCs and 50,000 autologous naive CD4+ T cells plated in a 96-well plate coated with 10,000 C. Albicans hyphae per well. Then EV pellets from 2.0 x 10^6 resting or activated neutrophils were added and as a control, no EV pellets or 100,000 autologous neutrophils were added. After 4 days of culture, cells were transferred to a 48-well plate (Costar) and refreshed with medium containing recombinant human IL-2 (Novartis AG, Basel, Switzerland) at a final concentration of 10 U/mL every 2 days. Cells were transferred to 24-well plates (Costar) 2 to 4 days later. After in total 11 to 13 days of culture, cytokine production (IL-17 and IFN-ɣ) of the cells was determined by flow cytometry.

DC maturation

On day 6 of moDC generation, moDCs were washed and matured in IMDM + 5% HI-FBS containing 10 ng/mL lipopolysaccharide (LPS) (Sigma-Aldrich) in the absence or presence of EV pellets from 2.0 x 10^6 neutrophils. After 48 hours, DCs were harvested and surface markers for maturation were determined by flow cytometry.

Flow cytometry

For analysis of T cells, cells were restimulated in a round-bottom 96-well plate (Costar) by a 5-hour incubation at 37 °C, 5% CO_2, in IMDM, supplemented with 5% HI-HS and PMA (100 ng/mL), ionomycin (1 µg/mL), and brefeldin A (10 µg/mL) (all Sigma-Aldrich). Cells were thereafter fixated in 3.7% formaldehyde in PBS and before staining and measurement permeabilized in 0.5% w/v saponine in PBA (PBS – 0.5% w/v BSA – 0.5% v/v azide). For analysis of moDCs and neutrophils degranulation, cells were harvested after stimulation and washed twice in cold PBA, followed by antibody labeling in PBA.

The following antibodies were used: αCD15-FITC (1:100; HI98), αCD16-PECy7 (1:1000; 3G8), αCD62L-APCCy7(1:25; Greg-56), αCD63-APC (1:100; H5C6), αCD66b-PE (1:100; G10F5), αCD14-PECy7 (1:100; M5E2), (all Biolegend, San Diego, CA, USA). αIFNɣ-FITC (1:10; 25723.11, BD Biosciences), αIL-17A-eFluor660 (1:20; eBio64DEC17, eBioscience), αCD1a-FITC (1:25; HI149, BD Pharmingen), αCD83-APC (1:25; HB15e, Sanbio), αCD86-BV421(1:100; 2331, BD Horizon), αHLA-DR-PERCP (1:10; L243, BD Pharmingen). For flow cytometric analysis 10,000 cells per condition were acquired in the live gate

on a FACSCanto instrument (BD Biosciences) and further analyzed using FlowJo Software (for Windows, Version 10.6.2., Ashland).

DC cytokine production analysis

Immature moDCs were seeded at a density of 4.0 x 10^4 cells/mL in a flat bottom 96-well plate and were stimulated with 10 ng/mL LPS with or without EV pellets from 2.0 x 10^6 neutrophils. After 24 h of culture, supernatants were collected and the levels of IL-6, IL-8, IL-10, IL-23, and TNF were assessed by specific solid-phase sandwich ELISA. In short, flat-bottom EIA/RIA 96-well plates (Costar, Corning Inc.) were coated at 4°C overnight with the corresponding antibody diluted in carbonate buffer (0.5M, pH9.6). After washing with PBS-0.1% v/v TWEEN 20 (PT) plates were blocked with PT-1% w.v BSA (PTB) for 1 h at 37°C. Plates were incubated with supernatants diluted in PTB for 1 hour at RT and thereafter washed in PT. Subsequently, plates were incubated for 1 h at RT with biotinylated detection antibody andafter washing with PT incubated for 45 min at RT with poly-streptavidin-horseradish peroxidase conjugate (1:10,000, M2032, Sanquin, Amsterdam, The Netherlands) diluted in PT containing 2% protifar (Nutricia, Utrecht, The Netherlands). Finally, plates were washed five times with PT and developed with 3,3',5,5'-tetramethylbenzidine (TMB, Merck, Germany). The reaction was stopped by adding 1M H_2SO_4. The absorbance was measured at 450 nm with reference at 655 nm using a VersaMax microplate reader (Molecular Devices, Silicon Valley, CA, USA).

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was done in GraphPad Prism Software (La Jolla, Ca, USA, version 8.3.0 for Windows). For analysis between pairs a paired t-test was used. For multiple comparisons, a one-way ANOVA was used with Tukey's post hoc test. Values of $P < 0.05$ were considered significant.

Results

Neutrophil-derived EVs contain Elastase but do not affect Th17 cell development

To study the functional effects of EVs from resting or activated neutrophils, first quantitative and qualitative analysis of the isolated EV batches were performed, as

described before [2,10]. Quantitative single EV-based flow cytometric analysis using fluorescence triggering showed a marked increase (2-fold for 10k EVs and 4-fold for 100k EVs) in the number of EVs present in the culture supernatant of activated neutrophils (**Fig. 1A**). The isolation of EVs was confirmed by Western blotting for common EV protein markers, i.e., CD9 and CD63, detected in both 10k and 100k EVs from resting and activated neutrophils (**Fig. 1B**). We previously showed that neutrophil-derived elastase (NE) is essential for DC-driven Th17 cell development from naïve T cells and demonstrated that 10^6 neutrophils activated by GM-CSF and fMLP release on average 124.2±42.7 ng/mL NE[2,5]. We here measured the amount of NE associated with EVs derived from $24x10^6$ neutrophils. Overall, more NE is released by activated neutrophils in soluble form than is present in EVs derived from activated neutrophils (**Fig 1C**). No significant differences were found in the amount of NE in 10k EVs from resting neutrophils compared to 10k EVs from neutrophils activated by GM-CSF and fMLP (**Fig. 1C**). In contrast, 100k EVs from resting neutrophils contained significantly less NE (p=0.05) compared to 100k EVs from GM-CSF and fMLP activated neutrophils, respectively 2.9±2.1 ng/mL per EVs and 21.9±20.2 ng/mL per EVs from 10^6 neutrophils (**Fig. 1C**). Because neutrophil-derived EVs contain NE we next analyzed whether these EVs could also support Th17 cell development. Th17 cell differentiation in the absence of neutrophils was negligible, whereas Th17 cell differentiation in the presence of neutrophils was promoted (**Fig. 1D**). Th17 cell differentiation was not increased in the presence of either 10k or 100k EVs derived from resting or activated neutrophil (**Fig. 1E and 1F**). As expected, Th1 cell development, as measured by IFNɣ expression, was also not affected by neutrophil-derived EVs (**Fig. 1E and 1F**). Taken together, we can conclude that while neutrophil-derived EVs do contain NE, they are not able to promote Th17 cell development.

Neutrophil-derived EVs do not affect moDC maturation

Though we observed no effect on Th17 cell development by neutrophil-derived EVs, we next investigated to what extent neutrophil-derived EVs affect the activation of moDCs. DCs were activated by LPS in the absence or presence of neutrophil-derived EVs derived from resting or activated neutrophils, and surface marker expression associated with maturation and involved in T cell activation (CD83,CD86, and HLA-DR) was measured by flow cytometry (**Fig. 2**). A representative graph is given in Fig. 2A and multiple experiments are summarized in Figs. 2B, C, D.

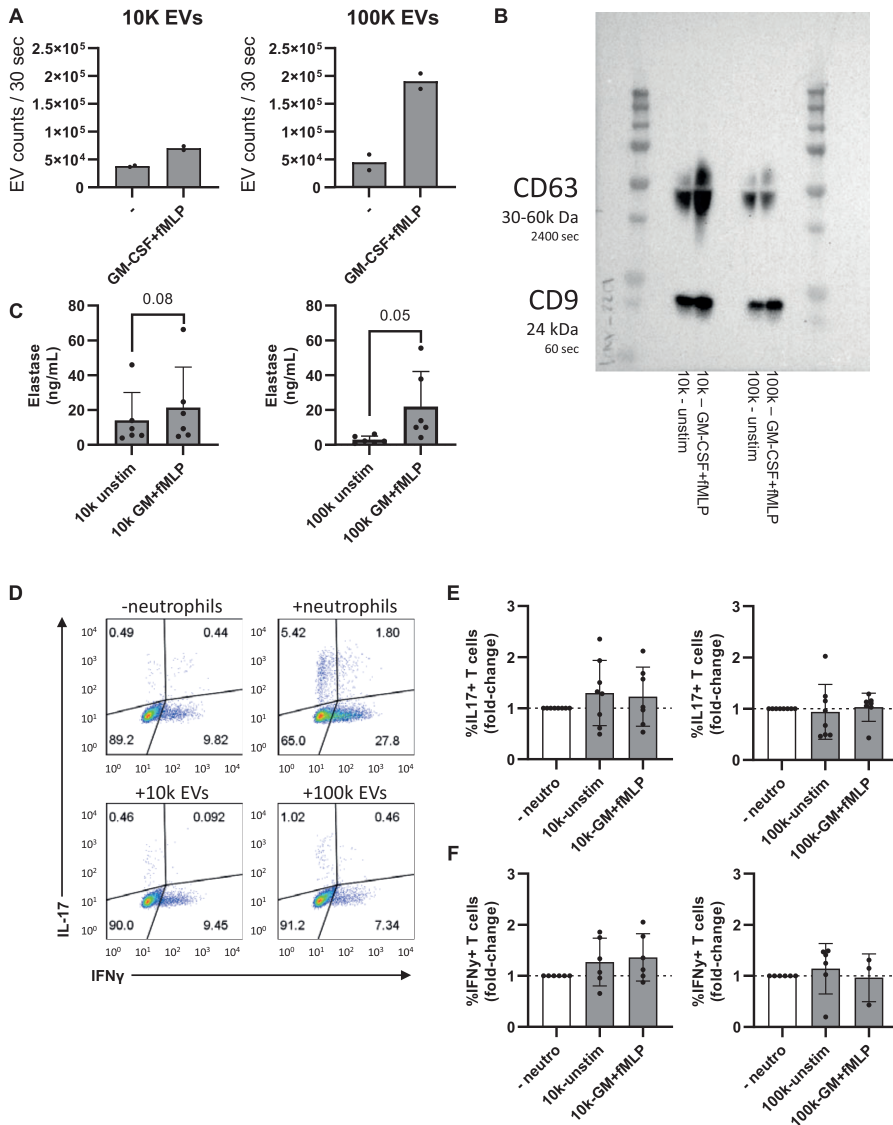

Figure 1: neutrophil-derived EVs contain elastase, but do not induce Th17 cell development

(**A**) Neutrophils were cultured for 2h in the absence or presence of GM-CSF (50 U/mL) and fMLP (1 µM). After 2h, culture supernatants were collected and EVs were isolated and fluorescently labeled and analyzed by single EV-based flow cytometric analysis. Quantification of EV release as determined by high-resolution flow cytometry. Indicated are the numbers of detected events within the fixed time frame of 30 s. Data is shown from two individual experiments. Each sample is a pool of sucrose fractions with a density between 1.10 and 1.22 g/mL. (**B**) CD9 and CD63 analysis as determined by western blotting of 10k and 100k EVs floated into a sucrose gradient

Figure 1: Continued

from neutrophils from a representative donor. (**C**) elastase levels in EVs derived from resting and activated neutrophils were determined by ELISA. (**D**) Representative flow cytometry plots of IL-17 and IFN-γ expression of restimulated T cells cultured in the presence of moDCs, C. albicans hyphae, and with or without neutrophils or neutrophil-derived EVs. Shown is a representative donor. (**E, F**) Percentage of IL-17^{+} cells and IFN-γ+ cells normalized to control moDCs without neutrophils or EVs. Data are presented as mean ± SD and are depicted of 6-8 independent experiments. Data were considered significant for p-value < 0.05. The p-values were calculated using one-way ANOVA with Tukey's post hoc test.

The preliminary data showed that EVs derived from both resting and activated neutrophils are able to increase and decrease CD86, CD83 and HLA-DR expression. On average the preliminary data indicates no effect on moDC maturation. Taken together, for moDC maturation measured by surface markers, no significant effect of neutrophil-derived EVs was observed. However, more data is necessary to validate these findings (**Fig. 2**).

EVs from activated neutrophils have an anti-inflammatory effect on DC cytokine release

Besides the expression of maturation markers, DCs produce many cytokines with various immune modulatory effects. To evaluate the impact of neutrophil-derived EVs on DC cytokine production, DCs were activated with LPS for 24 hrs in the absence or presence of neutrophil-derived 10k and 100k EVs and the presence of IL-6, IL-8, IL-10, IL-23, and TNF was measured in DC culture supernatant by ELISA. Without LPS stimulation the cytokine release by moDCs was below the detection level (**data not shown**). Using a suboptimal concentration of LPS (10 ng/mL) to stimulate cytokine production by moDCs, we observed significant effects of neutrophil-derived EVs on cytokine release by DCs (**Fig. 3**). For 10k EVs derived from resting neutrophils, a decreased production of IL-23 was observed while IL-8 was increased. No significant effects were observed on TNF, IL-6 and IL-10 production (**Fig. 3A**). Also 10k EVs derived from activated neutrophils significantly decreased the production of the pro-inflammatory cytokine IL-23 and in this case also TNF, while in contrast to 10k EVs from resting neutrophils no significant increase was observed on IL-8. Similarly as 10k EV from resting neutrophils activated neutrophil-derived EVs had no effect on the anti-inflammatory cytokine IL-10 (**Fig. 3A**). In the presence of 100k EVS from resting neutrophils no effects on IL-23, IL-6, IL-10 or TNF were observed, while IL-8

production was significantly increased (**Fig. 3B**). In contrast, 100k EVs derived from activated neutrophils significantly reduced the production of the pro-inflammatory cytokines TNF, IL-6, and IL-23 and increased the production of the anti-inflammatory cytokine IL-10, while no significant effects were observed on IL-8 production (**Fig. 3B**).

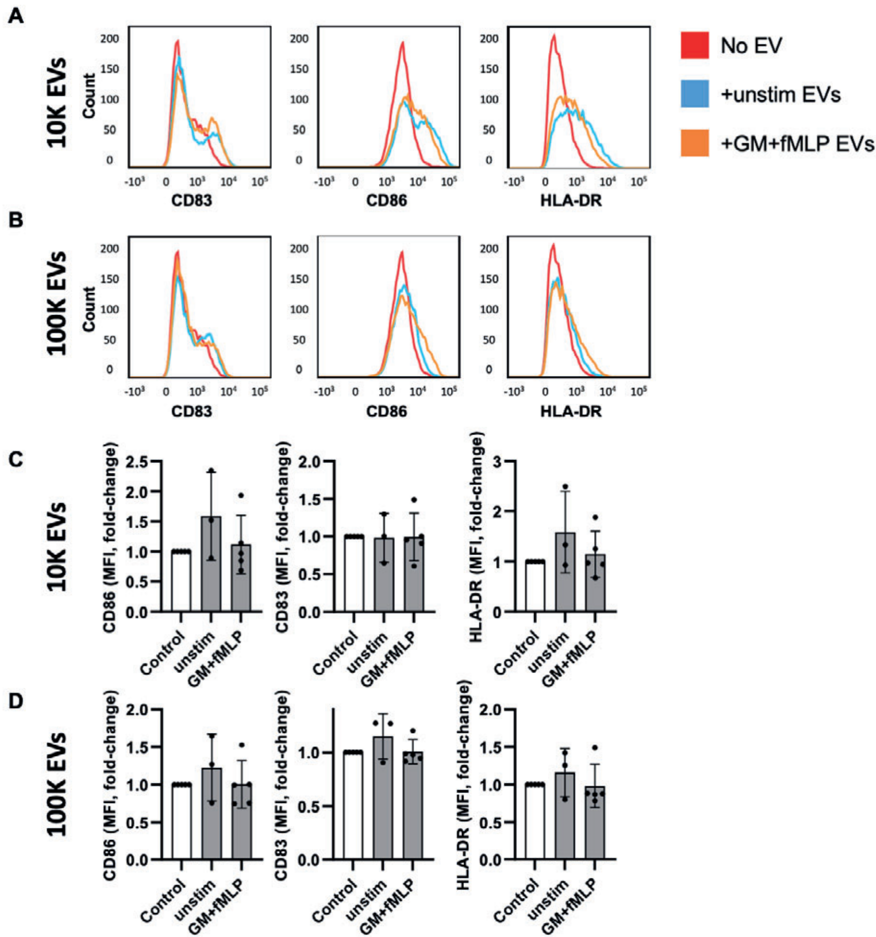

Figure 2: neutrophil-derived EVs have no effect on moDC maturation
moDCs were stimulated with LPS (10 ng/mL) and EVs derived from resting or GM-CSF+fMLP activated neutrophils. After 48h maturation was determined by flow cytometry. (**A, B**) Representative histograms of CD83, CD86, and HLA-DR expression for moDCs incubated with (**A**) 10k EVs and (**B**) 100k EVs. (**C, D**) CD83, CD86, and HLA-DR expression were shown in mean fluorescence intensity (MFI) for moDCs incubated with (**C**) 10k EVs and (**D**) 100k EVs. Data are presented as mean ± SD (N=3-5).

These data suggest that EVs derived from activated neutrophils have an anti-inflammatory effect on immune responses, while EVs derived from resting neutrophils do not have a pronounced anti-inflammatory effect, but can promote pro-inflammatory effects via stimulating IL-8 release.

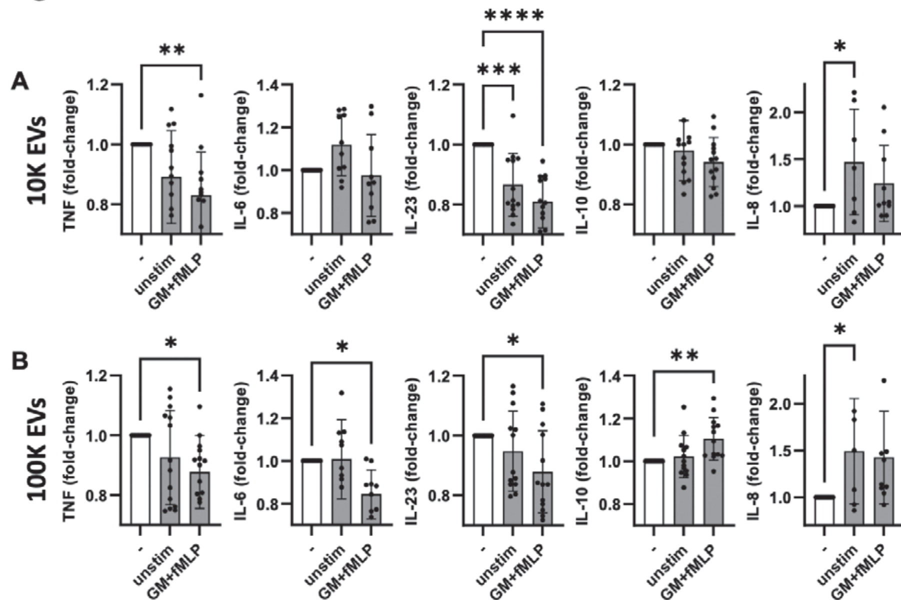

Figure 3: activated neutrophil-derived EVs induce an anti-inflammatory cytokine profile in LPS-stimulated DCs

moDCs were stimulated with LPS (10 ng/mL) and incubated with EVs derived from resting or GM- moDCs were stimulated with LPS (10 ng/mL) and cultured for 24h in the absence or presence of EVs derived from resting or GM-CSF+fMLP activated neutrophils. TNF, IL-6, IL-23, IL-10, and IL-8 concentrations were determined in supernatants from moDCs incubated with 10k EVs (**A**) and 100k EVs (**B**). Data are presented as mean ± SD. Data consist of 5-7 different moDC donors and 7-9 different neutrophil donors. Data were considered significant for p-value < 0.05. * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001. The p-values were calculated using one-way ANOVA with Tukey's post hoc test.

Discussion

Cross-talk between neutrophils and cells of the adaptive immune system is essential for regulation of many immunological processes. One mechanism of communication is via EV release. The role of neutrophil-derived EVs in various immunological processes remains poorly understood. In this study, we show that EVs derived from activated neutrophils have an anti-inflammatory effect on DC function as they reduce the secretion of the pro-inflammatory cytokines TNF, IL-23, and IL-6. In contrast, EVs derived from resting neutrophils can have a pro-inflammatory promoting effect, as they increase the release of IL-8 by DCs. These immunomodulatory effects were primarily induced by small EVs (pelleted after differential centrifugation at 100k xg) compared to the larger EVs (pelleted at 10k xg). This could be the result of different functional EV subsets, differing in EV-cargo composition in the 100k and 10k EV populations[11,12].

In general, activated neutrophils have a pro-inflammatory effect on DC function. Upon activation, neutrophils release many chemokines and cytokines including TNF, CCL3, CCL4, CCL5, and CCL20, to attract DCs to the sites of infection or inflammation[13,14]. Besides that, neutrophils are also able to stimulate DC activation, as demonstrated by LPS-stimulated murine neutrophils inducing murine BMDCs maturation by upregulating CD40, CD80, and CD86 and induced IL-12 and TNF production[15]. The pro-inflammatory effect of activated neutrophils on DC maturation was also shown in human cells. GM-CSF-stimulated neutrophils increased the membrane expression of CD40, CD86, and HLA-DR in human moDCs[16]. Furthermore, neutrophils enhance the ability of DCs to activate and polarize T cells, including Th1, Th2, Th17, and Treg development[5,17,18]. So, in contrast to the pro-inflammatory effects of activated neutrophils, we here demonstrated that the 100k EVs originating from these cells exert anti-inflammatory effects on DCs. Our findings corroborate a previous study showing that EVs from fMLP-stimulated neutrophils downregulated the release of the cytokines IL-8, IL-10, IL-12, and TNF and upregulated the release of TGFβ in LPS-stimulated moDCs[9]. However, in contrast to our findings, demonstrating that neutrophil-derived EVs on average did not affect DC maturation, they observed that these EVs inhibited DC maturation based on the analysis of surface markers CD40, CD80, CD83, CD86, and HLA-DR [9]. The samples that we did test show high variations, this can explain the differences found in other studies. Other reason for the different effects on DC maturation may be related to several differences between the study from Eken et al and this study. A major

difference is the isolation method applied, which could have consequences on the observed functions of EVs or the EV subsets that are isolated[19,20]. Where we used ultra-centrifugation at 10,000g and 100,000g, whereas they applied centrifugation with 4,000g followed by EV concentration steps with filters at 10,000 KDa cutoff. Ultracentrifugation is a common method for EV isolation[21]. Ultracentrifugation can lead to EV and protein aggregation, and this could possibly affect the function of the isolated EVs[22,23]. Secondly, the storage of EVs could affect EV function[24]. Although freshly prepared EVs are recommended for functional assays, due to practical limits, we stored our EVs at -80 °C for a maximum of a week, which may have limited the effect on EV function[24]. Thirdly, the number of EVs added to the functional assays may affect the outcome of the assays. Lastly, the way the neutrophils are stimulated may influence their effect on the function of their EVs. We chose the incubation time of 2 hours since this clearly shows a difference between resting and activated neutrophils[2]. Since neutrophils are rapid responders, it may be that shorter incubation times can lead to EVs with different effects.

Overall, multiple studies have shown an anti-inflammatory effect of neutrophil-derived EVs on various immune cells, including, amongst others, monocytes, macrophages, and neutrophils themselves[8,25,26]. However, recently it was suggested that neutrophils could produce both pro-inflammatory and anti-inflammatory EVs depending on their environment[8]. These observations fit into the concept that EVs are key players in homeostasis[27]. Interestingly, we here observed that EVs derived from resting neutrophils did not inhibit the release of pro-inflammatory cytokines by DCs and even stimulated IL-8 release. Given the fact that IL-8 is a common neutrophil recruitment cytokine[28], resting neutrophil-derived EVs might promote inflammation by promoting neutrophil recruitment.

In conclusion, we have demonstrated that in contrast to their cells of origin activated neutrophil-derived EVs do not induce Th17 cell development or DC maturation. Moreover, our study shows that EVs derived from neutrophils with different activation statuses have different effects on DC cytokine production and thus induce different inflammatory effects.

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I'm not like them, but I can pretend
The sun is gone, but I have a light
The day is done, but I'm having fun
I think I'm dumb

Or maybe I'm just happy

Dumb, Nirvana

CHAPTER 7

General Discussion

In this thesis, we have focused on the role of neutrophil activation and neutrophil communication with the adaptive immune system in the context of arthritis and Covid-19. We did this by answering the following questions:

1. How do different stimuli affect neutrophil activation and neutrophil-derived EVs?
2. What components present in SF from RA and SpA patients are associated with neutrophil activation and neutrophil-derived EVs?
3. How is DC function altered upon encountering neutrophil-derived EVs?
4. How do immunoregulatory drugs, that are used to treat COVID-19 patients, influence neutrophil activation?

The key findings of these studies are summarized in **Box 1**. In this chapter, we summarize and discuss the insights and results of our studies.

The key findings of this study are:

- Neutrophils require dual stimulation in order to get fully activated (chapter 3)
- Dexamethasone inhibits multiple neutrophil functions (chapter 4)
- Neutrophils derived from the synovial fluid (SF) of SpA patients are in an inactive state (chapter 5)
- Hyaluronic acid in SF inhibits neutrophil activation (chapter 5)
- EVs derived from activated neutrophils have an anti-inflammatory effect on DC function (chapter 6)
- EVs derived from resting neutrophils have a pro-inflammatory effect (chapter 6)

Box 1: Key findings of this thesis

Neutrophil activation

The primary role of neutrophils is to eliminate pathogens. As extensively described in **chapter 1** of this thesis neutrophils employ various functions in order to eliminate pathogens[1]. These functions include degranulation, reactive oxygen species (ROS) production, mediator release, neutrophil extracellular trap release (NETosis), phagocytosis, and the release of extracellular vesicles (EVs)[2–4]. Neutrophils are short-lived cells that live between 8 hours to a maximum of up to 5 days[5,6]. In circulation neutrophils are inactive, only upon encountering or being recruited to a site of infection they become activated[2]. Generally, it was thought that neutrophil activation was a two-step process. First neutrophils are pre-activated, also known as priming,

by a priming stimulus. Then neutrophils are activated by an activating stimulus[7,8]. In literature, the distinction between priming and activating stimuli is very unclear. For example, lipopolysaccharide (LPS) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) are sometimes reported as priming stimuli and other times as activating stimuli[7,9–13]. Furthermore, at the site of infection neutrophils encounter many different inflammatory stimuli at the same time. Therefore, we hypothesized that the distinction between priming and activating stimuli is not physiologically relevant and is an artificial concept. In **chapter 3**, we indeed demonstrated that dual stimulation effectively enhances most neutrophil functions, including degranulation, mediator release, ROS production, and EV release, as well as moderately increasing phagocytosis. NETosis was the only tested function that was not enhanced with dual stimulation. We could, however, induce NETosis in our assay with the non-physiological stimulus phorbol myristate acetate (PMA) (**chapter 3**), indicating that our assay functioned well and thus that NETosis could truly not be enhanced by two simultaneous stimuli, at least not with any combination of stimuli that we used (i.e. granulocyte-macrophage colony stimulating factor (GM-CSF), fMLP, tumor necrosis factor (TNF), and LPS). In our studies, we used PMA as a positive control for NETosis and this stimulus is by itself already capable of inducing neutrophil activation, but this is a very aggressive non-physiological stimulus. That we could not induce NETosis with the combinations of the four stimuli that we used was surprising since earlier research showed NETosis by stimulation to TNF and LPS[9,14]. In these studies, less than 0.5% serum was used in their assays, while we used 10% of serum in our assay. This could potentially play a role since previous research showed that serum and serum albumin can inhibit LPS-induced NETosis in vitro[15]. In vivo there is plenty of serum or serum albumin present and plenty of evidence indicates that NETosis happens in vivo[16–18]. Thus, we cannot know for sure if under other circumstances dual stimulation can enhance NETosis in vivo. However, since NETosis can cause such great harm, it does make sense that this is kept in check more than other neutrophil functions. While in vitro research is essential in gaining a better understanding of mechanisms surrounding processes, it does have its limitations. The results of in vitro studies may not always translate to the behavior of living organisms in vivo, where complex biological systems can have unexpected interactions and outcomes.

Based on these findings we concluded that the distinction between priming and activating stimuli, which are often used in literature for in vitro studies, is not correct. However, there are some limitations to our study. Our study was performed

in vitro on blood-derived neutrophils. In vivo neutrophils are first recruited to the site of infection[19]. For this migration, many stimuli play a role, including IL-8[20]. Then at the site of infection, many different activating stimuli that are the same or comparable to the stimuli used in our study (**chapter 3**) are present that fully activate neutrophils. The migration of neutrophils already changes the receptor expression on neutrophil surfaces[2]. For example, the expression of L-selectin (CD62L) is lost on neutrophils after they migrated[21]. Thus, if priming implicates migration this can definitely influence neutrophil activation. However, in in vitro studies, neutrophils used are often derived from healthy blood donors and therefore were circulating resting neutrophils that did not yet migrate. The challenge in in vitro studies is that these neutrophils need to be isolated from the blood before they can be used in assays and neutrophil isolation can influence neutrophil activation[22]. In our assays, isolated neutrophils seemed inactivated based on neutrophil degranulation (**chapter 3**). However, the influence of the isolation method is something to keep in mind during in vitro studies, as it might influence neutrophil activation.

In order to show that neutrophils require dual stimulation, we chose four common stimuli that are often involved in neutrophil activation. These were, GM-CSF, fMLP, TNF and LPS. These were chosen because they have different origins (human, bacterial) and they ligate different receptor types that are expressed during inflammation. Neutrophils have much more receptors that could potentially be involved in neutrophil activation. In **chapter 4** we used resiquimod (R848) and TNF in order to mimic a viral infection. R848 is a synthetic viral stimulus that ligates TLR7 and TLR8, TLR8 is present on neutrophils [23]. This dual stimulus was just as effective as any of the others tested. Although we did show that neutrophil activation requires dual stimulation, we did only show this for the five stimuli used in our studies.

Balancing neutrophil activation

Activation of neutrophils is helpful in many circumstances, as it is essential for eliminating pathogens and instructing other immune responses. However, uncontrolled neutrophil activation can cause tissue damage to the host. Hence it is essential that neutrophil activation is balanced. Also, neutrophils only need to be activated at the site of the infection and not in other areas. To prevent overactivation, neutrophil activation is regulated at multiple levels. First of all, the body prevents neutrophil activation by recruiting neutrophils to the site of infection or inflammation by

chemical and biological signals, called chemoattractants. Chemoattractants comprise for example lipids, chemokines, peptides, and parts of the complement system[24]. The concentration and distribution of chemokines help to direct neutrophils to where they are needed while avoiding healthy tissues[25]. Once neutrophils arrive at the site of infection, they migrate from the blood vessels into tissues using adhesion molecules for this. The expression of adhesion molecules is regulated to prevent excessive infiltration of neutrophils into healthy tissue[26]. Another strategy of the host body to prevent neutrophil activation is by needing more than one stimulus for activation. For most neutrophil functions dual stimulation is required (**chapter 3**), which also applies to other cell types. For instance, various functions of DCs are regulated by dual stimulation. DCs only produce abundant IL-12 after dual stimulation with a PAMP in combination with IFN-ɣ[27]. Also, for the production of high IL-23 and IL-1β levels, generated by DCs, two stimuli are needed: a PAMP in combination with an FC-receptor stimulation[28]. Neutrophil activation does not only require more than one stimulus but is also balanced by anti-inflammatory cytokines produced by neutrophils themselves and other immune cells or tissue cells, such as IL-4, IL-10, and TGF-β[29–31]. For instance, IL-10 is produced in various circumstances by regulatory T-cells, DCs, macrophages, and also neutrophils themselves, amongst others[32–34].

Furthermore, in this thesis, we showed that 100k EVs derived from activated neutrophils increased IL-10 production by DCs (**chapter 6**). In the same study, we showed that neutrophil-derived EVs can have different effects on DC cytokine production depending on EV isolation from resting or activating neutrophils (**chapter 6**). Our results are in line with previous research showing that neutrophils can produce both pro-inflammatory EVs and anti-inflammatory EVs depending on their environment[35]. EVs are an additional mechanism of the host body to balance neutrophil activation since multiple earlier studies have shown that EVs derived from various immune cells have an anti-inflammatory effect on different immune cells, including neutrophils[35–38]. EVs play an important role in homeostasis in many cellular processes[39].

Finally, neutrophils are cleared from the site of infection. They have a limited lifespan and undergo apoptosis. Also, they are cleared from the site of infection or inflammation by phagocytosis by other immune cells, such as macrophages[40]. Together these strategies prevent overactivation and excessive accumulation of neutrophils in tissues, thereby preventing damage done by neutrophils to the host.

Clinical implication of neutrophil activation in arthritis and COVID-19

As stated in the previous paragraph, balancing neutrophil activation is essential for good health. Neutrophils need to be activated in order to eliminate pathogens, but they should not be activated at the wrong place or time in order to avoid tissue damage. While the host body has many strategies and tools in place in order to prevent the overactivation of neutrophils, this is not always successful. This occurs in various diseases including in auto-immune diseases or severe infections. In this thesis, the role of neutrophils and neutrophil activation in the diseases of arthritis, especially RA and SpA, and COVID-19 were studied.

Neutrophils are present in high numbers in the synovial fluid (SF) and synovial tissue of inflamed joints of SpA and RA patients[41]. The severity of the diseases RA and SpA is associated with an increased neutrophil-to-lymphocyte ratio (NLR) in blood[42]. Although there is plenty of evidence showing that neutrophils play a role in the pathogenesis of arthritis, the exact role of neutrophils in various forms of arthritis is yet unclear. In **chapter 5,** we studied neutrophils from the SF of arthritis patients. Surprisingly, we found that these neutrophils were present in an inactive state based on the absence of signs of degranulation and ROS production and that these neutrophils were not exhausted but retained their ability to become activated. This was an unexpected observation, since many neutrophil-activating stimuli, including GM-CSF and TNF, are present in SF from inflamed joints[43–45]. Furthermore, we showed that the SF of arthritis patients inhibited the activation of healthy blood-derived neutrophils. Previous studies showed that SF-derived neutrophils from juvenile idiopathic arthritis (JIA) were in an active state based on degranulation[46,47]. Studies with SF from RA patients showed that 10-25% of SF-induced NETosis and ROS production of healthy blood-derived neutrophils[48–51]. Possible reasons for these differences could be that we looked at different time points or used different concentrations of SF. Also, the diagnosis, treatment, and possibly also disease severity were different and can account for the differences. In our study, we used samples from 20 SpA patients that all had different ages, gender, and use of medication. In addition, we had limited samples from RA and osteoarthritis (OA) patients. In all samples, we made the same observation, that neutrophils in SF from arthritis patients were present in an inactive state. Although this is a limited sample size, it is a very diverse sample size and in all of them we observed the same neutrophil-inhibiting

effect. This indicates that diagnosis, treatment, and disease severity possibly cannot account for the differences observed in these studies. As we demonstrated that the presence of hyaluronic acid (HA) in SF is the factor that inhibits neutrophil activation, difference in HA concentration in SF could be an explanation for the differences observed in these studies. The concentration of HA matters for the inhibition of neutrophil activation (**chapter 5**). HA is already used as a treatment for patients suffering from OA and has little to no side effects. In OA HA supplementation is used to restore lubrication of the joints and thereby stimulate the growth of cartilage and bone tissue[52,53]. Because we showed HA can also inhibit neutrophil activation, patients with other forms of arthritis than OA can potentially also benefit from HA supplementation. Intra-articular-HA injections have no significant side effects, and the treatment needs to be repeated only after 6 months, while intra-articular corticosteroid injection needs to be repeated after 1 to 2 months[52]. Only high molecular mass (HMM) HA has the potential to inhibit neutrophil activation and low molecular mass (LMM) HA does not have an inhibitory effect. SF from the joint of arthritis patients generally contains lower concentrations of HA and this HA has a reduced chain length compared to SF from healthy donors[54,55]. The exact molecular mass of HA to inhibit neutrophil activation is yet unknown. HA with a molecular mass of 1250 kDa or more was needed to inhibit the pro-inflammatory response of macrophages stimulated with LPS. HA with a lower molecular mass was not able to inhibit the pro-inflammatory response of macrophages[56]. Hence, we believe that HA supplementation may be beneficial for some arthritis patients. Possibly, in different populations of arthritis patients neutrophils are present in an activated state, since it depends on the molecular mass of HA if neutrophil activation can be inhibited.

In addition to arthritis, COVID-19 was also a topic of this thesis. In COVID-19, neutrophils are partly responsible for a cytokine storm, which contributes to the severity of COVID-19[57]. Furthermore, in deceased COVID-19 patients, neutrophil plugs enriched in NETs were found in their lungs and other inflamed organs[58,59]. In **chapter 4** we showed that dexamethasone (DEX), which is often given to patients suffering from severe COVID-19[60,61], dampens multiple functions of activated neutrophils and thus dampens neutrophil activation. DEX is a common corticosteroid that has a general anti-inflammatory effect. DEX and other corticosteroids, such as fluticasone, have been shown to inhibit various functions of neutrophil activation[62]. Corticosteroids have side effects that are harmful to the patients and could be a reason why some patients do not prefer to use these drugs. Developing more effective drugs

that influence neutrophil activation when necessary is useful for good patient care. By gaining a better understanding of when and how neutrophils are (over)activated we could develop more effective treatments for patients in the future.

Neutrophils and Neutrophil-derived EVs as orchestrators of the adaptive immune system

While traditionally neutrophils were considered to be first responders to infection with the primary role of eliminating pathogens, they also play a role in orchestrating adaptive immune responses. Neutrophils communicate with adaptive immune cells through a variety of mechanisms. One such mechanism is via direct cell-cell interactions. For instance, neutrophils interact with T-cells through the presentation of antigens[63]. Also, neutrophils can produce mediators that attract or activate other immune cells. For example, neutrophil-derived elastase is essential for Th17 cell development[64]. Another mechanism via which neutrophils communicate with the adaptive immune system is via the release of EVs. In **chapter 2** and **chapter 6**, we focused on the role of innate immune cell-derived EVs and neutrophil-derived EVs and their effect on adaptive immunity. In **chapter 2** we provide an overview of the effect of EVs derived from mast cells, neutrophils, macrophages, eosinophils, basophils, and NK cells on adaptive immunity. Here we concluded that most of these innate immune cell-derived EVs influence adaptive immunity via DCs (**chapter 2**). In **chapter 6** we focused on neutrophil-derived EVs and their effect on various DC functions, DC maturation, Th17 cell development, and DC cytokine production. We observed no significant effect of neutrophil-derived EVs on DC maturation and Th17 cell development. We did observe a significant effect of neutrophil-derived EVs on DC cytokine production. We found that EVs derived from resting or activated neutrophils had different effects on cytokine production by DCs. EVs derived from resting neutrophils increased IL-8 release by DCs. IL-8 is a neutrophil-recruiting cytokine and its release promotes inflammatory response by recruiting neutrophils[20]. EVs from activated neutrophils reduced the release of pro-inflammatory cytokines by DCs. Thus, neutrophils can produce EVs that have different effects on DC cytokine production, both pro-inflammatory and anti-inflammatory depending on their activation status (**chapter 6**). This is in line with previous research showing EVs from resting neutrophils have an anti-inflammatory effect on themselves by reducing ROS production, and cytokine release from neutrophils and that EVs from activated

neutrophils increase ROS production and cytokine release from neutrophils and endothelial cells[35]. While we found no effect on DC maturation, previous research did find that EVs from activated neutrophils inhibited DC maturation[11]. That we did not find an effect on DC maturation could be because our data is very preliminary and more experiments need to be conducted in order to draw a definitive conclusion. Nonetheless, our first experiments show little to no effect of neutrophil-derived EVs on DC maturation. Neutrophil and EV research are complex. Isolation methods of both neutrophils and EVs, EV storage, and treatment of neutrophils all have an influence on functional studies and this could be a reason for different effects on DC maturation found in different studies[65–68]. While more and more studies focus on neutrophil-derived EVs and the effect of neutrophil-derived EVs on the adaptive immune system, there are still many things about their functions and effects unknown. By gaining a better understanding of the capacity of neutrophil-derived EVs, we can unravel possible applications of neutrophil-derived EVs in therapies or as therapeutic targets.

Conclusion

In this thesis, we focused on neutrophil activation and its role in arthritis, COVID-19, and cellular communication. Our findings demonstrate that neutrophil activation requires dual stimulation for most neutrophil functions. Furthermore, we showed that neutrophils are present in an inactive state in the SF of SpA patients and that activation can be inhibited by various factors such as HA and DEX. Additionally, our results indicate that neutrophil-derived EVs have distinct effects on DC function depending on their activation state. Further studies could potentially focus on if differently activated neutrophils produce different subsets of EVs and the effects of these EVs on DCs and T-cell development. Our findings and future findings may contribute to the development of targeted therapeutic strategies aimed at modulating neutrophil function in arthritis and COVID-19. Further studies are required to explore the potential clinical implications of neutrophil activation and communication with the adaptive immune system.

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Then bow your head in the house of God
Little girl, who do you think you are?
You think you need it, you think you want love
You wouldn't want it if you knew what it was

Moderation, Florence and the Machine

APPENDICES

Summary
Samenvatting
Portfolio
Author contributions
List of publications
Dankwoord

Summary

The immune system is a complex system that protects us against pathogens. The complicated task of protecting the body is performed by a large variety of immune cells. The immune system can be divided into two different systems, the innate system and the adaptive system. Cells belonging to the innate system react quickly to an invading pathogen. Cells belonging to the adaptive system react slower, but are very specific. When an infection is over, a few specially trained cells of the adaptive immune system remain, which will recognize this pathogen more quickly during the next infection. As a result, we become immune to some pathogens.

Unfortunately, sometimes the immune system is inbalanced and then it recognizes the body's own healthy cells as wrong and attacks them. Why this happens is not entirely clear. Diseases in which this happens are autoimmune diseases, such as rheumatoid arthritis. It can also happen when the immune system has to work so hard to fight a disease that many healthy cells become victims in the process, as can happen in a serious infection with the coronavirus that causes COVID-19. In both cases, the immune system is out of balance and this can have unpleasant consequences: from painful knees in rheumatoid arthritis, to death in very severe COVID-19.

This thesis focuses on neutrophils. Neutrophils are important cells of the innate immune system and their main task is to eliminate pathogens. For this task, they have several mechanisms. These include degranulation, production of reactive oxygen species, phagocytosis, NETosis, mediator release, and extracellular vesicle release. It used to be thought that the only job of neutrophils was to destroy pathogens and that they didn't have other functions. However, in recent years it has become clear that they are important in the communication with other cells of the immune system, allowing them to perform their tasks properly. Communication between cells occurs in several ways. It can take place through direct cell-cell contact. It can take place by secreting mediators, or by secreting extracellular vesicles.

Communication by extracellular vesicles is, in addition to neutrophils, the focus of this thesis. Extracellular vesicles are small lipid membrane enclosed particles secreted by cells. The lipid membrane allows the contents of the vesicles, such as RNA, to be safely transported to other cells and thus they are an excellent communication mechanism employed by cells.

In this thesis, we investigated the role of neutrophil activation as well as the effect of extracellular vesicles of neutrophils on the function of cells of the adaptive immune system. The initial intention was to find out in the context of autoimmune diseases. In March 2020, the COVID-19 pandemic made conducting our research difficult since contact with the clinic was very limited during the pandemic. However, this unexpected turn of events also provided great opportunities to do research on neutrophil activation in COVID-19.

In **chapter 1**, we provide a general introduction to the work described in this thesis.

In **chapter 2**, we describe the role of extracellular vesicles secreted by various cells of the innate immune system and how these extracellular vesicles affect the adaptive immune system. We summarize relevant research by other researchers in this chapter.

In **chapter 3**, we examined how neutrophils become activated. It was thought that inactive neutrophils, or resting neutrophils, first needed to be primed with a priming stimulus. Then the neutrophil would be alert and an activating stimulus could cause them to actually start using their arsenal. Only, it was very unclear in the literature what exactly priming stimuli and activating stimuli were. We, therefore, figured out from the most common priming and activating stimuli how they activated the neutrophil. We found out that a neutrophil must be activated by two stimuli. The combination (and order) of which type of stimuli does not seem to matter in this regard.

In **chapter 4**, we looked at how different drugs that can be used in COVID-19 affect neutrophil activation. Neutrophils can become overactivated in COVID-19 and then they can damage surrounding healthy cells. This can be harmful, so it is desirable that neutrophil activation is limited in COVID-19. We found that dexamethasone inhibited neutrophil activation, that vitamin D had no effect, and that vitamin A actually stimulated neutrophil activation. Thus, that dexamethasone is given in COVID-19 patients is probably important to prevent excessive neutrophil activation.

In **chapter 5**, we looked at neutrophils in synovial fluid from primarily spondyloarthritis patients. Originally, our goal was to extract extracellular vesicles from the synovial fluid and look at the effect of those vesicles on other cells. Quite surprisingly, we found that the neutrophils from patients synovial fluid were not

activated at all. This was unexpected because the synovial fluid of these patients is full of neutrophil-activating stimuli. At first, we thought that perhaps these neutrophils were exhausted, that they had already been activated. We tested this by isolating the neutrophils from the fluid and then activating them with activating stimuli. It turned out that these cells were not exhausted at all and could be activated. Therefore we thought there is something in the synovial fluid that prevents activation. We then tested this by activating neutrophils from healthy blood donors while also adding cell-free synovial fluid. It turned out that these neutrophils were indeed not activated and so the conclusion was that there is indeed something in the synovial fluid that inhibits this activation. This turned out to be due to hyaluronic acid. With this knowledge, we may be able to better address the use of drugs in rheumatic diseases in the future.

In **chapter 6**, we examined the effect of extracellular vesicles of resting and of activated neutrophils on dendritic cells and T cells. We found that these extracellular vesicles have an effect on the secretion of mediators by dendritic cells. While activated neutrophils themselves have a pro-inflammatory effect on dendritic cells, the extracellular vesicles of activated neutrophils were found to have an anti-inflammatory effect. Thus, an opposite effect. Extracellular vesicles of resting neutrophils were found to have a slight pro-inflammatory effect. With this, we were able to elucidate a small piece of the action of extracellular vesicles of neutrophils.

In **chapter 7**, we summarized and put all our results and conclusions from the previous chapters into perspective. Neutrophils are certainly not simple cells that are only part of the innate immune system. They are important cells that play a role in rheumatic diseases and COVID-19. In this thesis, we were able to contribute to elucidating the role of neutrophil activation within these diseases.

Samenvatting

Het immuunsysteem is een complex systeem dat de taak heeft ons lichaam te beschermen tegen ziekteverwekkers en het ontstaan van kanker. Ziekteverwekkers zijn bacteriën, schimmels, virussen en parasieten die niet thuishoren in ons lichaam en die ons ziek maken. Het immuunsysteem is een schild tegen al deze ziekteverwekkers en ruimt ook kwaadaardige cellen op. Deze taak is ingewikkelder dan in eerste instantie gedacht. Het immuunsysteem kan niet zomaar alles aanvallen wat lichaamsvreemd of afwijkend is. Het is bijvoorbeeld belangrijk dat het immuunsysteem niet reageert op normaal, gezond voedsel. Ook zijn er nuttige bacteriën is ons lichaam, bijvoorbeeld in de darm, en het immuunsysteem moet deze dan ook als niet-ziekteverwekkend herkennen. Zolang er een balans is en zij zich niet verder verspreiden in het lichaam.

De ingewikkelde taak om ons te beschermen wordt uitgevoerd door een groot leger aan immuuncellen. Verschillende cellen van het immuunsysteem hebben een eigen specialistische rol. Het immuunsysteem kan je indelen in twee verschillende systemen, het aangeboren systeem en het adaptieve systeem. Cellen die horen bij het aangeboren systeem reageren snel op een binnendringende ziekteverwekker. Cellen van het adaptieve systeem reageren een stuk trager, maar zijn daarentegen wel heel specifiek. Zij hebben exact de juiste wapens om een bepaalde ziekteverwekker op te ruimen. Als een infectie achter de rug is blijven er een paar specialistische getrainde cellen van het adaptieve immuunsysteem over, die bij een volgende infectie deze ziekteverwekker sneller zullen herkennen. Hierdoor raakt het lichaam immuun voor bepaalde ziekteverwekkers.

Soms gaat het helaas mis met het immuunsysteem en herkent het immuunsysteem lichaamseigen, gezonde cellen als fout en valt deze aan. Waarom dit gebeurt is niet helemaal duidelijk. Ziekten waarbij dit gebeurt zijn auto-immuunziekten, zoals verschillende vormen van reuma. Ook kan het voorkomen dat het immuunsysteem zo hard moet werken om een ziekte te bestrijden dat hierbij veel gezonde cellen slachtoffer worden, zoals kan gebeuren bij een zeer ernstige infectie met het coronavirus dat COVID-19 veroorzaakt. In beide gevallen is het immuunsysteem uit balans en dit kan nare gevolgen hebben: van ontstoken, pijnlijke gewrichten die beschadigd raken bij patiënten met reuma tot een dodelijke afloop bij zeer ernstige COVID-19.

A

Dit proefschrift richt zich op neutrofielen. Neutrofielen zijn belangrijke cellen van het aangeboren immuunsysteem en hun belangrijkste taak is het elimineren van ziekteverwekkers. Voor deze taak hebben zij verschillende mechanismen tot hun beschikking. Het eerste is degranulatie, het uitscheiden van antimicrobiële eiwitten. Het tweede is het produceren van reactieve zuurstofcomponenten, deze richten schade aan bij ziekteverwekkers. Het derde is fagocytose, het proces waarbij de ziekteverwekker wordt 'opgegeten'. De eerste twee wapens kunnen schade aanrichten aan omliggende gezonde cellen. Fagocytose is een stuk veiliger voor omliggende cellen. Het vierde wapen van neutrofielen is de vorming van web-achtige structuren die bestaan uit DNA en antimicrobiële eiwitten. Deze worden neutrofiel extracellulaire traps genoemd, oftewel NET's. In dit web blijven ziekteverwekkers plakken en zo kan de neutrofiel ze makkelijk vernietigen. Als vijfde wapen kunnen neutrofielen stoffen uitscheiden die andere immuuncellen helpen om de weg naar de ziekteverwekker te vinden. Ook deze laatste twee wapens kunnen schade aan omliggende gezonde cellen aanrichten.

Vroeger werd gedacht dat de enige taak van neutrofielen het vernietigen en opruimen van ziektemakers was en dat ze verder niet veel deden. Echter is de afgelopen jaren duidelijk geworden dat zij wel degelijk belangrijke andere taken hebben. Zo communiceren zij met andere cellen van het immuunsysteem, waardoor deze hun taken goed kunnen uitvoeren. Communicatie tussen cellen gebeurt op verschillende manieren. Het kan plaatsvinden via direct cel-cel contact, maar ook door het uitscheiden van boodschappermoleculen die bepaalde signalen overbrengen naar andere cellen of het uitscheiden van extracellulaire blaasjes.

Communicatie door extracellulaire blaasjes staat, naast neutrofielen, centraal in dit proefschrift. Extracellulaire blaasjes zijn kleine ronde structuren die worden uitgescheiden door cellen. Ze zijn een stuk kleiner dan cellen, met een diameter van 20 nanometer tot 1 micrometer. Net als cellen hebben zij een membraan van een dubbelle lipiden laag. Hierdoor kan de inhoud van de blaasjes, bijvoorbeeld RNA, veilig worden getransporteerd naar andere cellen. Als RNA los zou reizen tussen cellen zou het snel worden afgebroken en komt het nooit bij de juiste bestemming aan.

In dit proefschrift hebben wij onderzoek gedaan naar de rol van neutrofielactivatie én het effect van extracellulaire blaasjes van neutrofielen op de functie van cellen van het adaptieve immuunsysteem. De bedoeling was in eerste instantie om dit uit te

zoeken in de context van auto-immuunziekten. Wij gebruikten hiervoor vocht uit ontstoken gewrichten van patiënten met verschillende reumatische aandoeningen, zoals reumatoïde artritis en spondyloartritis. Dit vocht wordt synoviaal vocht genoemd. Tot onze verbazing bleken de neutrofielen in het synoviaal vocht van deze patiënten helemaal niet geactiveerd te zijn. Dit was een interessante observatie en bood veel nieuwe inzichten. Geheel onverwacht gooide daarnaast de COVID-19 pandemie roet in het eten van het onderzoek. Het werd heel lastig om onderzoek te doen op patiëntmateriaal. Deze onverwachte wending bood echter ook mooie kansen om interessant onderzoek te doen naar neutrofielactivatie bij COVID-19.

In **hoofdstuk 1** geven wij een algemene introductie van het werk beschreven in deze thesis.

In **hoofdstuk 2** beschrijven wij de rol van extracellulaire blaasjes die uitgescheiden worden door verschillende cellen van het aangeboren immuunsysteem en hoe deze extracellulaire blaasjes het adaptieve immuunsysteem beïnvloeden. Wij vatten in dit hoofdstuk relevant onderzoek van andere onderzoekers samen.

In **hoofdstuk 3** hebben wij onderzocht hoe neutrofielen geactiveerd raken. Er werd gedacht dat voor een goede activatie van neutrofielen, deze eerst 'wakker' gemaakt moesten worden met een priming stimulus. Dan zou de neutrofiel alert zijn en zou een activerende stimulus kunnen zorgen dat ze werkelijk hun wapenarsenaal gingen gebruiken. Alleen was het in de literatuur zeer onduidelijk wat nou precies priming stimuli en activerende stimuli waren. Wij hebben daarom van de meest voorkomende priming en activerende stimuli uitgezocht hoe ze de neutrofiel activeerden. Wij kwamen erachter dat een neutrofiel door twee stimuli moet worden geactiveerd. De combinatie (en volgorde) van welke soort stimuli lijkt hierbij niet uit te maken.

In **hoofdstuk 4** hebben wij gekeken hoe verschillende medicijnen die vaak gebruikt worden bij COVID-19 invloed hebben op de activatie van neutrofielen. Neutrofielen kunnen bij COVID-19 overgeactiveerd raken en dan brengen zij schade aan bij omliggende eigen cellen. Dit kan zeer gevaarlijk zijn en het is dus wenselijk dat activatie van neutrofielen wordt geremd bij COVID-19. Wij vonden dat dexamethason neutrofielactivatie remde, dat vitamine D geen effect had en dat vitamine A juist neutrofielactivatie stimuleerde. Het feit dat dexamethason wordt gegeven bij COVID-19 patiënten is daarom waarschijnlijk van belang om overmatige neutrofielactivatie te voorkomen.

In **hoofdstuk 5** hebben wij gekeken naar neutrofielen in synoviaal vocht van voornamelijk spondyloartritis patiënten. Heel verassend kwamen wij erachter dat de neutrofielen uit het synoviaal vocht van patiënten helemaal niet geactiveerd waren. Dit vonden wij ontzettend vreemd, want het synoviaal vocht van deze patiënten zit vol met neutrofiel activerende stoffen. In eerste instantie dachten wij dat deze neutrofielen misschien uitgeput waren, dat zij al geactiveerd waren geweest en daarom niet nogmaals geactiveerd konden worden. Dit hebben wij getest door de neutrofielen te isoleren uit het vocht en vervolgens te activeren met activerende stimuli. Het bleek dat deze cellen niet uitgeput waren en prima geactiveerd konden worden. Dus toen dachten wij dat er iets in het synoviaal vocht moest zitten dat activatie tegengaat. Dit hebben wij vervolgens getest door neutrofielen van gezonde bloeddonoren te activeren terwijl wij hier ook cel-vrij synoviaal vocht aan toe voegden. Het bleek dat deze neutrofielen dan inderdaad niet geactiveerd werden en dus was de conclusie dat er inderdaad iets in het synoviaal vocht zit dat deze activatie tegen gaat. Dit bleek te komen door hyaluronzuur. Deze kennis zou kunnen leiden tot nieuwe behandelstrategieën bij bepaalde vormen van reuma.

In **hoofdstuk 6** hebben wij onderzocht wat het effect is van extracellulaire blaasjes van rustende en van geactiveerde neutrofielen op dendritische cellen en T-cellen van het adaptieve immuunsysteem. Wij ontdekten dat deze extracellulaire blaasjes effect hebben op de uitscheiding van stoffen door dendritische cellen. Terwijl geactiveerde neutrofielen zelf een ontstekingsbevorderend effect hebben op dendritische cellen, bleken de extracellulaire blaasjes van geactiveerde neutrofielen een tegenovergesteld, ontstekingsremmend effect te hebben. Extracellulaire blaasjes van rustende neutrofielen bleken een licht ontstekingsbevorderend effect te hebben. Hiermee hebben wij meer inzicht gekregen in de effecten van extracellulaire blaasjes afkomstig van neutrofielen.

In **hoofdstuk 7** hebben wij al onze resultaten en conclusies uit de eerdere hoofdstukken samengevat en in perspectief gezet. Neutrofielen zijn zeker geen simpele cellen die alleen onderdeel zijn van het aangeboren immuunsysteem. Het zijn belangrijke cellen die bij reumatische aandoeningen en COVID-19 een rol spelen. In dit proefschrift hebben wij een bijdrage kunnen leveren aan het verhelderen van de rol van activatie van neutrofielen binnen deze ziekten.

Portfolio

Courses	Year	ECTS
Laboratory Safety	2018	0.3
The AMC world of Science	2018	0.7
Advanced Immunology	2019	2.9
Didactic skills	2020	0.4
Scientific writing	2020	0.6
Seminars, symposia, meetings, and masterclasses		
Weekly EXIM seminars	2018-2022	2.0
Weekly meeting de Jong group	2018-2022	2.0
AMC EXIM retreat 3x	2018-2021	1.5
Masterclass by Muzlifah Haniffah	2018	0.2
I&I symposium Utrecht	2018	0.5
Masterclass by Edit Buzas	2019	0.2
AIII symposium 2x	2019, 2021	1.0
Presentations		
ECI conference Amsterdam – poster	2018	0.2
NLSEV 2018 – poster	2018	0.2
NVVI annual meeting – poster	2019	0.2
NVVI annual meeting – online poster	2020	0.2
The Neutrophil – online poster	2021	0.2
AIII symposium – oral presentation	2021	0.2
(Inter)national conferences		
ECI conference Amsterdam	2018	1.5
NLSEV	2018, 2019	1.0
NVVI lunteren symposium 3x	2018-2021	1.5
NVVI annual meeting 3x	2018-2021	1.5
The Neutrophil (online)	2021	1.0
ECI conference (online)	2021	1.5
Supervising		
Supervising research internship bachelor student – 5 months	2019	1.7
Supervising research internship master student – 7 months	2020-2021	2.3
Supervising practicals master students	2018-2021	1.0
Awards		
NLSEV 2018 poster award	2018	
NVVI 2020 poster prize	2020	

Author contributions

Chapter 1

Writing original draft, S.M.; Writing review and editing, T.G.K., S.W.T., M.H.M.W., E.C.d.J.

Chapter 2

Writing original draft, T.G.K, S.M.; Writing review and editing, T.G.K., M.H.M.W., E.C.d.J.

Chapter 3

S.M., S.W.T., M.H.M.W., T.G.K., and E.C.d.J. were involved in study conception and design. S.M., F.M.J.H., L.V., N.S. and E.W.T.-K. performed experimental work. S.M. performed statistical analysis and figure production and wrote the initial draft of the manuscript. S.W.T., M.H.M.W., T.G.K., and E.C.d.J. revised the manuscript. All authors participated in interpreting results and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

Chapter4

Conceptualization: Mol S, Hafkamp FMJ, De Jong EC; Investigation: Mol S, Hafkamp FMJ, Waqué I; Formal analysis: Mol S, Hafkamp FMJ; Supervision: De Jong EC; Writing – original draft: Mol S, Hafkamp FMJ; Writing – review: Waqué I, De Jong EC.

Chapter 5

Conceptualization: Mol S, Groot Kormelink T, Van de Sande M, Tas SW, Wauben MHM, De Jong EC; Investigation: Mol S, Taanman-Kueter EW, Van der Steen BA, Groot Kormelink T; Formal analysis: Mol, S; Supervision: Wauben MHM, Tas SW, De Jong EC; Writing – original draft: Mol S; Writing – review: all authors; Final approval of the manuscript: all authors

Chapter 6

S.M., T.G.K., S.W.T., M.H.M.W., and E.C.d.J. were involved in the study conception and design. S.M., F.M.J.H., L.V., and E.W.M.T-K. performed experimental work. S.M. performed the statistical analysis and figure production and wrote the initial draft of the manuscript. T.G.K., S.W.T, M.H.M.W., and E.C.d.J. revised the manuscript. All authors participated in interpreting the results and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

Chapter 7

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List of publications

This Thesis

Groot Kormelink, T., **Mol, S.**, de Jong, E. C., & Wauben, M. H. (2018, September). The role of extracellular vesicles when innate meets adaptive. In Seminars in immunopathology (Vol. 40, pp. 439-452). Springer Berlin Heidelberg.

Mol, S., Hafkamp, F. M., Varela, L., Simkhada, N., Taanman-Kueter, E. W., Tas, S. W., ... & de Jong, E. C. (2021). Efficient neutrophil activation requires two simultaneous activating stimuli. International Journal of Molecular Sciences, 22(18), 10106.

Mol, S., Hafkamp, F. M., Waqué, I., & De Jong, E. C. (2021). Dexamethasone, but Not Vitamin D or A, Dampens the Inflammatory Neutrophil Response to Protect At-risk COVID-19 Patients. Immune Network, 22.

Mol, S., Taanman-Kueter, E. W., van der Steen, B. A., Groot Kormelink, T., van de Sande, M. G., Tas, S. W., ... & de Jong, E. C. (2023). Hyaluronic Acid in Synovial Fluid Prevents Neutrophil Activation in Spondyloarthritis. International Journal of Molecular Sciences, 24(4), 3066.

Mol, S., Varela, L., Hafkamp, F. M., Taanman-Kueter, E. W., Tas, S. W., ... & de Jong, Extracellular vesicles from resting or activated neutrophils differentially affect DC function. Manuscript in preparation

Other

Zwarthoff, S. A., Berends, E. T., **Mol, S**., Ruyken, M., Aerts, P. C., Józsi, M., ... & Gorham Jr, R. D. (2018). Functional characterization of alternative and classical pathway C3/C5 convertase activity and inhibition using purified models. Frontiers in immunology, 9, 1691.

Driedonks, T. A., **Mol, S**., de Bruin, S., Peters, A. L., Zhang, X., Lindenbergh, M. F., ... & Nolte-'T Hoen, E. N. (2020). Y-RNA subtype ratios in plasma extracellular vesicles are cell type-specific and are candidate biomarkers for inflammatory diseases. Journal of Extracellular Vesicles, 9(1), 1764213.

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Het leven bestaat absoluut niet alleen maar uit werken en ontwikkelen. Ontspanning en gezelligheid zijn minstens net zo belangrijk, zo niet nog belangrijker.

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Amber en **Maaike**, we go way back. Jullie waterpoloën dan wel niet meer, maar gelukkig zie ik jullie nog vaak. Allemaal doen we iets totaal anders. Ontzettend bedankt dat jullie altijd geïnteresseerd waren in mijn onderzoek. Ik vond het leuk om het te kunnen uitleggen aan mensen die niet in de richting van life sciences zaten. Gelukkig zijn jullie net zo'n grote fan van Harry Potter en net zo enthousiast over leuke dingen als ik. De bezoekjes aan de Sauna, het lekkere en vele eten, de spelletjes, en bezoekjes aan foodfestivals en de Efteling zijn altijd gezellig. Ik kijk uit naar ons volgende uitje.

De erlenmeyers, **Manon**, **Romy**, **Robin** en **Elrik**. Met jullie begon ik mijn wetenschapsreis en nu zijn we alweer 11 jaar bevriend. Allemaal gesetteld en een aantal van ons zijn zelfs al begonnen met nieuwe jonge wetenschappers opleiden. Hoewel we allemaal begonnen in dezelfde richting, zijn onze banen van nu toch behoorlijk verschillend. Nu nog maar twee over die op het lab willen blijven werken. **Elrik**, wat fijn dat wij samen de master in Utrecht zijn begonnen. Het was een goede keuze om daar verder te gaan studeren. Dankjewel ook voor het doneren van neutrofielen voor mijn experimenten. **Manon**, mega bedankt voor het nakijken van vele van mijn stukken. Super fijn dat je dat hebt willen doen. Jij bent altijd secuur en netjes in je werk, daar kan ik nog wat van leren! Ook straal jij vertrouwen en rust uit, enorm fijn dat ik af en toe mijn verhaal bij je kwijt kon.

Esther en **Simon**. Tijdens mijn grote masterstage heb ik jullie leren kennen en wat was die stage gezellig met jullie. Wat kunnen wij (vooral jullie) verhit filosoferen over het leven zeg! Allemaal worstelen we soms met ontzettend ambitieus zijn en tegelijkertijd vanuit onze eigen waarden willen leven. Het is ontzettend fijn om met jullie te reflecteren over de grote zaken in het leven en een beetje te klagen over de PhD's.

Mijn waterpolo team. **Alyssa**, **Dide**, **Eline**, **Fleur**, **Hanna**, **Jody**, **Josien**, **Lianne**, **Lotte**, **Marinda**, **Melina**, **Sanne**, **Renske**, **Yvonne ,** en favoriete coach **Dennis**. Jullie zijn mijn halve sociale leven. We zijn allemaal best wel anders, maar toch werkt het extreem goed. We zijn ook echt wel een life science team en veel begrijpen ook hoe shit een PhD kan zijn. Ontzettend bedankt voor jullie steun, gezelligheid, en het zijn van een sociaal vangnet. Ik weet niet wat ik zonder jullie zou moeten doen. **Marinda**, wat ben jij dapper om nog met een PhD te starten na al het geklaag van Joost, Lex, en ondergetekende. Dat jij dit aandurft getuigd ook dat je het heel graag wilt. Ik weet zeker dat als iemand het kan jij het kan! Mocht het toch ooit een beetje of erg shit worden. Wij zijn er voor je! **Alyssa**, als iedereen aan het, in mijn ogen, moeilijk doen was, dan kon ik in jou een maatje vinden. Ik waardeer je nuchterheid en je vermogen om alles aan te pakken. **Lotte**, dankjewel dat je altijd herkende wanneer ik mijzelf dreigde te verliezen in de passie voor de sport tijdens een wedstrijd. Fijn dat je zo goed op mij let. Ik ga je missen nu je komend seizoen niet meer gaat spelen. Hopelijk kom je nog vaak kijken. **Sanne** en **Dide**, de twee-eenheid binnen het team. Betrokken, enthousiast en geïnteresseerd zijn kernwoorden die bij jullie beiden goed passen. Dank jullie wel voor jullie interesse in mijn leven en jullie steun tijdens mijn PhD en daaromheen. **Yvo**, dankjewel voor jouw vrolijkheid. Het woord glitter koppel ik aan jou. Jij bent overal enthousiast over en kan van alles een feestje maken, maar waar nodig kan je ook heel serieus zijn en goed advies geven. Dankjewel ook voor al je tips over het moederschap, fijn om een goede vriendin te hebben die het allemaal al gedaan heeft. **Melina**, waar moet ik beginnen met jou bedanken. Wat heb jij ongelooflijk veel voor mij betekend de afgelopen jaren. Gewoon gezellig doen, advies geven over sociale situaties, steun in moeilijke tijden, een top ceremoniemeester zijn. Jij kan en bent het allemaal. Dat heb je allemaal gedaan en ondertussen ook nog 'eventjes' je opleiding afgemaakt en zo goed bezig met je sport. Ik heb diep respect voor jou. Ik geloof niet dat ik zou staan waar ik nu sta zonder jou. Een vriendin zoals jij gun ik iedereen. Jij bent echt goud waard! Ik kijk uit naar ons gezamenlijk tripje naar Marrakesh. **Josien**, ook jij bent een top ceremoniemeester. Jij hebt samen met Mel de trouwdag van mij en Arjan onvergetelijk gemaakt. Jij bent de perfecte combinatie van serieus en enorm gezellig. Er was altijd ruimte om met jou te praten over ingewikkelde zaken en te klagen over het afronden van de PhD. Dankjewel voor al je adviezen, die waren en zijn ontzettend nuttig. Daarnaast heb je ook altijd ruimte voor gezelligheid en als er weer iets georganiseerd moest worden dan heb jij 100 leuke ideeën. **Lianne**, wij begonnen ongeveer tegelijkertijd aan een PhD. Onze PhD's liepen zo anders en toch zo hetzelfde. Wat fijn om elkaar er door heen te

slepen. Jij stond altijd klaar om te sparren over PhD situaties. Net als onze PhD's zijn ook wij zo anders en toch zo hetzelfde. Waar ik iets doe of zeg zonder dat ik er goed en wel over nadenk, doe jij alles weloverwogen en serieus. Verfrissend om te zien dat we allebei komen waar we willen zijn. Er zijn gelukkig meerdere wegen naar Rome leiden. Je bent ontzettend loyaal en goed in alles wat je doet. Je straalt vertrouwen uit. Als ik een team zou moeten samen stellen voor het oplossen van een probleem, zou ik jou daar als eerste in willen hebben. Gelukkig lossen we samen regelmatig grote escape room problemen op! Wat fijn dat je op mijn verdediging naast mij staat als paranimf. Mooi om het PhD hoofdstuk samen te kunnen afsluiten.

Vrienden waren belangrijk, maar familie minstens net zo. Ik ben gezegend met twee families, dat eigenlijk ook weer één familie is.

Mijn schoonfamilie, wat zijn jullie toppers. Bruisend van gezelligheid. Het motto van mijn schoonfamilie is volgens mij het volgende: "wij kennen geen koude kant." Vanaf het eerste moment dat ik alweer 13 jaar geleden bij jullie over de vloer kwam voelde ik mij welkom. Zo welkom dat ik bij het eerste Pasen, zonder Arjan bij de familie Baar heb gebruncht, nog met oma en opa Peek. Later volgde nog de vrouwen-diners ook met mijn eigen moeder erbij. Iedereen is betrokken met elkaar en dat is erg fijn. Extra dank aan **Anne & Marjolein**, wat fijn om van jullie ervaringsverhalen over het doen van een PhD te horen. **Bart**, **Mark**, en **Marieke**. Wat zijn jullie fijne ooms en tantes voor onze lieve Isabella. **Ruud**, dankjewel voor je oprechte interesse in mijn werk. **Petra**, bij jou is er altijd ruimte voor gezelligheid en een luisterend oor. Liefde heb jij in overvloed te geven. Dankzij jou snap ik de verhalen over schoonmoeders nooit, jij bent de allerfijnste schoonmoeder die ik mij maar kan wensen.

Mijn eigen familie is een stuk rustiger dan mijn schoonfamilie, maar zeker niet minder leuk. Mijn familie kan ik beschrijven als heerlijk nuchter. Niemand is snel onder de indruk en dat is fijn, dat houd iedereen met de benen op de grond.

Alex, ik heb bewondering voor de manier hoe jij in het leven staat. Stress staat niet in jouw woordenboek. Jij bent de perfecte combinatie van zorgeloos zijn, kinderlijk enthousiast zijn en het hebben van een "dat doe ik wel even"-mentaliteit. Eigenschappen die typerend zijn voor ons gezin, maar sterk bij jou naar voren komen. Jij bent altijd in voor gezelligheid en een goed spel. **Rianne**, wat fijn dat je met Alex samen bent. Dank jullie wel, voor jullie interesse en jullie ruimte en rust. Jullie oordeelden nooit als ik weer eens midden in de drama zat, bedankt daarvoor.

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Isa, je bent nog maar kort op deze wereld, maar jouw impact is nu al zo groot. Jij laat mij vertragen en laat mij inzien wat echt belangrijk is in het leven. Niks maakt mij op dit moment gelukkiger dan jouw schaterlach. Ik kijk er naar uit om jou te zien opgroeien en ik ben zo ontzettend dankbaar dat ik getuige mag zijn van jouw ontdekkingsreis van deze wereld.

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Nobody said it was easy
It's such a shame for us to part
Nobody said it was easy
No one ever said it would be this hard
Oh, take me back to the start

The Scientist, Coldplay