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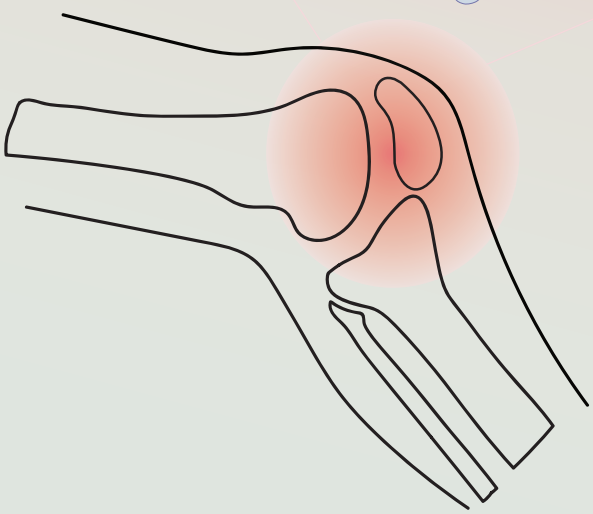

Monocytes and Neutrophils in Juvenile Idiopathic Arthritis

Tobias Schmidt

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University, Department of Clinical Sciences in Lund.

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Abstract:

Juvenile idiopathic arthritis (JIA) is a heterogenous inflammatory joint disease and the most common rheumatic disease in children. Oligoarticular JIA (oJIA) is the major subgroup, which mainly affects few and large joints, such as the knee. The immunological processes and key players driving inflammation within the affected joints are not well characterised. Research has primarily focused on adaptive immunity, and little is known of the contribution of the innate immune system. Neutrophils and monocytes are central members, with crucial roles as phagocytes, cytokine producers and regulators of inflammation. Given the limited knowledge of the role of innate immunity in oJIA, we aimed to characterize the phenotype and function of monocytes and neutrophils in the arthritic joint.

We found that synovial monocytes had both regulatory and pro-inflammatory features. For example, at the surface level, they expressed markers of clearance and antigen presentation. This was correspondingly reflected at the mRNA level. Functionally, the synovial monocytes showed resistance to cytokine production upon further activation and had an increased efferocytosis. Additionally, they also promoted activation of healthy T-cells. Interestingly, we found that healthy monocytes acquired the regulatory features of synovial monocytes (both phenotypical- and fucntional features) through exposure to patient synovial fluid. This was primarily through the IL-6/JAK/STAT pathway. Furthermore, we showed that the monocytes obtained the inflammatory features through cell-cell interactions, such as the ability to promote T-cell activation. Specifically, we found that synovial fibroblasts induced this activation in healthy monocytes in co-cultures in a contact-dependent manner, especially if the synovial fibroblasts were priorly exposed to synovial fluid. Indeed, this exposure to synovial fluid resulted in cytokine production and an enhanced ability to induce immune cell chemotaxis by the fibroblasts.

Furthermore, we found that synovial neutrophils displayed signs of activation at the surface level, and they had acquired a monocyte-like phenotype. This phenotype correlated with impaired effector functions, primarily decreased phagocytosis ability and reactive oxygen species production. Interestingly, their phenotype could not be induced by stimulation of healthy neutrophils with synovial fluid, nor in co-cultures with synovial fibroblasts, suggesting that different mechanisms drive the neutrophil phenotype.

Taken together, the results of this thesis describe the phenotype and role of synovial monocytes and neutrophils in the pathogenesis of oJIA, and emphasize potential underlying mechanisms driving their phenotypes that could be utilized to develop therapies.

Key words: Monocytes, neutrophils, juvenile idiopathic arthritis, inflammation, rheumatology, immunology

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“Remember kids, the only difference between screwing around and science is writing it down.”

Adam Savage

Table of Contents

Abbreviations

ANA – Anti-nuclear antibodies

APC – Antigen presenting cell

BCR – B-cell receptor

CCR – Chemokine receptor

CCL/CXCL – Chemokine

CD – Cluster of differentiation

CTV – CellTrace Violet

DAMP – Damage associated molecular pattern

DC – Dendritic Cell

DMARD – Disease modifying anti-rheumatic drug

ECM – Extracellular matrix

FLS – Fibroblast-like synoviocytes

HLA – Human leukocyte antigen

IC – Immune complexes

IFN – Interferon

IL- – Interleukin-

JAK – Janus kinase

JIA – Juvenile idiopathic arthritis

LC-MS – Liquid chromatography mass spectrometry

LPS – lipopolysaccharide

NADPH – Nicotinamide adenine dinucleotide phosphate

NETs – Neutrophil extracellular traps

NSAID – Non-steroid anti-inflammatory drug

MCP-1 – Monocyte chemoattractant protein 1

M-CSF – Macrophage colony stimulating factor

MHC – Major histocompability complex

mRNA – messenger ribonucleic acid

MPO – Myeloperoxidase

MØ – Macrophage

OA – Osteoarthritis

oJIA – oligoarticular juvenile idiopathic arthritis

PAMP – Pathogen associated molecular pattern

PBMC – Peripheral blood mononuclear cells

PMA – Phorbol 12-myristate 13-acetate

PMN – Polymorphonuclear neutrophils

PRR – Pattern recognition receptor

PsA – Psoriatic Arthritis

RA – Rheumatoid arthritis

RF – Rheumatoid factor

ROS – Reactive oxygen species

Ser – Serum

SF – Synovial fluid

S-Fib – Synovial fibroblasts

sJIA – systemic juvenile idiopathic arthritis

SNP – Single nucleotide polymorphism

SpA - Spondylarthritis

STAT – Signal transducer and activator of transcription

TCR – T-cell receptor

TGF-β – Transforming growth factor-β

Th – Helper T cell

TLR – Toll like receptor

TNF – Tumor necrosis factor alpha

Treg – Regulatory T cell

Populärvetenskaplig sammanfattning

Det är väl bara gamla människor som får problem med lederna, inte barn?

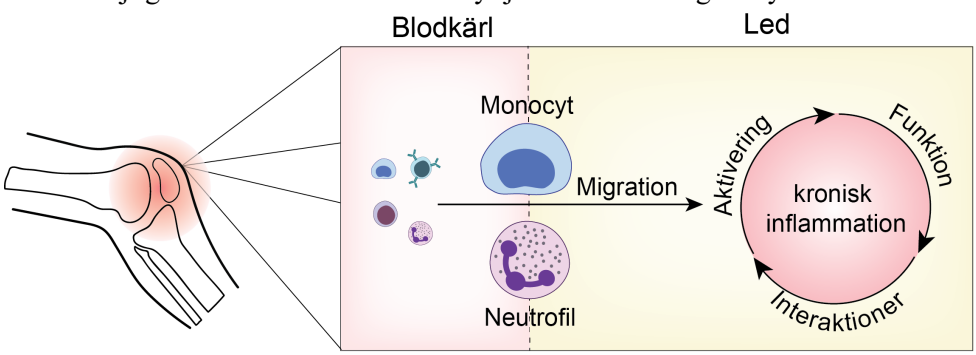
Kanske något överraskande så är inflammation i lederna den vanligaste reumatiska sjukdomen som drabbar barn. Ungefär 200–250 barn drabbas varje år i Sverige. Denna sjukdom kallas för *juvenil idiopatisk artrit (JIA)*. *Juvenil* står för att den drabbar barn, *idiopatisk* för att orsaken till sjukdomen inte är klarlagd, och *artrit* för ledinflammation. *Inflammation* är kroppens reaktion på en skada eller infektion, och kan beskrivas som slaget där kampen mellan kroppen och skadan utkämpar sig. JIA tros uppstå på grund av att de så kallade vita blodkropparna, som ingår i vårt försvarssystem, misstar kroppens egna celler för att vara en inkräktare och påbörjar en attack. Detta leder till något som kallas för en *autoimmun sjukdom*, där kroppen helt enkelt försöker bryta ner och oskadliggöra kroppens egen vävnad. Eftersom ”inkräktaren” inte går att göra sig av med så tar inte inflammationen slut, utan i stället uppstår *kronisk inflammation*. I JIA så angrips lederna – vilket leder till smärta, ömhet och rörlighetssvårigheter. Tack vare modern behandling, som syftar till att bromsa immunförsvaret, så ser förutsättningarna för patienterna ljusare ut. Men trots att sjukdomen är så pass ”vanlig” så vet vi väldigt lite om hur den uppstår, och om vi kan återställa immunförsvaret. Historiskt har JIA ansetts vara en barnvariant av ledsjukdom hos vuxna (t.ex. reumatoid artrit), vilket forskning dock har visat inte är helt sant. Därför vet vi i dagsläget väldigt lite om varför ledinflammation uppstår hos just barn. Vilka delar av immunförsvaret är involverade? Hur orsakar de sjukdom? Kan vi bromsa – eller stänga av – den onödiga autoimmuna attacken?

I den här avhandlingen djupdyker vi i just dessa frågor, och söker reder ut vilka – och hur – immunförsvarets celler bidrar till JIA. Vi fokuserar framför allt på två av immunförsvarets mest förekommande celler: neutrofiler och monocyter. Neutrofiler är våra vanligaste immunceller som utgör upp till 70% av de vita blodkroppar i blodet. De är vanligtvis först på plats vid en infektion eller skada, och är väldigt bra på att oskadliggöra inkräktare och driva på inflammationen. Monocyterna har många olika funktioner, men framför allt är de väldigt bra på att styra och aktivera andra delar av immunförsvaret. De producerar olika signalmolekyler som kallar på andra delar av immunförsvaret och aktiverar dessa. Båda celltyperna kan också hjälpa till att bromsa och stänga av inflammation (de har så kallade regulatoriska egenskaper). Detta är minst lika viktigt, då en obalans mellan inflammation och reglering av denna leder till kronisk inflammation, till exempel vid JIA. När

avhandlingen påbörjades visste man väldigt lite om dessa cellers roll i JIA. Syftet var således att undersöka hur monocyter och neutrofiler är aktiverade, vad deras funktion är och hur de kan bidra till sjukdom i den inflammerade leden.

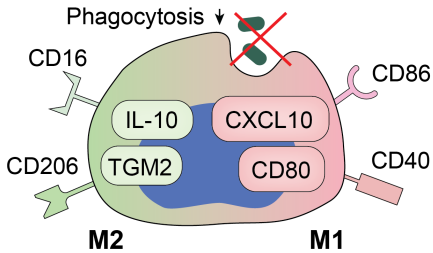
I det första och andra arbetet så beskriver vi monocyternas roll i leden. Vi jämförde monocyterna i blodet med monocyterna i patientens led, och undersökte hur de skiljde sig åt. Bland annat så kom vi fram till att monocyterna har genomgått aktivering och är funktionellt påverkade på ett sätt som gynnar kronisk inflammation i leden. Till exempel så fann vi att de driver aktivering av andra immunceller. Slutligen så identifierade vi hur monocyterna blev aktiverade, vilket visade sig vara delvis av den inflammerade ledvätskan, men också via interaktioner med andra lokala celler. I det tredje arbetet undersöker vi på ett liknande sätt neutrofilerna. Precis som monocyterna så visade neutrofilerna i leden tecken på aktivering. Bland annat var neutrofilerna sämre på att städa upp, vilket tros leda till en förmånlig miljö för fortsatt inflammation. I det fjärde och sista arbetet så studerar vi hur de lokala ledcellerna från patienterna (så kallade fibroblaster) driver aktivering av monocyter och neutrofiler. Vi fann att fibroblasterna inducerar aktivering av monocyter, men inte av neutrofiler. Detta var särskilt tydligt när fibroblasterna tidigare har utsatts för inflammerad ledvätska.

Sammanfattningsvis så har vi i de fyra olika arbetena identifierat aktiveringsmönster och funktionella förändringar hos monocyter och neutrofiler i den inflammerade leden hos patienter med JIA. Arbetena stärker tesen om en obalans i den immunologiska miljön, och ger oss delvis mer kunskap om sjukdomsförloppet, och delvis möjliga mekanismer som kan utnyttjas för utveckling av nya läkemedel.



Bilden visar en kort sammanfattning av huvudfynden i avhandlingen. Vi visar att monocyter och neutrofiler infiltrerar leden och genomgår aktivering, t.ex. via interaktioner med de lokala cellerna eller via den inflammatoriska miljön. Detta innebär att monocyternas och neutrofilernas funktionella egenskaper förändras, vilket i sin tur resulterar i ökat bidrag till inflammation. Slutligen så interagerar de aktiverade monocyterna och neutrofilerna med andra celler i leden, vilket leder till ytterliggare aktivering av andra delar av immunförsvaret och således en loop av kronisk inflammation.

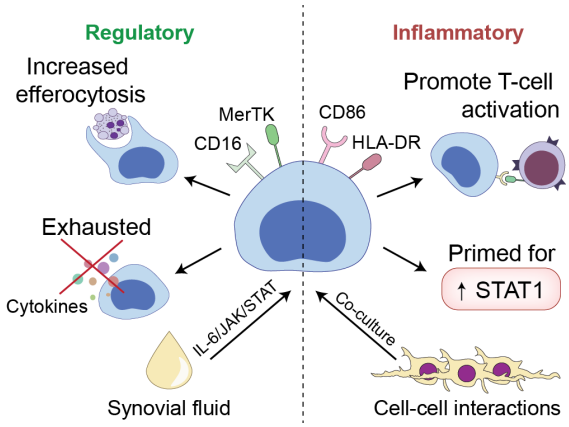
Thesis at a glance



Paper I. *Aim:* To map the polarization pattern of synovial monocytes from patients with oligoarticular JIA (oJIA).

Key findings: Synovial monocytes, compared to circulating monocytes, displayed a state of mixed polarization, with both pro-inflammatory (M1) and regulatory (M2) features at the surface and mRNA level. *In vitro*, synovial fluid induced the expression of M2 markers in healthy monocytes, but not M1 markers. The synovial monocytes were also functionally affected, as they had reduced phagocytosis and reactive oxygen species (ROS) production.

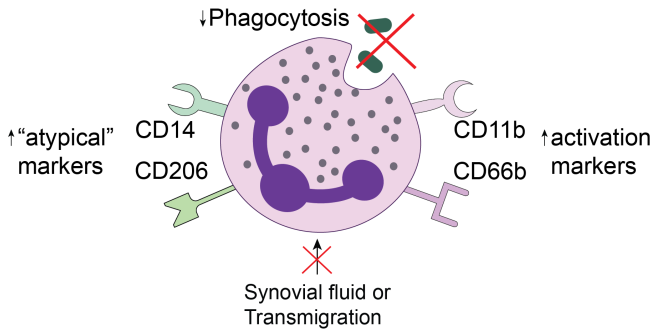
Conclusion: Synovial monocytes are activated and functionally impaired, which highlight their potential role in the pathogenesis of oJIA.



Paper II. *Aim:* To characterize the function of synovial monocytes and unravel the mechanisms behind how they obtain their phenotype in oJIA.

Key findings: Monocytes from joints of patients with oJIA had both pro-inflammatory and regulatory functions, displaying increased efferocytosis, resistance to cytokine production following activation and an increased ability to induce T cell activation. Synovial fluid induced the regulatory features in healthy monocytes, driven by IL-6/STAT signaling. The magnitude of IL-6/STAT activation in monocytes was reflected in circulating cytokine levels. On the other hand, the pro-inflammatory aspects required cell-cell contact and could be induced in co-cultures with fibroblast-like synoviocytes.

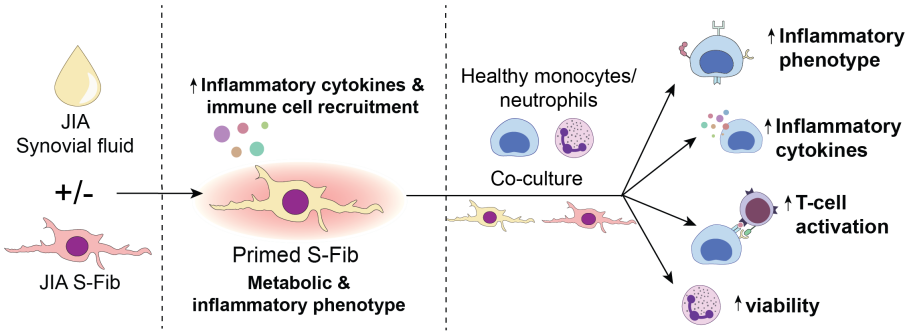
Conclusion: Synovial monocytes have a dual role in the pathogenesis of oJIA and obtain their features through the inflammatory environment and cell-cell interactions.



Paper III. *Aim:* To describe the phenotype and function of neutrophils in the synovium of patients with oJIA.

Key findings: Compared to circulating neutrophils, synovial neutrophils displayed an activated phenotype. Furthermore, they also expressed "atypical" markers that are not usually found on neutrophils, such as CD206. This atypical phenotype correlated with impaired effector functions, specifically phagocytosis and ROS production. Finally, the neutrophil phenotype could not be replicated *in vitro* using healthy neutrophils and inflamed synovial fluid, nor was it a result of transmigration alone.

Conclusion: Synovial neutrophils are phenotypically and functionally different compared to circulating neutrophils.



Paper IV. *Aim:* To investigate the influence of synovial fluid on synovial fibroblasts (S-Fib) from oJIA patients, and their ability to induce activation in healthy monocytes and neutrophils.

Key findings: Stimulation of S-Fib with inflammatory synovial fluid induced cytokine production and enrichment of metabolic- and inflammatory processes. Co-culture between S-Fib and healthy monocytes induced pro-inflammatory changes, such as increased cytokine production and ability to induce T-cell activation, which was further pronounced if the S-Fib were activated with synovial fluid prior to co-culture. Effects on the neutrophil phenotype and function were minor, but co-culture with primed S-Fib prolonged their survival.

Conclusion: S-Fib contribute to synovial inflammation by inducing inflammatory monocytes and prolonging neutrophil survival, processes amplified by prior activation of the S-Fib with inflammatory synovial fluid.

List of publications and manuscripts

I. **Schmidt, T**., Berthold, E., Arve-Butler, S., Gullstrand, B., Mossberg, A., Kahn, F., Bengtsson, A. A., Månsson, B., & Kahn, R. (2020).

Children with oligoarticular juvenile idiopathic arthritis have skewed synovial monocyte polarization pattern with functional impairment- a distinct inflammatory pattern for oligoarticular juvenile arthritis. *Arthritis research & therapy*, *22*(1), 186.

II. **Schmidt, T**., Dahlberg, A., Berthold, E., Król, P., Arve-Butler, S., Rydén, E., Najibi, S. M., Mossberg, A., Bengtsson, A. A., Kahn, F., Månsson, B., & Kahn, R. (2023).

Synovial monocytes contribute to chronic inflammation in childhood-onset arthritis via IL-6/STAT signalling and cell-cell interactions. *Frontiers in immunology*, *14*, 1190018.

III. Arve-Butler, S., **Schmidt, T**., Mossberg, A., Berthold, E., Gullstrand, B., Bengtsson, A. A., Kahn, F., & Kahn, R. (2021).

Synovial fluid neutrophils in oligoarticular juvenile idiopathic arthritis have an altered phenotype and impaired effector functions. *Arthritis research & therapy*, *23*(1), 109.

IV. **Schmidt, T**., Mossberg, A., Berthold, E., Król, P., Bengtsson, A. A., Kahn, F., Månsson, B., & Kahn, R. (2023).

Synovial fluid potentiates local fibroblasts to drive inflammatory monocytes in childhood-onset arthritis. *Manuscript.*

Publications, published during doctoral studies, not included in this thesis

- Kahn, R., **Schmidt, T**., Golestani, K., Mossberg, A., Gullstrand, B., Bengtsson, AA., Kahn, F. (2021) Mismatch between circulating cytokines and spontaneous cytokine production by leukocytes in hyperinflammatory COVID-19. *J Leukoc Biol*. Jan;109(1):115-120.
- Arve-Butler, S., Mossberg, A., **Schmidt, T**., Welinder, C., Yan, H., Berthold, E., Król, P., Kahn, R. (2022) Neutrophils Lose the Capacity to Suppress T Cell Proliferation Upon Migration Towards Inflamed Joints in Juvenile Idiopathic Arthritis. *Front Immunol*. Jan 13;12:795260.
- **Schmidt, T**., Kahn, R., Kahn, F. (2022) Ascorbic acid attenuates activation and cytokine production in sepsis-like monocytes. *J Leukoc Biol*. 2022; 1– 8.
- Izadi, A., Hailu, A., Godzwon, M., Wrighton, S., Olofsson, B., **Schmidt, T**., Söderlund-Strand, A., Elder, E., Appelberg, S., Valsjö, M., Larsson, O., Wendel-Hansen, V., Ohlin, M., Bahnan, W., Nordenfelt, P. (2023) Subclass-switched anti-spike IgG3 oligoclonal cocktails strongly enhance Fc-mediated opsonization. *Proc Natl Acad Sci*. Apr 11;120(15):e2217590120.
- **Schmidt, T**., Neumann, A. (2023). Analysis of Neutrophil and Monocyte Inflammation Markers in Response to Gram-Positive Anaerobic Cocci. In: Nordenfelt, P., Collin, M. (eds) Bacterial Pathogenesis. Methods in Molecular Biology, vol 2674. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-3243-7_14.

Introduction to the field

Overview of the immune system

Introduction

The word *immune* originates from the Latin word *immunis*, meaning "exempt from public service", which was adopted in medicine to signify one being "exempt from disease". Accordingly, the role of our immune system is exactly that, to protect us from disease. The immune system functions as our bodies' sophisticated defence system, designed to quickly eradicate any potential threat to our health and subsequently restore homeostasis. Therefore, it is evident that without this system, we would not be alive. Additionally, it is important to note that the immune system serves to protect us not only from intruders (e.g., bacteria, viruses) but also from other breaches of our body, such as injuries and cancer. The immune system consists of several components, such as cells (white blood cells), proteins (e.g., complement and antibodies), mucosa and organ systems (e.g., the lymphatic system). When members of the immune system sense an intruder or an injury, one of their primary roles is to initiate *inflammation*.

Inflammation

Inflammation can be described as the immune system's response to various stimuli, such as pathogens, damaged cells/tissues, or toxic composites. Inflammation is initiated to combat the threat and can be identified based on five pillars: *redness, swelling, heat, pain,* and *loss of function.*

The initial phase of inflammation is referred to as *acute inflammation*. Here, local- and recruited immune cells initiate various processes focused on: limiting damage, protecting tissues, clearing debris, and/or eliminating pathogens. Acute inflammation lasts for only a couple of days and is then followed by a second phase, commonly referred to as the *proliferation phase*. In this phase, tissues are rebuilt through the processes of cell migration, proliferation, and collagen formation. As such, the focus of the immune system at this phase shifts from controlling damage to reversing it and lasts for approximately 1-3 weeks. Finally, the last phase, termed the *remodelling phase,* is activated. Here, the repaired tissue is adapting – scars are

being established, collagen crosslinking is forming, and blood vessels become organized.

So how does the immune system know where the damage is? In an endogenous setting, such as an injury, damaged and destroyed cells release molecules called damage-associated molecular patterns (DAMPs). These are common molecules of intracellular origin that should not be found outside of the cell, which include molecules such as histones, heat-shock proteins, or DNA. Similarly, during an infection, the immune system instead encounters molecules called pathogen-associated molecular patterns (PAMPs). These PAMPs are of foreign origin, such as the bacterial components lipopolysaccharide (LPS) or lipoteichoic acid. Both DAMPs and PAMPs can interact with surface receptors on immune cells, called pattern recognition receptors (PRRs) (**Figure 1**). PRRs are comprised of several families, such as the toll-like receptors (TLRs) or Nod-like receptors (NLRs). Regardless of the source, both DAMPs and PAMPs quickly encounter cells of the immune system, such as macrophages (MØs), which are scattered throughout the tissues. Upon interaction of PRRs with DAMPs or PAMPs, these cells become activated and start to produce signalling molecules of their own, termed cytokines and chemokines, which attract more immune cells from the circulation.

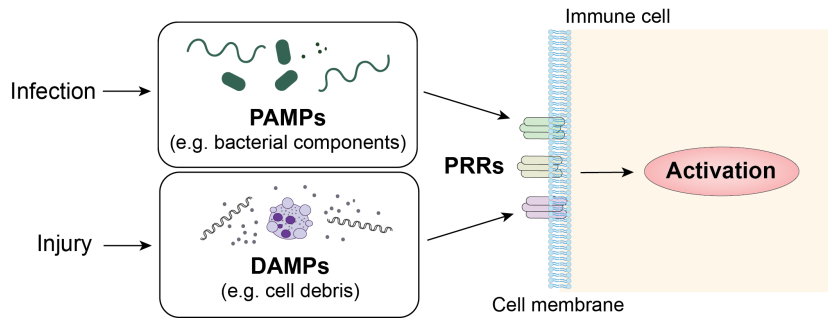


Figure 1. Identification of danger. When the body is threatened, such as during an infection or following an injury, PAMPs or DAMPs, respectively, are released and interact with PRRs on immune cells. Subsequently, the immune cells become activated, start to recruit more immune cells and initiate inflammation. *PAMP – Pathogen associated molecular pattern, DAMP – Damage associated molecular pattern, PRR – Pattern recognition receptor.*

The two main branches of the immune system

The immune system is commonly separated into the innate- and adaptive immune system (**Figure 2**). The *innate immune system* represents the first line of defence, whose purpose is to engulf and eliminate pathogens and damaged cells. This system exists in most multicellular organisms and reacts to an extremely broad range of pathogens.

However, jawed vertebrates have developed an additional immune response – the *adaptive immune system*. This part of the defence system is often referred to as being slow but having a specific memory with extremely precise weapons – antibodies.

Importantly, these two systems are highly integrated as, for example, cells of the innate immune system may activate members of the adaptive immune system, thus mounting the next level of the defence. Together, they form our protection system.

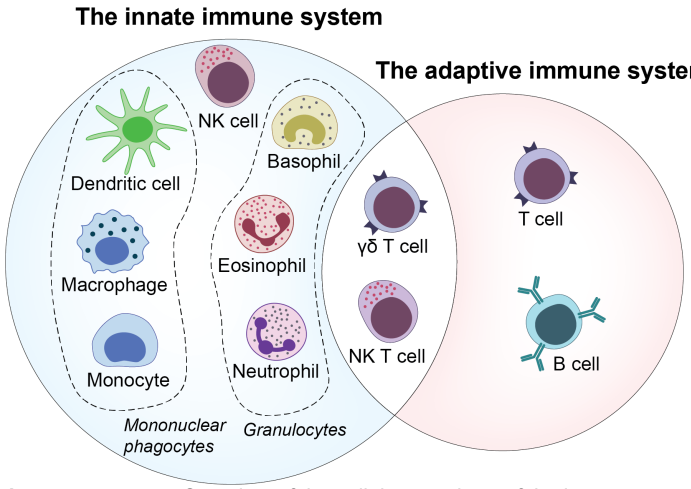


Figure 2. The immune system. Overview of the cellular members of the immune system.

The innate immune system

The innate immune system consists of an arsenal of cells, proteins, barriers, and surfaces that protects us from pathogens entering the body. The innate (and adaptive) immune system has two main parts: the *humoral immunity* and *cell-mediated immunity*. The humoral part is involved in signalling processes and the labelling of pathogens for elimination, whilst the cellular part is involved in direct elimination of the intruders. Since monocytes and neutrophils are the focus of this thesis, they are discussed more in detail below.

Humoral immunity

The most integral component of the innate humoral system is that of the complement system. The complement system is a protein network that was discovered nearly 100 years ago, that "complements" the cellular immunity. It is mainly present in the circulation and can be activated in several ways, such as through the binding of microbes. The complement system is involved in a multitude of processes, including opsonization (the process in which peptides and proteins bind pathogens, marking them for phagocytosis), chemotaxis (attracting cells), lysis and signalling. Hence, it is important for the clearance of pathogens and cells, in addition to cell communication. It can also act as a bridge between the innate and the adaptive immune system. However, as complement is not the focus of this thesis, it will not be discussed further in detail. Other members of the innate humoral immunity include signalling molecules, such as pentraxins and cytokines, which are created

during inflammation and interact with multiple parts of the immune system and resident cells to prolong or inhibit inflammation.

Cell-mediated immunity

Most cells of the innate immune system originate from the same precursor cells located within the bone marrow, termed common myeloid progenitor cells. These cells continuously proceed to differentiate and specialize, resulting in the different aforementioned members of the innate immune system. These newly differentiated cells are then released into the circulation, where they mainly reside until an injury or infection occurs. Innate immune cells have developed a wide range of functions to both maintain homeostasis, eliminate potential threats, and heal any affected areas. For example, the process of *phagocytosis* is considered to be one of the most prominent of these functions, whereby immune cells termed *phagocytes* devour and digest any invading bacteria and dead cells.

Mononuclear phagocytes

Mononuclear phagocytes include monocytes, dendritic cells (DCs), and MØs. DCs, and to a lesser extent MØs, are also classically termed professional APCs (antigen presenting cells). Monocytes can be found in the circulation, whilst MØs and DCs mainly reside within different tissues. Monocytes are the precursor to most DCs and MØs, which they can differentiate into either of these two cell types. Furthermore, it is common for monocytes and their derivatives to be specialized in phagocytosis, production of signalling molecules (such as cytokines) and antigen presentation. Upon digestion of pathogens, debris and other material, mononuclear phagocytes present small peptides from their digested targets, called antigens (hence the name APC), on receptors termed the major histocompatibility complex II (MHC II). Using this complex, they can subsequently activate members of the adaptive immune system. This process will be discussed more in detail further on.

Granulocytes

Granulocytes are a subtype of phagocytes that contain granules, which are small vesicles/particles within the cytoplasm of the cell. These granules contain a multitude of enzymes and peptides that are designed to eliminate or opsonize pathogens. The most abundant granulocyte is the neutrophil (which will be discussed in detail below), followed by mast cells, eosinophils, and basophils. Granule content varies between the different cells, highlighting the specialized nature of each cell type to respond to diverse types of danger.

Memory

Memory is referred to as the ability of immune cells to recognize a pathogen which has previously infected the body, thus responding substantially quicker upon re-infection than if it was a newly recognised pathogen. The innate immune system is

historically considered as being devoid from a long-term memory to previous pathogens. This is due to 1) the short lifetime of most innate immune cells (in contrast, the adaptive immune system contains cells that can live for many years), and 2) the broad immunological response to DAMPs and PAMPs. However, it is becoming increasingly clear that innate immune cells form their own versions of a trained response, reminiscent of a memory (1, 2). This means that innate immune cells display a greater response upon encountering the same pathogen. This theory is supported by epigenetic- and metabolic alterations in these cells following activation (1, 2). Furthermore, MØs may reside within tissues for years. Indeed, some sub-populations of MØs are non-hematopoietic, and originates from various places, such as the yolk sac, highlighting the presence of long-lived and proliferating members of the innate immune system (3). Thus, innate immune cells are able to form some sort of memory or prepared response to re-infections, which has also been suggested as a possible mechanism contributing to autoimmune diseases (4).

The monocyte

Monocytes represent roughly 3-7% of the leukocytes (white blood cells) found in blood (5). They often display a round morphology, with an indented nucleus. In humans, at least three subtypes of monocytes are recognized based on expression of the two surface markers CD14 and CD16 (**Figure 3**) (6). The most prevalent subtype consists of '*classical*' monocytes, which constitutes 80-90% of all circulating monocytes (6). These are defined as $CD14^{+}CD16^{-}$. Next are the '*intermediate*' subtype representing 5-10% of monocytes and are defined as $CD14^{+}CD16^{+/-}$. Finally, the last subtype of '*alternative*' monocytes is defined as $CD14^{-/+}CD16^{+}$ and represents 5-10% of monocytes.

Monocytes typically have a half-time of 1-3 days in the circulation (7). Classical monocytes are shorter lived, whilst intermediate and alternative monocytes may live up to a week (8). Some studies, including mathematical models, suggest that a proportion of classical monocytes undergo a subtype transition to replenish the intermediate- and alternative monocyte reserves over time (8, 9). During steady state (health), monocytes are either cleared from the circulation, or migrate into tissues to differentiate. Here, they participate in maintaining tissue integrity, such as clearance of dead cells. If circulating monocytes encounter DAMPs, PAMPs or signalling molecules as they patrol the vessels, they extravasate into the surrounding tissues to combat the source. Accordingly, monocytes have an arsenal of available tools and effector functions to aid against injury or infection.

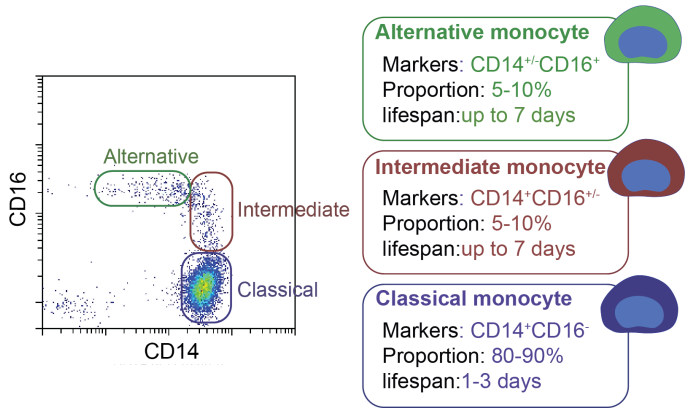


Figure 3. Monocyte subtypes. An overview of the three different monocyte subtypes in humans, as visualized by CD14/CD16 expression using flow cytometry.

Effector functions

Monocytes have several crucial effector functions necessary to eliminate pathogens, clear debris, and initiate resolution. The main functions of monocytes are summarized in **Figure 4**. As monocytes are a phagocytosing cell, one of their main functions is the clearance of bacteria (through phagocytosis) or apoptotic cells (through a subtype of phagocytosis termed efferocytosis) (10). Apoptosis is the term for controlled cell death, describing the procedure of a cell killing itself due to a multitude of reasons. Monocytes initiate phagocytosis when they encounter an ”eat me” signal, usually from opsonized pathogens or apoptotic cells presenting markers for efferocytosis. The cell membrane forms a vesicle by invagination (termed a phagosome) encapsulating the pathogen, which is then transported into the cell and combined with lysosomes, forming a phagolysosome (11). These acidic lysosomes contain specific enzymes designed to digest the engulfed material into small peptides to be recycled. The cells can then use these digested peptides, as previously mentioned, and present them as antigens to the adaptive immune system via MHC II.

In addition to phagocytosis, monocytes also produce reactive oxygen species (ROS). Used primarily during an oxidative burst following phagocytosis to eliminate pathogens, ROS can also act as a signalling substance (12). Furthermore, monocytes express high levels of MHC II, and co-stimulatory molecules, such as CD80 and CD86 (6, 13, 14). Thus, monocytes can activate antigen-specific T- and B-cells and mount an adaptive immune response. Finally, monocytes are master cytokine producers, of both pro- (TNF, IL-1ß, IL-6, IL-8 and IL-12) and anti-inflammatory cytokines (IL-10 and transforming growth factor (TGF-ß)) (6, 13, 14). These cytokines are essential for signalling to other immune cells, resulting in initiation, skewing, or resolution of inflammation.

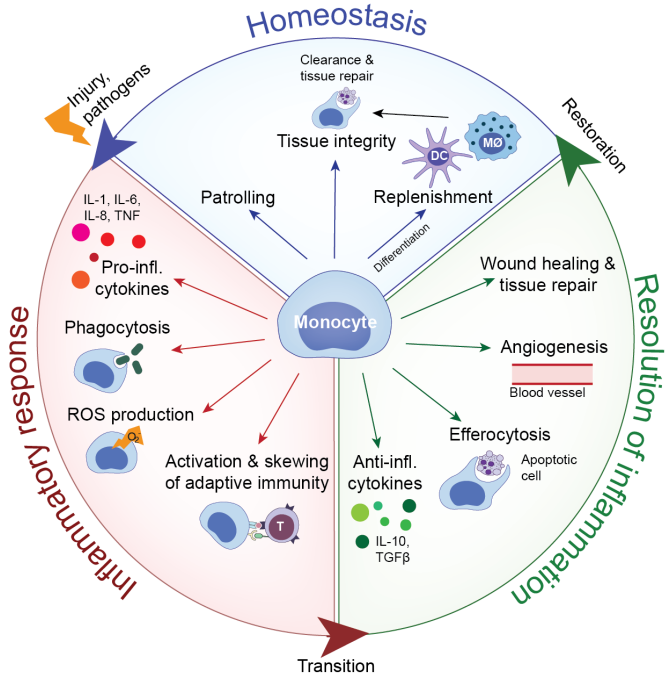


Figure 4. The role of monocytes. An illustration of the most common monocyte functions during homeostasis, inflammation, and resolution. *ROS – Reactive oxygen species, infl – Inflammatory, TGF– Transforming growth factor, DC – Dendritic cell, MØ – Macrophage.*

The extent of how much the monocyte subtypes (classical, intermediate, and alternative) differ from one another functionally is still under debate. Purification and isolation techniques are believed to influence subsequent analysis of the subsets, and current available data is conflicting (15-17). Yet, at the transcription level, they share a multitude of markers and expression patterns, but some differences still exist.

Firstly, classical monocytes seem to express a higher degree of chemokine receptors (CCRs) and produce more cytokines such as IL-1ß, IL-6 and TNF (15, 18, 19). Furthermore, gene expression analysis supports a heightened inflammatory nature of this subtype (20). On the other hand, intermediate monocytes display higher expression of MHC II as well as a gene expression profile related to antigen processing (17, 18). Additionally, there is some evidence suggesting pro-angiogenetic properties of this subtype (21). Lastly, alternative monocytes seem to have an increased cytoskeletal rearrangement (18). Indeed, $CD16^{+}$ monocytes appear to be more mobile than their $CD16^{-}$ counterparts and may thus have a patrolling behaviour on endothelium (22).

Furthermore, there is evidence that the different monocyte subtypes are related to different diseases (15, 23). For example, circulating alternative monocytes are

enriched and functionally influenced in both sepsis and autoimmune diseases (24). In rheumatoid arthritis (RA), circulating classical monocytes are believed to differentiate into intermediate and alternative monocytes (24, 25). Still, mainly classical monocytes seem to enter the joint, acquiring an activated phenotype, including CD16 expression (26). Nevertheless, as with the function of the different subtypes, there are contradicting data on the presence, role, and phenotype of the monocyte subtypes in disease (25, 26).

In conclusion, despite the existence of multiple monocyte subtypes, the functional properties between them are largely similar. Nonetheless, differences between the subtypes do exist but should be interpreted carefully due to dissimilarities in isolation protocols and methods. Further research is needed to clarify the functional roles.

Differentiation

Monocytes are the precursors to MØs and DCs. As aforementioned, most MØs and DCs reside within different tissues, in contrast to monocytes who mainly circulate in the bloodstream. These cells survey tissues for pathogens and are potent initiators of inflammation. They additionally have the potential to perform anti-inflammatory functions, such as the initiation of wound healing (27).

MØs are very diverse. Indeed, MØs may not only originate from monocytes, but as mentioned previously, some are also believed to originate from different stem cell populations, ranging back the fetal state (28, 29). For example, MØs of the liver are called Kupffer cells, and are believed to originate from the yolk sac, with one of their main roles being to clear the circulation from debris through phagocytosis (29). In short, MØs may be both monocyte-derived and not, with specialized roles depending on their residing tissue.

DCs are considered specialized and efficient antigen presenters. As with MØs, there are several DC subtypes scattered among the tissues, with a significant proportion located in the lymphatic system. Some DCs originate from monocytes, others from bone marrow progenitors. The most common subtypes are that of plasmacytoid DCs, conventional DCs and monocyte derived DCs (30, 31). The plasmacytoid DCs mainly circulate but can also be present in lymphoid organs, and are primarily involved in viral defense as a major producer of type 1 interferons (IFNs) (32). Conventional DCs reside within the tissues and are specialized in antigen presentation (33). Upon antigen encounter, they will migrate to the lymph nodes to initiate an adaptive response, which will be discussed later.

So, is it the monocytes' sole role to differentiate? Not necessarily. Differentiation to DCs and MØs do represent a major part of the purpose of monocytes. Yet, some studies suggest that certain monocytes may migrate into tissues, with no differentiation occurring or even emigrate back out of tissues into the circulation (34-36). For example, a tissue reservoir of monocytes exists in the splenic red pulp,

which can be released upon distant injury to populate those tissues (37). Additionally, monocytes are capable of executing similar effector functions to DCs and MØs, albeit less effectively. This is especially true during inflammation, as monocytes constantly enter the affected tissues and replenish diminished populations, were monocytes also, for example, serve as a key source of cytokines.

Polarization

During infection, inflammation or resolution, monocytes and MØs come across signaling molecules that alter their phenotype that leads to activation, a process called *polarization*. Historically, the monocytes are classified into pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes (**Figure 5**) (38). Pro-inflammatory phenotypes drive inflammation, whilst anti-inflammatory alterations try to slow down or inhibit inflammation. *In vitro*, cytokines can be used to induce clearly defined phenotypes. However, the literature surrounding this field remains confusing as a wide range of concentrations and combinations have been tested. Generally, there are three phenotypes that are frequently encountered (39-41). An M1 phenotype, induced by IFNγ and/or LPS (termed M1(IFNγ) or just M1); an M2 phenotype, induced by IL-4 and/or IL-13 (termed M2(IL-4) or M2a); and an M2 phenotype, induced by IL-10 and/or glucocorticoids (termed M2(IL-10) or M2c).

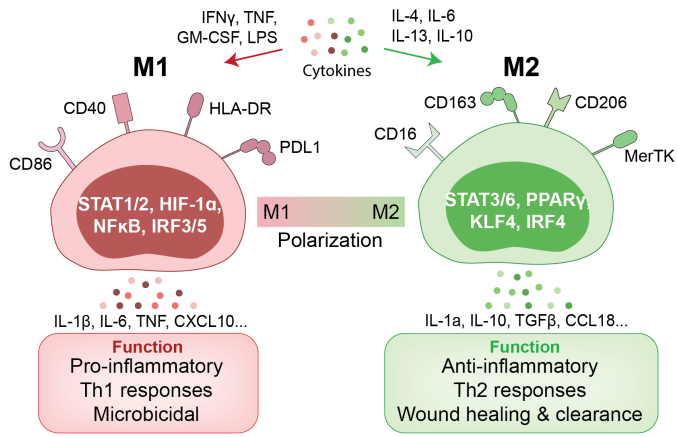


Figure 5. Polarization. An overview of the two endpoints, M1 and M2, representing the continuum of monocyte/macrophage polarization.

These phenotypes are then characterized mainly by the expression of surface markers, but also through their function. Accordingly, this is why polarization has emerged as a major field in monocyte/MØ biology, as it highly influences the function of these cells.

M1 cells express markers related to antigen presentation, such as CD40, and produce pro-inflammatory cytokines (TNF, IL-6, IL-8 and so on). They are often coupled with Th1 T-cells and an increased microbicidal activity (39, 42). On the other hand,

M2 cells express markers of clearance, such as CD163 and MerTK, and produce anti-inflammatory cytokines (such as IL-10 and TGF-ß). These cells are thus involved in phagocytosis, wound healing, and extracellular matrix (ECM) production (39, 42). In a classic infection, timing is a crucial factor, as early monocytes seem to acquire an M1 phenotype, and monocytes entering at the later stages of inflammation acquire an M2 phenotype (43, 44). This is most likely reflecting a shift in the presence of M1 driving cytokines to M2 ones, resulting in the resolution of inflammation. Another contributing factor is likely that prolonged exposure to inflammatory stimuli exhaust the monocytes and drive them to an M2-like state.

Finally, it is important to know that monocytes and MØs in patients are influenced by a multitude of different stimuli, such as cytokines, cell-cell contact and time, rather than defined cytokines as in a laboratory setting. Thus, cells from patients rarely fall in the specific classification of M2a, M2c and so on. Indeed, there exist polarized subtypes expressing markers of both M1 and M2, or a mixed population of both subtypes, in disease settings (45, 46). For example, in sepsis, monocytes expressing the M2 marker CD163 are the main producers of pro-inflammatory cytokines (47). Thus, in reality, polarization is a continuum, and attempts to rigorously define a subtype and subsequently base the function of the cell on this classification, are irrelevant. Instead, the phenotype should be investigated in its specific environment and condition, especially in regard to disease, and should not be classified based on a clearly controlled *in vitro* setting.

In conclusion, polarization of monocytes is a complex process controlled by a multitude of factors which influence their function, and thus contribution to disease.

The neutrophil

Neutrophils represent the majority of white blood cells, roughly 60-70% of the immune cells in blood (5). The dogma is that they are circulating cells with a short lifespan of 6-12 hours. Yet, this lifespan can be extended upon activation, allowing neutrophils to further execute some of their effector functions (48, 49). In addition, some data suggest that that neutrophils can re-circulate, exemplified by their ability to enter, and exit lymph nodes (50). Additionally, some neutrophils are located in the tissues, and are extremely fast responders to danger signals (51).

Neutrophils, in contrast to monocytes, are terminally differentiated. The general role of neutrophils is to combat infections, and they have an arsenal of tools to do so (**Figure 6**). Neutrophils have a nucleus that is lobulated, and their cytoplasm contains granules, pre-loaded with multiple enzymes, anti-microbial peptides, and other substances to be released upon inflammation (52). Upon encounter with pathogens or damage, neutrophils quickly migrate from the blood stream to initiate inflammation.

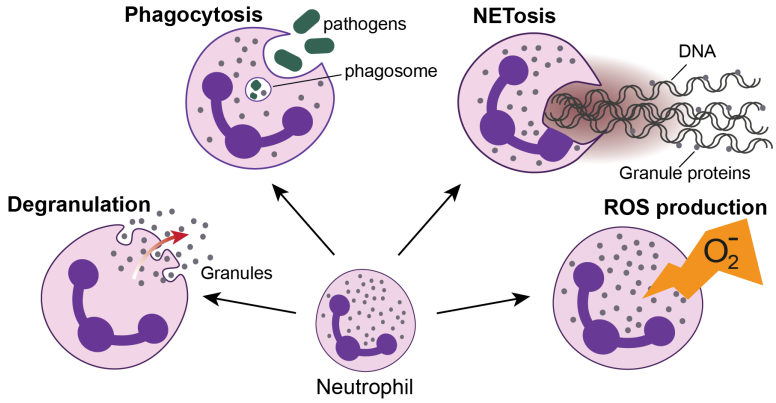


Figure 6. Neutrophil function in health. An illustration of the most common neutrophil functions. *ROS – Reactive Oxygen Species, NET – Neutrophil extracellular trap.*

Neutrophil recruitment

The recruitment of neutrophils to a site of injury or infection represents a crucial step in the initiation of inflammation. This process can be divided into the following five main parts: tethering, rolling, adhesion, crawling and transmigration through the endothelium (53, 54).

Upon an encounter with PAMPs or DAMPs, local cells begin producing cytokines and chemokines, that activate the endothelial cells (the cells lining blood vessels), to express selectins and chemokines themselves. Circulating neutrophils are attracted to the chemokines and the activated endothelial cells, resulting in binding of the neutrophils to the selectins expressed by the endothelial cells (termed tethering). This interaction then permits neutrophils to roll across the endothelial cells. As the neutrophils become more adherent, integrins expressed by the neutrophils get involved, finally resulting in firm adhesion. Subsequently, the crawling neutrophils follow the chemokine gradient through crawling until they reach a preferential site of transmigration. Transmigration occurs at cellular junctions, and in combination with integrins and enzymes, results in the movement of neutrophils to the site of injury, where they can perform their effector functions.

Effector functions

The most common effector functions of neutrophils include phagocytosis, degranulation, production of ROS and the release of neutrophil extracellular traps (NETs). Phagocytosis and ROS has been discussed above, but they will be briefly discussed below from a neutrophil perspective. There are several types of ROS that can be generated by neutrophils, and as with monocytes and MØ, one of the main functions of ROS is to kill pathogens. ROS is also important for NET formation, which will be discussed below, as well as in cell-cell communication (55). ROS is mainly generated from a class of enzymes called nicotinamide adenine dinucleotide

phosphate (NADPH) oxidases. These generate O_2^- from molecular oxygen (O_2) by passing electrons through the enzyme complex in different steps. O_2^- can then be further used in several reactions, for example, to generate hydrogen peroxide by myeloperoxidase (MPO) (55). ROS can also be generated in the mitochondria. Additionally, some ROS-generating enzymes are inducible, such as inducible nitric oxide synthase (iNOS) (56). Interestingly, phagocytosis in neutrophils is believed to differ from that in monocytes and MØ, as it occurs much faster (57). For example, granule delivery in neutrophils to the phagosome is believed to be an important difference, as is more powerful NADPH oxidase activation (57).

Degranulation

Neutrophils mainly contain four kinds of vesicles (see **Figure 7A**): primary (azurophilic)-, secondary (specific)-, tertiary (gelatinase)- and secretory granules (58-60). Their names originate from the order in which they are formed during neutrophil maturation (61). Azurophilic granules are one of the main storages of anti-microbial products and have an abundance of toxic mediators, including molecules such as MPO, proteases, and neutrophil elastase. The specific granules are also primarily anti-microbial in nature, containing cytotoxic enzymes and peptides such as lysozyme, lactoferrin and LL-37 (62). Additionally, they also contain ECM degrading products, such as collagenase. Next, we have the gelatinase granules, which also contain enzymes designed to degrade the ECM, such as matrix metallopeptidases (MMPs) and collagenases (62). Despite specific- and gelatinase vesicles sharing common proteins and functions, granule type separation can be performed through density gradients (61). Finally, the secretory granules contain mainly plasma proteins and membrane receptors, which can be rapidly delivered to the plasma membrane and is thus important in migration and phagocytosis. Notably, during degranulation, granules are released in reversed order from which they are created, thus starting with the secretory vesicles (61). Finally, neutrophil granules also contain an array of cytokines (63).

NETosis

NETosis is the process in which neutrophils sacrifices themselves to release their cellular content, forming DNA and chromatin spiderweb-like structures termed NETs (64). NETs are also scattered with anti-microbial enzymes and peptides from the granules. The resulting structure creates a trap to capture and eliminate pathogens (64). Thus, it is a specific and controlled mechanism of cell death, different from necrosis and apoptosis (65). The steps of NETosis are as follows: 1) the nuclear membrane is dissolved, 2) the content is combined with the cytoplasmic granules, 3) membrane integrity is lost and 4) the NET product is released (see **Figure 7B**). There are several pathways that can induce NETosis, which can primarily be separated into ROS dependent or independent pathways (64). The main pathway involves the activation of NADPH oxidase (E.g., through protein kinase C (PKC) signalling) and subsequent ROS generation. Classic activators of this

pathway involve phorbol 12-myristate 13-acetate (PMA), N-Formylmethionyl-leucyl-phenylalanine (fMLP) and Ca^{2+}. This ROS mediated process leads to chromatin de-condensation, nuclear envelope rupture and subsequent NET release (66, 67). Alternatively, mitochondrial ROS (mtROS) production represents a NADPH oxidase independent pathway to generate NETs (68). However, NETosis does not always result in immediate cell death, with other pathways observed resulting in NETosis without neutrophil death (often termed vital NETosis). In these poorly-described pathways, NETosis occurs much faster, and nuclear content is released through nuclear blebbing and vesicle release, leaving an intact plasma membrane, that still has some phagocytic capacity (69). Mechanistically, PAD4 (which is also involved in ROS dependent NETosis) has been suggested as an activator, as it can be triggered through ROS independent mechanisms, resulting in histone citrullination and chromatin de-condensation (70, 71) Finally, at the receptor level, signalling through TLRs (e.g. TLR4), is suggested to be a main inducer of vital NETosis (69). NETs have also been the focus for autoimmune diseases, as excess NETosis is believed to contribute to chronic inflammation (64).

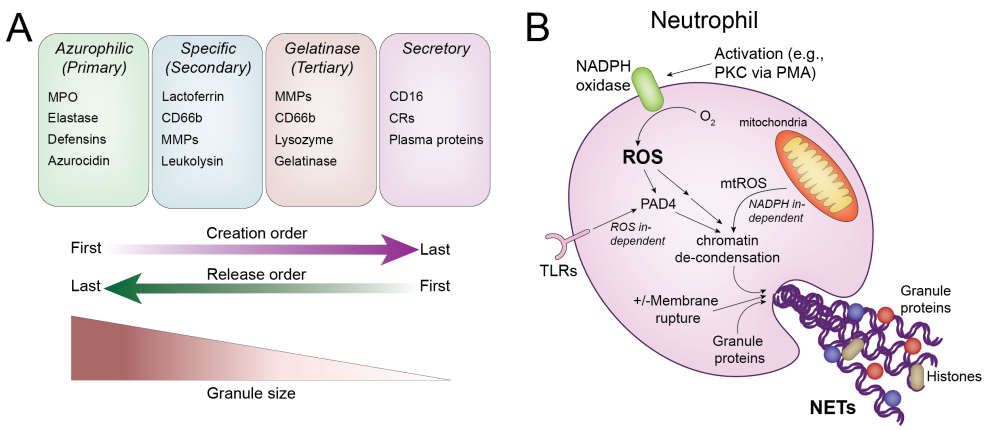


Figure 7. Simplified overview of the relationship between degranulation, ROS and NETosis in neutrophils. (A) Shows the four main types of vesicles identified in neutrophils, their main components and their order of creation and release upon activation. (B) An illustration of the process of ROS production, as well as different mechanisms of NETosis. *MPO – Myeloperoxidase, MMPs – Matrix metalloproteinases, CRs – Complement receptors, NADPH – Nicotinamide adenine dinucleotide phosphate, ROS – Reactive Oxygen Species, NET – Neutrophil extracellular trap, TLRs – Toll-like receptors, PMA – Phorbol 12-myristate 13-acetate, PKC – Protein kinase C, mtROS – mitochondrial ROS.*

Regulatory role

Even though neutrophils are mainly known for their pro-inflammatory role in combating pathogens, they also have the capacity to regulate inflammation. Neutrophils have been described as both suppressors and activators of other branches of the immune system (72, 73). On one hand, neutrophils may crosstalk with T- and B-cells to present antigens and provide co-stimulatory signals to induce adaptive immunity, skew monocytes and MØs to M1 and influence DC responses

(72-74). On the other hand, neutrophils have the capacity to suppress T cells through production of extracellular ROS and secretion of arginase 1, to produce anti-inflammatory cytokines and to induce M2 monocytes and MØs (72, 74-76).

As with monocytes, neutrophils are more complex than historically thought, and studies suggest that neutrophils also have several subtypes with specialized roles (52). For example, a proportion of neutrophils that reside within tissues will express different markers compared to circulating ones, with others appearing to be specialized in inducing angiogenesis or preferentially migrate to lymph nodes (72, 77, 78).

Finally, recent findings (mainly in cancer) suggest that neutrophils may also polarize to different subtypes (termed N1 and N2) that are preferentially pro- or anti-inflammatory, similar to monocytes and MØs (79, 80). The N1 neutrophils are more prone to ROS and cytokine production/release (e.g., TNF) compared to N2 neutrophils, thus they are likely more inflammatory in nature (79). On the other hand, N2 neutrophils have pro-longed survival, promote angiogenesis and may be immunosuppressive (79, 80). These findings collectively highlight a long-overlooked plasticity of neutrophils and suggest that modulating neutrophil phenotype and function can be a potential way of controlling inflammation. In conclusion, neutrophils are specialized in combating pathogens, but they also have a regulatory role and may drive both pro- and anti-inflammatory processes.

The adaptive immune system

Each cell of the adaptive immune system is developed and selected to respond to a specific type of antigen, in addition to collectively responding to all antigens imaginable (excluding our own cells). The main cells of the adaptive immune system are lymphocytes, mainly consisting of T-cells and B-cells. Generally speaking, T-cells, which are developed in the thymus and represent 20-26% of the white blood cells, are responsible for the cellular response. B-cells on the other hand, which are developed in the bone marrow and represent 2.5-5% of all white blood cells, are responsible for the humoral response (5). As with other immune cells, lymphocytes patrol the circulation and the lymphatic system.

Members and function of the adaptive immune system

Beginning with T-cells, this lymphocyte subtype mainly consists of $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ T-cells are often termed T helper cells (or Th cells for short). Their role is to aid other branches of the immune system through cytokine production and receptor interactions (81). In the lymph node, these cells provide support for B-cell activation and maturation, in addition to migrating to inflammation sites to provide support for innate immune cells.

Interestingly, Th cells acquire different subtypes during the course of inflammation. Th1 cells drive cell-mediated responses, produce proinflammatory cytokines and in particular, drive the elimination of intracellular pathogens (81, 82). Contrastingly, Th2 cells drive humoral responses, and the elimination of extracellular pathogens, with a focus on parasites (81, 82). Th2 cells may also be anti-inflammatory in nature, counteracting Th1 responses. A third subtype termed Th17 cells produce IL-17, a cytokine that has been linked to autoimmunity. Finally, the fourth subtype termed Tregs are regulatory T-cells, whose purpose is to suppress immune responses. Tregs do this through production of anti-inflammatory cytokines and expression of inhibitory co-stimulatory receptors (81, 82). As such, these cells are crucial in preventing immune activation in response to self-antigens, which could cause autoimmunity, a condition which will be discussed below.

The main purpose of $CD8^+$ T-cells is to eliminate intracellular pathogens, which is supported by Th1 cells. *So how does it work?*

As previously mentioned, APCs present antigens of engulfed pathogens on MHC II receptors to the adaptive immune system. However, nearly all cells in the body have a similar receptor called MHC I. On this receptor, all bodily cells present antigens from themselves. Normally, all antigens on MHC I are from the host. But if cells are infected with an intracellular pathogen, they will try to present antigens from this pathogen on MHC I instead. The purpose of $CD8^+$ T-cells therefore is to eliminate cells that present foreign antigens, as they have been trained to not recognize self-antigens (83). If it finds a foreign antigen, the T-cell induces cell death through several mechanisms (83). Thus, they are also known as cytotoxic T-cells.

Regarding the other lymphocyte subtype, B-cells have several functions, such as cytokine production and antigen presentation. As with T-cells, B-cells can also skew the immune response through the production of either pro- or anti-inflammatory cytokines. However, arguably the most classic function of B-cells is their differentiation into plasma cells, whose main function is to produce antibodies. Different subclasses of antibodies exist (termed immunoglobulins, such as IgG and IgA), with subclass production influenced by the surrounding environment. Antibodies are extremely specific and are primarily used to opsonize pathogens for phagocytosis, markedly increasing phagocytosis efficiency (84). Other functions including neutralization, whereby antibody binding inhibits the function of their target, in addition to aiding other parts of the immune system, such as the complement system (84). Thus, the adaptive immune system has an arsenal of tools, but is highly dependent on initial triggering by the innate immune system which is discussed in the following section.

At the crossroads between innate and adaptive immunity

This section will focus on how the innate immune cells activate the adaptive immune cells, as well as the interaction between these two branches.

Immune tolerance

Overall, the innate immune system, and in particular APCs, are instrumental in the activation of the adaptive immune system. In a nutshell, T- and B-cells cells are developed to recognise any antigen imaginable but are selected to only recognize *foreign* antigens, and *not* self-antigens. To do this, each cell contains membrane-bound receptors: the T-cell receptor (TCR), found on T-cells to recognise antigens; and the B-cell receptor (BCR), located on B-cells. The unresponsiveness of lymphocytes to self-antigens is called immune tolerance (i.e. the lymphocytes should be *tolerant* to self) and is crucial in protecting the body from being attacked by its' own immune system (85). This is partly ensured during the development stages of these cells, termed central tolerance (which occurs in the thymus for T-cells and in the bone marrow for B-cells), in which cells positive for self-antigens are either eliminated or set to undergo receptor editing (for B-cells) (**Figure 8**) (85, 86). However, some T-cells that are weakly positive to self-antigens will be destined to become Tregs (87). Ultimately, some cells do escape the central tolerance into the periphery, even in normal situations. Thus, another mechanism, termed peripheral tolerance, prevents the activation of circulating lymphocytes. One of the main contributors of this process are Tregs, which can inhibit and induce anergy in autoreactive T-cells (88). The lack of co-stimulation from APCs also ensure to limit autoreactivity (89). Ultimately, peripheral tolerance results in apoptosis, anergy and suppression of the autoreactive lymphocytes. Circulating lymphocytes who survive the tolerance mechanisms are then termed naïve cells and are now awaiting activation through encountering their foreign antigens.

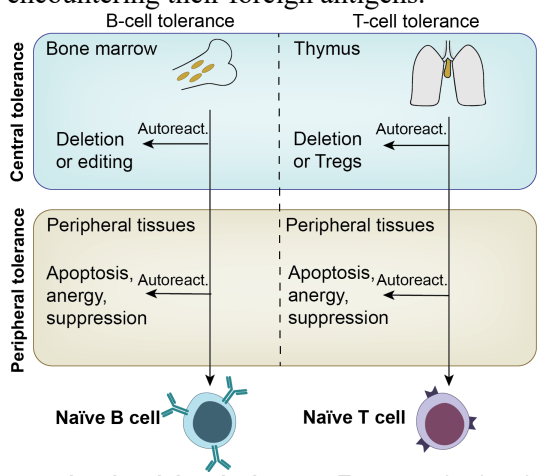


Figure 8. Overview of central and peripheral tolerance. To ensure that lymphocytes do not recognize self-antigens and become autoreactive, cells undergo both central and peripheral tolerance.

Antigen encounter and activation

Once a foreign body is identified, APCs digest their targets, present small antigens of this target on MHC II receptors, and then migrate to the lymph nodes where they have the biggest chance to encounter T- and B-cells. An extreme minority of lymphocytes that reside here express receptors that recognize these antigens. To activate, a $CD4^+$ T-cell requires 3 signals: antigen exposure (through an APC), co-stimulatory signals (by interacting with different types of receptors on the APC), and proliferation/maturation signals (from cytokines) (**Figure 9A**) (90, 91). Once a T-cell has received all of these signals, it proliferates, expands, and starts to execute its functions. Whether it ends up as a Th1, Th2, Th17 etc depends on factors such as the cytokine environment (81, 90). $CD8^+$ T-cells also require co-stimulation for full activation, in order to prevent the unintentional elimination of cells (83).

Conversely, B-cells are activated when an antigen binds to the BCR (which is basically a membrane antibody). This binding trigger uptake of the antigen and the subsequent presentation to activated T-cells through MHC II (a B-cell is thus also an APC) (92). Upon interaction with a matching T-cell that also recognizes the antigen, the B-cell receives its second signal (co-stimulatory) from the T-cells and starts to mature and proliferate (**Figure 9B**) (92, 93). However, a proportion of B-cells may activate without the help of T-cells (92). Recognition of the BCR to the antigen is usually weak initially, but as the B-cell proliferates, it undergoes a process called somatic hypermutation – resulting in small changes to the BCR and its affinity maturation (higher intensity binding to the antigen by the antibody) (94). This means that the B-cell clone with the strongest affinity to the antigen is selected for further proliferation. This results in the production of highly specific antibodies. Following the elimination of the threat, a subpopulation of T- and B-cells then remain as memory cells, and can quickly mount this intensified, specific response upon reencounter with the known antigen (95).

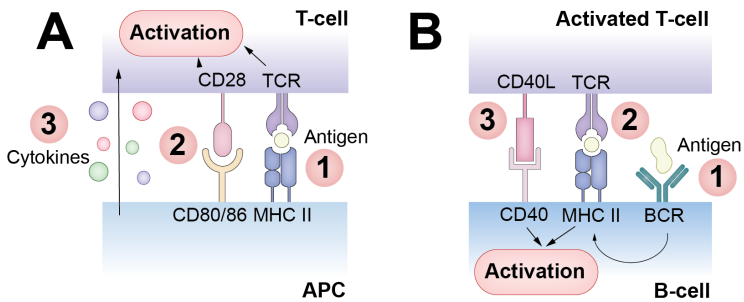


Figure 9. The process of lymphocyte activation A. Shows the interaction between an APC and a naïve T-cell. (1). The APC presents its digested antigen on MHC II, which interacts with the TCR on the T-cells if it recognizes the antigen. (2). In parallel, upon antigen recognition, the T-cells are also activated with co-stimulatory receptors such as CD80/86 that binds to CD28 on the T-cells. (3). Cytokine release from the APC further activates the T-cell and skews its activation. **B. (**1). An antigen that binds the BCR on the B-cell is internalized and (2). presented on the MHC II of the B-cell. Upon interaction with a matching TCR, and (3). subsequent co-stimulation, the B-cell becomes activated. *BCR – B-cell Receptor, TCR – T-cell Receptor, MHC II – Major histocompatibility complex II.*

As discussed previously, monocytes and neutrophils have different mechanisms to suppress or accelerate the response of T-cells and B-cells, through cytokine production and cell-cell crosstalk. Undifferentiated monocytes are not professional APCs, even though they are capable of presenting antigens. However, they are potent cytokine producers and can express high levels of co-stimulatory molecules (e.g., CD80/86). Hence, they are capable of accelerating an adaptive immune response. On the other hand, they may also inhibit inflammation through production of anti-inflammatory cytokines, or through induction of anergy (96-98). Neutrophils normally do not express MHC II, but its' expression can be induced in certain settings, resulting in APC related functions (99). Neutrophils also have the capacity to induce B-cell activation through the production of factors such as B-cell activating factor (BAFF) (100). On the other hand, several mediators of neutrophil origin, such as ROS, MPO and arginase 1, can mediate a suppressive effect on lymphocytes (76, 101, 102). Thus, even though the adaptive immune system is imperative for a robust defense response, it is initiated, skewed, and controlled by the innate immune system.

Autoimmunity

The projects of this thesis are focused on oligoarticular juvenile idiopathic arthritis (oJIA), a rheumatic disease that arises partly due to defects in the immune system. In order to understand oJIA, we have to first take a look at diseases caused by a dysfunctional immune system – autoimmune- and autoinflammatory diseases.

Breach of tolerance

Autoimmunity arises when the immune system *mistakes self as non-self*, resulting in an immune response towards self-antigens. Principally, this means that the immune system targets what it is meant to protect – the body. This gives rise to numerous diseases, all involving the immune system mistakenly recognizing the body as foreign. But how does this happen?

As discussed in the previous section, the lymphocytes recognize foreign antigens – but has undergone a selection process to not recognize self-antigens. Thus, if they come across APCs presenting self-antigens, this will not lead to activation. However, due to genetic-, environmental- or other factors, some lymphocytes escape the selection process. This results in lymphocytes that are capable of mounting an immune response against the body, as they consider self-cells as foreign which in turn leads to a *breach of immune tolerance*. This can be both autoreactive T-cells that target specific host cells, or B-cells producing autoreactive antibodies and fooling the rest of the immune system to mount an immune response

(103). Environmental factors may then trigger APCs to present co-stimulatory receptors, that eventually can result in an autoimmune response (**Figure 10**) (104). Autoimmunity can also arise from molecular mimicry. Molecular mimicry describes the phenomenon in which a foreign antigen highly resembles a self-antigen (105). Hence, when APCs present this antigen as part of the normal response, lymphocytes recognising this foreign antigen may cross-react with the self-antigen. In addition, other factors have been linked to autoimmunity, such as smoking and the gut microbiome (104). Finally, post-translational modifications, such as citrullination, are also involved in the pathogenesis of autoimmunity (106, 107). Nevertheless, the results of these various mechanisms can range from a single affected organ (such as type 1 diabetes) to a multisystem- and multifactorial disease (such as systemic lupus erythematosus). Roughly 100 autoimmune diseases have currently been identified (108, 109). But who is at risk?

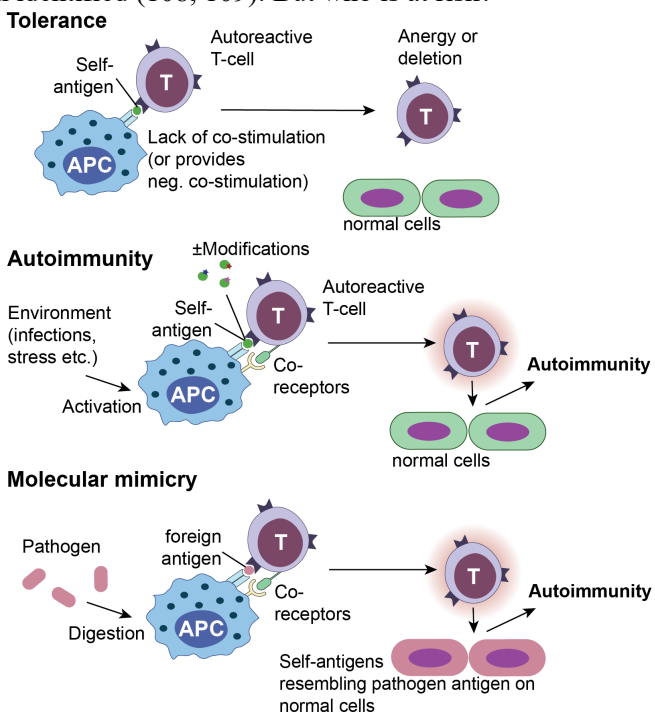


Figure 10. Breach of tolerance. A schematic overview of some ways that autoreactive T-cells are devoid of activation (due to tolerance) or become activated and contribute to autoimmunity (breach of tolerance). *APC – Antigen presenting cell.*

Aetiology of autoimmunity

There is a clear genetic component underlying autoimmune diseases, which is often complex and dependent on multiple factors and genes (108). These genetic alterations may result in variations of the protein coded by the affected gene, and thus variations in disease susceptibility. A common variant associated with

autoimmune diseases are variations in the MHC region, which influence antigen presentation. Variants in other genes affect other functions of the immune cells, ranging from signalling pathways to receptors and cytokine production (110). Given that there are shared genetic components, some autoimmune diseases tend to cluster together (111). However, genetic susceptibility often also requires a secondary trigger to cause disease, such as environmental factors (pollutions, smoking, metals, gut microbiota, infections etc) (112). Thus, all individuals with genetic susceptibility are not doomed to develop disease. Diseases can be split into those caused by a single gene mutation (termed monogenic, although these diseases are relatively rare) and those caused by several factors (termed polygenic).

Generally, autoimmune diseases affect women more often than men, at a 2:1-10:1 ratio. (108, 113). This is possibly due to the fact that women tend to have a more active immune response and thus, a better defence against infections (114). For example, women have an increased expression of TLRs, APC efficiency and higher B- and T-cell counts (114, 115). In addition, a recent study found that men have a higher frequency, as well as an increased suppressive ability of Tregs (116). One explanation for these differences between the sexes may lie in either the X chromosome, as many genes related to immune function are located within it, or with hormone production, which can also affect immune function (115).

Innate immunity in autoimmunity

The innate and adaptive immune system work closely together. Antibodies opsonize pathogens for phagocytosis, and T-cells produce cytokines that attract innate cells. The adaptive immune system is typically dependent on activation by the innate immune cells. Indeed, activation by the innate immune system is believed to play a vital role for the initiation of autoimmunity (as it does in other situations, such as an infection) (104). Furthermore, cytokines produced by innate immune cells sustain and skew the adaptive response. Several drugs used in autoimmune diseases target cytokines produced by innate immune cells (e.g., TNF and IL-6) (117). In addition, several commonly associated gene changes in autoimmunity are related to either the ability of the innate immune system to activate the adaptive immune system, or cell-cell communication (118, 119). Thus, the innate immune system has a crucial role in diseases believed to be driven by autoreactive lymphocytes.

However, some diseases arise due to defects in genes related to the innate immune system. This also leads to diseases involving the immune system, but these are instead termed auto-inflammatory diseases. *But what is really the difference?*

Autoinflammation and autoimmunity

Diseases not primarily caused by autoreactive lymphocytes are considered to be autoinflammatory rather than autoimmune. Simply, autoinflammation can be

viewed as diseases devoid of autoreactive lymphocytes (i.e., no breach of tolerance), thus being caused by the innate immune system (e.g. an overproduction of cytokines) (120). It was originally portrayed in 1999 and was then used to describe periodical fever syndromes (121). Autoinflammatory diseases, compared to autoimmune diseases, do not display a clear sex dominance (117). In general, symptoms often include periods of fever devoid of infection and can affect a multitude of organ systems (117, 122). Since the innate immune system does not discriminate between self and non-self, but rather senses general danger (PAMPs or DAMPs), autoinflammatory diseases are believed to arise from hyperactivity of the immune system (120). Rather than lymphocytes, innate immune cells and the cytokines IL-1, IL-6 and IFNs, are key players (117). Yet, there is no clearcut definition or factors to thoroughly categorize diseases as autoimmune or autoinflammatory, and most represent a spectrum (117) (**Figure 11**). There are monogenic autoinflammatory diseases (e.g., familial Mediterranean fever (FMF) and monogenic autoimmune diseases (e.g., monogenic systemic lupus erythematosus (117, 123, 124). Most, however, are influenced by both sides of the spectrum. For example, Type 1 IFNs, produced by DCs, are believed to have a crucial role in the pathogenesis of systemic lupus erythematosus (125). Furthermore, healthy individuals may also have autoantibodies, and innate components are unsurprisingly present in well-defined autoimmune diseases (117, 126). Thus, autoimmune- and autoinflammatory diseases are complex and multifactorial, and should be investigated for all aspects of immunity to thoroughly identify mechanisms and treatment targets. The next section will take a closer look at arthritis, the focus of the projects in this thesis.

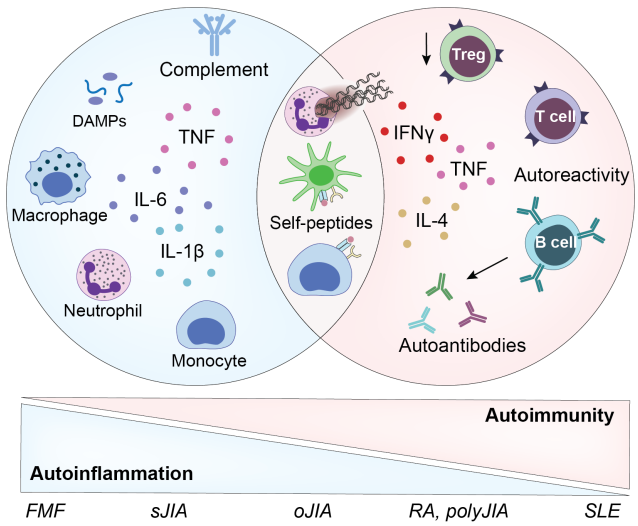


Figure 11. Autoinflammation vs autoimmunity. The traditional components of the immune system in autoinflammation (blue), autoimmunity (red) and associated diseases are highlighted. *FMF – Familial Mediterranean fever, JIA – Juvenile idiopathic arthritis, sJIA – systemic JIA, oJIA – oligoarticular JIA, RA – Rheumatoid arthritis, SLE – Systemic lupus erythematosus, TNF – Tumor necrosis factor, IFN – Interferon.*

Rheumatic diseases with a focus on arthritides

Autoimmune and autoinflammatory diseases encompasses a vast spectrum of diseases – ranging from monogenic to multifactorial diseases. This section will briefly cover an introduction to rheumatism and focus on arthritic diseases.

What is rheumatism and arthritis?

Rheumatology, or rheumatism, refers to diseases affecting primarily joints, connective tissues, and the musculoskeletal system, which are often autoimmune in nature (122). The concept of rheumatic diseases has been around for millennia. The word *rheum* originates from Greek and means "flow", which was used to describe how pain flows through the patient's body (122, 127). Rheumatic diseases often involve inflammation of the joints, termed *arthritis* (also derived from Greek – "disease of the joints") (128). There are over 100 types of arthritic diseases. The most common one is osteoarthritis (OA) which historically has not been considered a classic inflammatory arthritis, but rather a degenerative disease resulting in cartilage loss and bone damage. However, it is becoming increasingly clear there is an inflammatory component. The incidence increases with age, and it affects roughly 10% of the population above 60 years of age (129). Inflammatory arthritis can be caused by crystal deposition (e.g., gout) or infections, which could result in septic arthritis (128). However, it must be noted that this thesis will continue to focus only on the autoimmune arthritic diseases.

The healthy joint

The healthy joint consists of the joint capsule that encapsulates the synovial fluid (SF). The inner layer is called the synovium, or synovial membrane. In turn, the synovium is made up of an inner layer (closest to the SF) and an outer layer (130). The inner layer consists mainly of two cell types, the SF producing cells, called fibroblast-like synoviocytes (FLS), and MØs. SF is a viscous liquid used to lubricate the joint and aid in movements by reducing cartilage friction (131). The outer layer mainly consists of extracellular matrix containing blood vessels, fat, fibroblasts and other local cells (130). Besides the local MØs, the healthy joint is basically devoid of immune cells.

In arthritic disease, the synovial membrane is the main part affected, which is accompanied by infiltration of immune cells into the joint and disruption of tissue homeostasis (**Figure 12A and B**).

Autoimmune arthritic diseases

The most common autoimmune arthritic disease is rheumatoid arthritis (RA), affecting roughly 0.5-1% of the population (132). RA is often symmetric (also affecting the joint in the opposite body part) and influence several small joints (polyarthritis) which can then spread to larger joints (133). RA can also progress to

affect other organs, such as the skin and kidneys, thus being systemic in nature. Left untreated, the disease is destructive and aggressive, resulting in the degradation of both cartilage and bone structure, and subsequent deformation of the joints (133). Most patients afflicted with RA tend to have autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated peptide (133). However, the aetiology of RA is largely unknown, but it is believed to be caused by a combination of genetic and environmental factors. As such, treatment options aim to slow or avert the progression of joint damage. Common treatments include corticosteroids and disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate; or biologicals, such as anti-TNF or anti-IL-6 drugs (134).

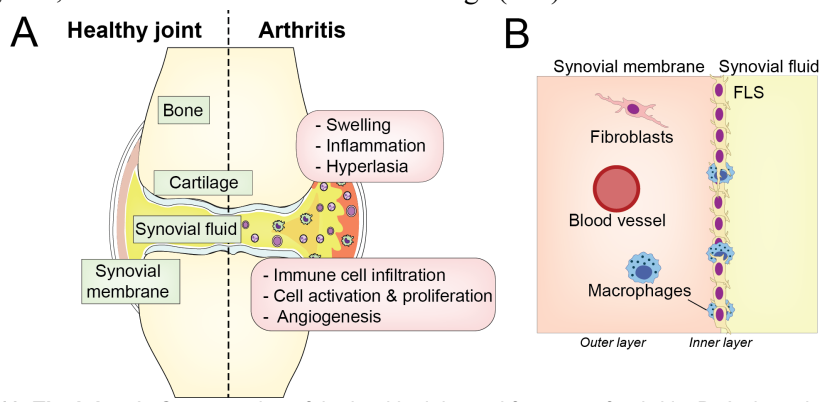


Figure 12. The joint. A. Cross section of the healthy joint and features of arthritis. B. A closer look at the synovial membrane and the local cells of the healthy joint. *FLS – Fibroblast-like synoviocytes.*

Spondylarthritis (SpA) is an umbrella term for several arthritic diseases that share some common features (135). Most affected patients have a strong link to a specific human leukocyte antigen (HLA) allele, HLA-B27 (136). In addition, IL-17 in particular is believed to play a major role in the pathogenesis (137). SpA is commonly classified as either axial (affecting the spine) or peripheral. For example, ankylosing spondylitis affects mainly the spine, but also other joints and enthesis (138). Again, HLA-B27 and IL-17/IL-23 are believed to play key roles (139).

Psoriatic arthritis (PsA) represents another form of arthritis (although some argue it is part of the SpA group) (140). PsA have several distinctive features, including psoriasis (chronic inflammatory skin condition) and nail dystrophy (141). Furthermore, PsA is often RF- and anti-citrullinated peptide -negative (141). Treatment for PsA remains relatively similar to RA, with DMARDs and anti-TNF agents being common, but several other options exist (140).

Lastly, other types of arthritis include reactive arthritis, which is the emergence of arthritis following an infection in other parts of the body (142). Examples of common infections that could induce arthritis include urinary tract infections or borrelia. If left untreated, reactive arthritis can become chronic and destructive.

Juvenile Idiopathic Arthritis (JIA)

Introduction to JIA

Juvenile idiopathic arthritis (JIA) is an inflammatory joint disease in children. It is defined as persistent arthritis of unknown origin (hence idiopathic) with an onset before 16 years of age (143). It is the most common rheumatic disease in children (144). JIA is an umbrella term encompassing seven different subgroups and is defined according to the international league of associations for rheumatology (ILAR) (145) (**Figure 13**). JIA was given its current international name in 2001 and was previously termed juvenile chronic arthritis (mainly used in Europe) or juvenile rheumatoid arthritis (mainly used in America)

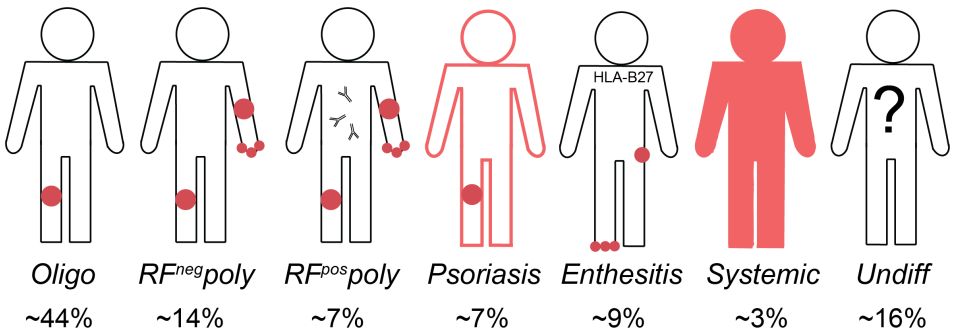


Figure 13. Overview of the different JIA subgroups. Schematic of the common joints affected in the different subgroups and other common features. The percentages show the distribution of each subgroup in southern Sweden (146). *RF – Rheumatoid factor.*

The seven subgroups of JIA

Oligoarticular JIA

Oligoarticular JIA (oJIA) is the most common subgroup in the western world and affects roughly half of the patients with JIA, and thus is the main focus of this thesis (143). It primarily affects larger joints asymmetrically, affecting females more often than males (147). "Oligo" indicates that the arthritis affects few joints, specifically four or less within the first six months. Often, only one knee is affected. However, the number of affected joints can increase over time (following the initial six months) to include five or more joints, at which point the disease is classified as "extended oJIA" (with fewer than five joints termed persistent oJIA) (147). Extended oJIA affects roughly half of all oJIA patients. The debut of oJIA often occurs at preschool age, with the majority of patients being anti-nuclear antibody (ANA) positive (143). The most common extra-articular manifestation associated with oJIA is uveitis (inflammation of the eye), affecting up to 30% of the patients

(143). Finally, some argue that oJIA is JIA subgroup that is most unique for children, with no clear counterparts in adults, though there is still ongoing debate regarding this (148, 149).

RF-negative polyarticular JIA

RF-negative polyarticular JIA (pJIA) affects five or more joints within the first six months, with patients being negative for RF. This subgroup is relatively heterogenous, as some patients resemble extended oJIA (uveitis and ANA are common manifestations of this subgroup), and others display more symmetric arthritis in several joints (similar to seronegative RA) (144). Thus, some argue that the patients resembling extended oJIA share similar characteristics with actual extended oJIA and therefore should be considered the same subgroup (150).

RF-positive polyarticular JIA

Similarly, RF-positive polyarticular JIA (pJIA) affects five or more joints within the first six months but the patients are positive for RF. These patients often have symmetric arthritis affecting several smaller joints, and several are anti-citrullinated protein antibody (ACPA) positive (144). RF-positive pJIA typically debuts in adolescence with a female predominance (143). Additionally, this subgroup is highly similar to RA in adults.

Juvenile psoriatic arthritis

Juvenile psoriatic arthritis (JPsA) affects 3-10% of the patients with JIA, which often presents with arthritis and psoriasis (or display dactylitis, nail involvement or have first-degree relatives with psoriasis) (144). However, these symptoms do not necessarily develop at the same time and roughly half of the patients lack the typical lesions (151). Two subgroups of JPsA have been observed, with one group of patients being similar to oJIA, such as a disease onset before the age of six and a positive ANA (144). The other group however, seems to share similarities with SpA and enthesitis-related arthritis (143).

Enthesitis-related JIA

Enthesitis-related (ERA) JIA is suggested to belong to spondyloarthropathies in adults and accounts for 5-10% of the patients, and it is unique amongst the JIA subgroups as it has a male predominance with a typical onset during adolescence (144). Up to 90% of cases are HLA-B27 positive, with the disease having a strong genetic connection (144). Most patients have enthesitis; inflammation of the entheses (connective tissue of tendons, bones, and ligaments). Arthritis often affects the hip or lower extremities, but can also involve the spine, resembling ankylosing spondylitis (143, 147). Most patients are negative for ANA, and ERA is also often linked to inflammatory bowel disease (143).

Systemic JIA

Systemic JIA has several systemic manifestations and differs significantly from the other subgroups. It is an autoinflammatory disease that is believed to be largely driven by the innate immunity, and is the same disease as Still's disease in adults (147). It accounts for 5-10% of the cases in the western world but is more prevalent in Asia (144). Symptoms include recurrent, quotidian fever as well as rashes and organ involvement. Potential arthritis is often symmetric and polyarticular (144). Roughly 10% of patients develop macrophage activation syndrome, which is a potentially life-threatening condition in which MØs devour the bone marrow (144).

Undifferentiated JIA

Finally, this group of JIA patients contain patients that fulfil the criteria for several subgroups, do not meet the criteria for any group, or fulfil an exclusion criteria (such as family history) (143). For example, one of the most common reasons for this classification is a family history of psoriasis (152).

Epidemiology

The incidence of JIA varies throughout the world. In Europe, the incidence rate varies greatly, but has been suggested to be between 2-23/100'000 and the prevalence rate to be 4-400/100'000. (153). In the Nordic countries, the incidence rate is reported to be between 12.8-24.1/100'000 (146, 154, 155). However, the incidence rate in southern Europe is markedly lower, with suggestions of a north-south gradient existing (156, 157). Additionally, subtype distribution differs in various parts of the world. In southeast Asia for example, ERA and sJIA are more common than in the western world (158).

Aetiology

It is mostly unknown how JIA develops, with it largely believed to be multifactorial. A combination of genetic predisposition and environmental factors seem at least to be necessary to develop disease. As such, this section will briefly describe the contribution of genetics and environmental factors.

Genetics

There is a clear genetic component influencing JIA development. The concordance in monozygotic twins is roughly 25-40% (159). In addition, siblings usually develop the same subtype if they also develop JIA (160). At the genetic level, multiple single nucleotide polymorphisms (SNPs) have been identified through GWAS (genome-wide association studies), with roles in immune function, such as STATs, IL-6R and PTPN22 (161-163). Interestingly, several of these SNPs overlap with other

autoimmune diseases. Unsurprisingly, there are also differences between JIA subgroups (144, 161). Maybe most well studied are HLA variants (as HLA variations are present is in most autoimmune diseases) (144, 161). These variants imply antigen presentation to be involved in JIA susceptibility, with some of the identified loci and variants being unique to JIA, whilst others overlap with other diseases (164, 165). However, the relevance of the GWAS studies is limited and few large studies exist (144). Additionally, a recent study using TWAS (transcriptome WAS) identified enriched pathways related cytokine signalling and the immune system (166). Taken together, there is a clear genetic component influencing the susceptibility of JIA, including both HLA- and non-HLA genes.

Environmental factors

Genetic associations cannot fully explain the aetiology of JIA, and several environmental factors have been suggested to contribute to disease. A substantial proportion of studies investigating environmental factors in JIA are limited in size, with several conflicting studies existing. However, it is clear that there are several factors that are potentially associated with an increased risk of developing JIA. For example, a recent meta-analysis found that caesarean section delivery is weakly associated with an increased risk of developing JIA, whilst the presence of siblings seems to be protective against JIA development (167). Infections in early life are also suggested to be linked to an increased risk of developing JIA (168) but evidence is conflicting (169). An example of a suggested infectious agent is Parvovirus B19 (170, 171). Additionally, antibiotic use during the early period of life has been associated with a higher risk of developing JIA (169). There is also weak support for the role of dietary factors (172). Finally, a short duration of breast feeding has also been suggested to be a risk factor, but conflicting evidence exists (173, 174). Thus, a combination of genetics, environmental factors and, as discussed before, a breach in tolerance, all influence the risk of JIA development (**Figure 14**).

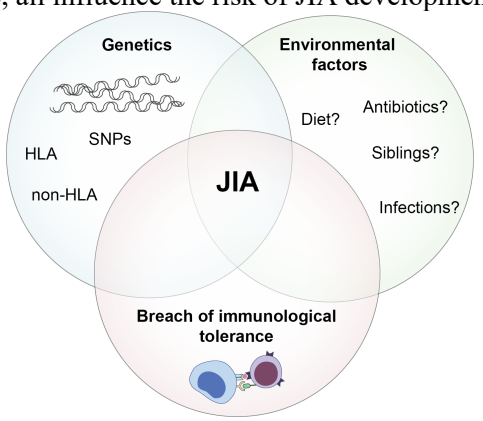


Figure 14. Schematic summary outlining the etiology of JIA. JIA is believed to arise through a combination of genetic- and environmental factors, which predispositions a breach of tolerance and subsequent disease. *SNPs – single nucleotide polymorphisms, HLA – human leukocyte antigen.*

Treatment

JIA is a chronic disease and there is currently no available cure. Therefore, the goal of treatment is to limit symptoms and prevent joint damage. If left untreated, the arthritis might develop and cause substantial damage to the joint structure. As young adults, roughly half of JIA patients are still in active disease, with even some patients in inactive disease still requiring treatment (146, 175). Thus, despite significant advancements in drug development, there is still a major need for new interventions.

The current first-line of treatment for oJIA consists of non-steroid anti-inflammatory drugs (NSAIDs) and intra-articular corticosteroid injections (144, 176). If these fail, the patients are treated with disease modifying anti-rheumatic drugs (DMARDs). DMARDs can be further divided into conventional DMARDs and biological DMARDs (bDMARDs). The most common conventional DMARD is methotrexate, which is used in low doses for its' anti-inflammatory properties and is the first DMARD of choice. It has multiple mechanisms of actions, and it is known to act as an analogue of folic acid and block various enzymes (177). However, its precise mechanism in arthritis is not known. bDMARDs consist of several types of drugs that target different inflammatory processes. The most common bDMARDs are TNF inhibitors, but other drugs include IL-6 inhibitors and anti-CD80/CD86 antibodies (176). In oJIA, primarily TNF inhibitors and, to a lesser extent, IL-6 inhibitors are used. Recently, JAK/STAT inhibitors, such as tofacitinib, have also shown promise in the treatment of JIA, which offers an advantage over bDMARDs as they can be administered orally (178).

However, there are few studies looking into long-term follow-up and the progression of JIA into adulthood. One relatively recent Norwegian study found that roughly half of JIA patients had active disease, with the other half having inactive disease (roughly a third were in remission on treatment) (175). Notably, the selected population was quite young (mean age of 24 years). Still, this is in line with other studies, that also found that roughly half of patients after long-term follow-up are in active disease (179, 180). Finally, in one study, the majority (70%) of patients who were in remission at a 15 year follow-up mark, were also still in remission at the 30 year follow-up (181). More studies are needed to evaluate the long-term prognosis.

Pathogenesis

Introduction

Similar to the aetiology, the pathogenesis of JIA is not fully understood. As oJIA is the focus of this thesis, and it shares some features with RF-negative pJIA, this section will thus focus on these subgroups only. The role of monocytes and neutrophils specifically will be discussed in detail further on.

An overview of the synovium and immunity driven inflammation

In oJIA, the current understanding is that there is first an initial breach of tolerance, resulting in an influx of immune cells into the joint, followed by local inflammation, abnormal activation, and tissue disruption (144, 147) (**Figure 15**). This subsequently triggers local swelling, thickening of the synovial membrane and excess SF production. During this process, most types of immune cells can be found within the joint, including lymphocytes and myeloid cells (144). In particular, there is evidence of tertiary lymphoid follicles in the synovial tissue, containing lymphocytes and APCs (182). In addition, FLS proliferate and have an altered phenotype and transcriptome in oJIA (183). Finally, in the SF, there is an accumulation of several cytokines, chemokines, and other mediators, of both innate and adaptive origin (184, 185).

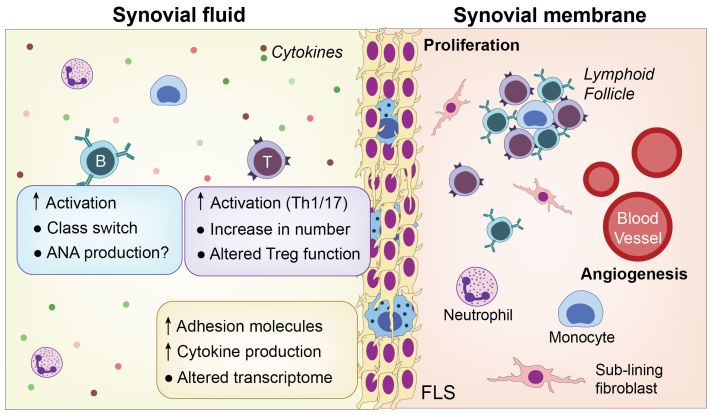


Figure 15. Overview of the synovium and immunological alterations in JIA. Summary on the major immune changes in the synovial fluid and synovial membrane, with a focus on B-cells, T-cells and fibroblast-like synoviocytes (FLS). *ANA – Anti-nuclear antibodies.*

Role of adaptive immunity

Most of the available literature on the pathogenesis of JIA has focused on adaptive immunity, mainly that of T- and B-cells. Thus, it is known that these cells are clearly present, activated and involved in the synovial inflammation. Though, knowledge of potential autoantigens remains limited. It has been previously shown that circulating T-cells react to a broad range of synovial antigens, such as aggrecan, fibrillin and MMP-3, and that a proportion of circulating T-cells are enriched in synovial clonotypes (186-188). Accordingly, a recent study using a single-cell RNA approach identified several heterogenous clonally expanded subpopulations of T-cells in the joint (189). Another study also found that T-cell clonality was similar within several affected joints (188). In contrast, reactivity to HSP60 has been suggested as a protective mechanism, possibly through the induction of Tregs (190-192). Tregs are present in the inflamed joint and show a stronger suppressive capacity than their circulating counterparts (193). However, Tregs are heterogenous

in nature, and other studies have identified subpopulations that contribute to the inflammatory environment with impaired suppressive capacity, indicating a dual role (194-197).

T-cells have been extensively studied in the synovium of patients with oJIA. Most studies suggest that an expansion and activation of heterogenous populations of Th1-, Th17- and Th1/Th17 cells occurs, that produce pro-inflammatory cytokines (e.g., IFNγ and IL-17) and correlate in various ways to clinical parameters (196, 198-202). Interestingly, these cells are resistant to suppression (203). T-cells are also prevalent in affected tissues, with extended-to-be oJIA patients having more lymphocytes than persistent oJIA patients (204). Furthermore, the number of DCs correlates with TNF levels and $CD4^+$ T cells in the SF (205). In the tissue, HLA-DR- and IFNα positive cells have also been shown to cluster around T cells (206, 207). Recently, it has been suggested that a certain population of memory T-cells is involved in mediating flares (208, 209). Furthermore, T-cells can also influence the activation of other cells, such as B-cells (210).

Similarly, B-cells are also found in the inflamed synovium. The SF and tissue contain class-switched B-cells and plasma cells that are IgG^+, and the presence of lymphoid follicles in the tissue has been linked to circulating ANA, implying local production of autoantibodies (182, 211). B-cells also display markers of activation and changes in chemokine receptor expression (211, 212). Despite the majority of oJIA patients being ANA positive, the role of ANA in the pathogenesis is still unclear (146). A recent study found that clonally expanded T-cells preferentially accumulate in ANA positive compared to ANA negative patients (213). ANA positivity has also been repeatedly associated with an increased risk of developing uveitis, with several studies reporting an association of ANAs with eye tissue (214-216). Interestingly, the eyes of uveitis patients also contain plasma cells which correlate with ANA, raising the question on whether potentially pathogenic ANA originates from the joint or eye (217). In addition, ANA has been suggested as a tool to define JIA subgroups more homogenously, but the use of ANA positivity for this purpose is still under dispute (150, 218).

Taken together, there is clear evidence for the involvement of lymphocytes in the pathogenesis of oJIA, as these cells are recruited, activated, and sustain inflammation in the joint.

Role of fibroblast-like synoviocytes

Beyond the immune system, a handful of studies have looked into the role fibroblasts in the pathogenesis of JIA (183). Synovial fibroblasts are found in the lining layer (usually referred to as fibroblast-like synoviocytes (FLS)) as well as the sub-lining layer in the synovial membrane (usually referred to as sub-lining-fibroblasts). Indeed, the origin of these cells is diverse, and some are believed to originate from embryonic precursors (219). There are also data on distinctive

markers in adults to distinguish different fibroblast populations, such as cadherin-11, CD90 or lubricin (220, 221).

The importance of these cells in arthritis is highlighted within animal studies showing that fibroblasts can initiate and sustain arthritis (222). Indeed, synovial fibroblasts can be activated independently of immune cells, such as TLR ligands, and in turn can drive immune cell infiltration and activation (223). There is clear evidence of the different roles of lining and sub-lining populations in adult disease and animal models (221, 224, 225). Simplified, sub-lining fibroblasts are described as inflammatory and proliferative, and FLS as invasive, destructive and mediators of cartilage and bone destruction (224). Collectively, synovial fibroblasts can produce cytokines and chemokines which recruit immune cells, they sustain and promote proliferation of the immune cells and they also have the capacity to present antigens (226-228). Additionally, synovial fibroblasts also support the formation of tertiary lymphoid organs (229). Finally, studies suggest that notch signalling and intracellular complement drives a metabolic shift in synovial fibroblasts upon repeated stimuli, which in turn drives inflammation independent of adaptive immunity (230, 231). However, as stated earlier, not much is known about these cells in JIA (**Figure 16**).

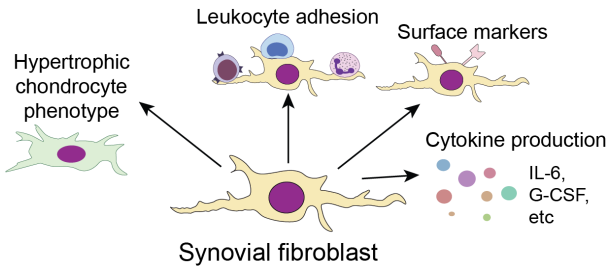


Figure 16. Schematic of the role of synovial fibroblasts in JIA. Synovial fibroblasts have been shown to produce cytokines, express various surface markers, facilitate leukocyte recruitment and acquire a hypertrophic chondrocyte phenotype.

In JIA, as in adults with arthritis, several studies have identified different fibroblast populations (183). JIA synovial fibroblasts produce several cytokines, chemokines, and enzymes (232). However, bone erosion and cartilage degradation are not frequent manifestations in oJIA. Instead, growth disturbances are more commonly observed. Synovial fibroblasts have been shown to interact with chondrocytes, cells involved in cartilage formation (233, 234). FLS in JIA show a TGF-β induced transcriptome, and through BMP4, are believed to acquire a hypertrophic chondrocyte-like phenotype, suggesting a role in the observed growth disturbances (233-235). However, there is an early study that has identified some degradation properties of JIA fibroblasts (236). Furthermore, one study has identified an increased expression of VCAM-1, with a corresponding increase in leukocyte attachment, suggesting ongoing crosstalk between cells (237). Indeed, co-cultures of leukocytes with synovial fibroblasts have also been shown to induce cytokine

production by T-cells (238). Thus, there is limited knowledge regarding synovial fibroblasts in JIA, but given the non-destructive phenotype in oJIA, fibroblasts may have different roles in this disease compared to adult arthritis.

Systemic features

It is generally believed that oJIA is not a systemic disease as the majority of symptoms are local. However, it is clear that circulating immune cells differ in various ways compared to control and disease states (239-242). For example, circulating T-cells share clonality with populations found the joint (188). In addition, elevated levels of cytokines are also present in the circulation of oJIA patients, with the cytokine pattern being related to disease activity (243). Furthermore, the circulating cytokines are not necessarily mirrored by the distribution of cytokines in the inflamed SF, as the cytokine pattern of oJIA is distinct from other subtypes of JIA and adults with arthritis (185, 243). In addition, several markers have been suggested to be used as biomarkers, such as MRP8/14, which has been proposed to predict methotrexate response and is related to disease activity and uveitis (244-247). Thus, even though most symptoms are restricted to the joint, it is clear that there are immunological alterations present in the circulation.

Monocytes and Neutrophils in Arthritis & JIA

Rationale

Lymphocytes have an established role in driving inflammation of the joint. However, many parts of the pathogenesis are still unknown, and we know little of the role of the innate immune system. The innate immune system sustains and skews the adaptive response, which could have crucial implications in the disease course. Indeed, the main bDMARDs used in JIA target cytokines that are mainly produced by innate immune cells, as opposed to drugs that directly target adaptive immunity (144). Additionally, several genetic polymorphisms identified are not specific to lymphocytes, and some are crucial for the function of the innate immune system (161-163). Activation of the adaptive immune system does not necessarily include antigens and clonal expansion. As mentioned previously, T-cells can be activated to proliferate and produce cytokines without an antigen (248). Finally, the role of ANA in the pathogenesis has yet to be determined, as healthy children also have ANA, but an association of ANA positivity to uveitis incidence does exist (126, 144, 249). Thus, there is a dire need to increase our understanding of how other cells may contribute to the pathogenesis in order to identify disease mechanisms and better tailor treatments. This section will focus on the two main cells of the innate immune system that are the focus of this thesis: the monocyte, and the neutrophil.

Monocytes & Neutrophils in Arthritis

Introduction

Monocytes and neutrophils have been studied significantly more in adult arthritic diseases than in oJIA. This might partly be due to historical assumptions that JIA is a childhood-version of adult diseases, but also due to ethical reasons. Still, there is a lot to learn from adult studies, and this section will give a brief overview of these cells in adult diseases, and how they potentially differ between disease settings.

Monocytes in arthritis

In RA, there is extensive research into monocytes/MØs and their role in disease (**Figure 17**). Interestingly, the number of synovial monocytes/MØs correlate with disease activity in RA (250). Circulating monocytes are also hyperactive, adhesive, and metabolically altered (251, 252). During disease progression, monocytes are recruited to the joint by cytokines and chemokines (e.g., MCP-1) produced by the local cells, such as fibroblasts and MØs (253). These "synovial" monocytes then acquire a $CD16^{+}CD14^{+}$ phenotype and express several markers of activation, including CCRs, TLRs, HLA-DR, CD163 and CD80 (26, 254-256). Conversely, the synovial monocytes drive T-cell responses, produce pro-inflammatory cytokines, and induce activation of other cells (256, 257). Furthermore, the synovial monocytes produce ROS which is believed to drive inflammation and the induction of pro-inflammatory cytokines (258). In addition, synovial monocytes and MØs are believed to be of an M1 phenotype and be prone to osteoclastogenesis, which in turn can lead to increased bone erosion (257, 259). Importantly, infiltrating monocytes can also differentiate into MØs, further sustaining the inflammation through cytokine production and cell activation (257, 260).

However, during remission, synovial monocytes are believed to acquire an M2 phenotype, favouring resolution (257). Deficiencies in the clearance of apoptotic cells has been typically associated with several autoimmune diseases, such as systemic lupus erythematosus, but macrophages in RA and animal models have sustained efferocytosis, which is believed to prevent further inflammation (261, 262). However, some studies have observed impaired phagocytosis of RA monocytes and macrophages, resulting in the accumulation of immune complexes that drive activation and inflammation (263, 264). Finally, there is data which suggests that monocytes may be affected already in the bone marrow with evidence of increased turnover (26).

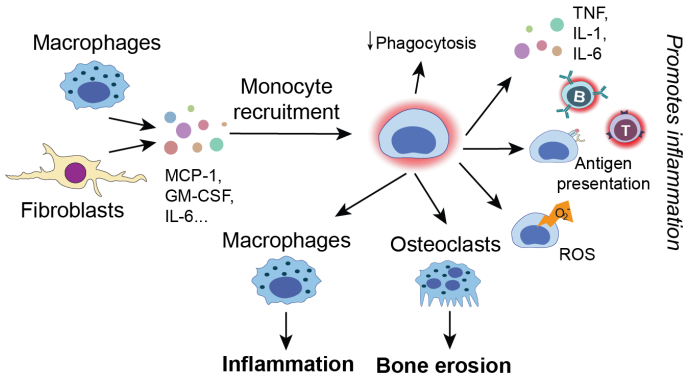


Figure 17. Monocytes in arthritis. Shows an overview of the general role of monocytes in adult arthritis, as they are recruited, activated, and differentiated in the inflamed joint.

Similar to RA, the circulating monocyte number in SpA correlates with disease activity, and the monocytes show an increased expression of surface marker (e.g., TLRs) and production of pro-inflammatory cytokines (265-267). However, there is enrichment of different populations of circulating monocytes in SpA compared to RA (7, 268). Interestingly, the number of MØs is similar between the two diseases (269). However, synovial monocytes and MØs of patients with SpA display a higher expression of CD163 than RA patients (270). Furthermore, SF from SpA preferentially induces M2-like features in MØs *in vitro,* with the SF containing less M1-derived cytokines than SF from RA patients (271). Indeed, SpA synovial tissue has more prominent vascularization compared to RA patients (272). Hence, MØs and monocytes in SpA might be of an M2 phenotype compared to RA. Similar to SpA vs RA, oJIA and ERA patients have more vascularization compared to pJIA patients (273). Importantly, it is worth mentioning that an M2-like phenotype is not necessarily beneficial, as it can result in increased angiogenesis, cell influx and hypertrophy (274, 275). Indeed, the MØs in SpA patients are potent TNF producers and also express high levels of HLA-DR (276). Thus, M2 monocytes will be referred to as regulatory, rather than anti-inflammatory, in the studies below, as an M2 phenotype is not necessarily equal to inhibition of inflammation.

Taken together, monocytes and MØs drive the pathogenesis in arthritis through cytokine production, antigen presentation and differentiation. Interestingly, there are also phenotypic variations between arthritic diseases, suggesting that despite some similarities, distinct mechanisms may exist for each disease.

Neutrophils in arthritis

Neutrophils, as monocytes, are also known to contribute to inflammation in adult arthritis (**Figure 18**). In RA, circulating neutrophils are primed for ROS production (possibly through immune complexes) and have a delayed apoptosis but show similar transcriptomic- and surface expression profiles to healthy controls (277-

282). At the site of inflammation, neutrophils show a prolonged survival, increased expression of activation markers and increased ROS production (278, 279, 283, 284). Regarding expression markers, the presence of HLA-DR and release of BAFF suggest a role in antigen presentation and the promotion of an adaptive response (278, 285). An increased production of ROS is thought to cause tissue damage and induce activation of other cells, such as fibroblasts (286). Lastly, the degranulation and release of proteases and enzymes are believed to promote joint degradation. Furthermore, synovial neutrophils typically have an altered transcriptome related to several cytokine signalling pathways (278). Additional mechanisms believe to be overactive in synovial neutrophils include citrullination, which is believed to be related to the generation of citrullinated autoantigens in RA (287). Furthermore, NET formation in the joint has been suggested to induce inflammatory responses in other cells, exposing potential autoantigens and leading to the release of degrading enzymes (288, 289). Additionally, neutrophils have the potential to degrade cartilage (290). Underscoring the importance of these cells, depletion of neutrophils in animal models of arthritis results in less severe arthritis (291).

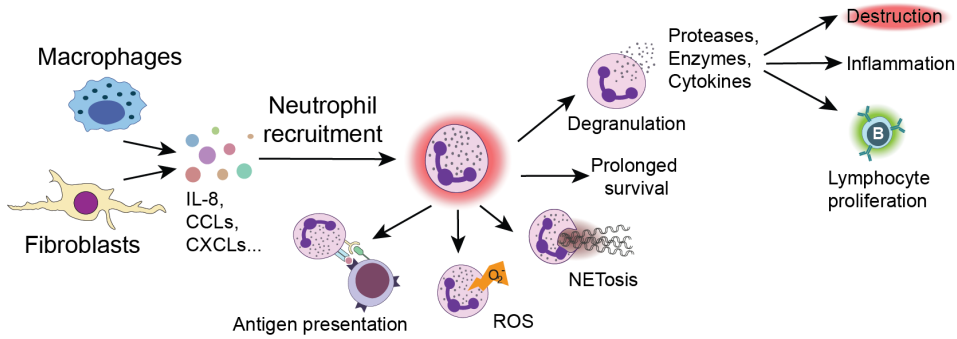


Figure 18. Neutrophils in arthritis. Displays the general role of neutrophils in adult arthritis, highlighting key processes believed to contribute to the pathogenesis.

In SpA, the role of neutrophils is believed to be similar to RA, as synovial neutrophils display increased NETosis, cytokine release and increased neutrophil-lymphocyte ratio that correlate with disease severity (292, 293). In addition, neutrophils have increased mRNA of MRP8 and MRP14 and can produce cytokines such as IL-23 and IL-17 (294, 295). However, synovial neutrophils in SpA do not display a clear activated phenotype, with the SF of SpA patients containing less neutrophil derived proteins, suggesting less activation occurring in the SF (296, 297). The SpA synovial tissue, however, is enriched in neutrophils and neutrophil-derived molecules compared to RA (272, 298). Levels of intracellular citrullinated proteins, however, are less compared to RA patients (272).

In summary, neutrophils have a prominent role in the pathogenesis of arthritis as they produce excessive ROS, degranulate and undergo NETosis. As with monocytes, there are notable differences in distribution and phenotype between diseases, highlighting different mechanisms.

Monocytes in JIA

Monocytes in JIA are not as well studied as in adults. Monocytes have been shown to be enriched in the circulation compared to controls, where they express increased levels of surface markers, such as TLR4 and CD86, and are hypersensitive to activation (299-302). At the genetic level, peripheral mononuclear cells have altered expression profiles, both within JIA and compared to controls (303, 304). Interestingly, in a study comparing methotrexate responders and non-responders, a monocyte signature was prominent within the non-responders (305). Indeed, a recent study analysing chromatin data showed enrichment of multiple genes related to immune function in circulating monocytes (306). This is supported by another recent study, looking into epigenetic changes, which found enrichment of an inflammatory signature driven by IFN, which was blocked by JAK inhibition (307).

In the joint, early studies in juvenile rheumatoid/chronic arthritis have shown that synovial monocytes produce higher levels MRP8 and MRP14 and are prone to bone degradation (308, 309). Synovial monocytes also express several activation markers, such as CD64 and PDL-1, and produce various chemokines and cytokines believed to contribute to the pathogenesis (310-314). For example, they produce VEGF, which is believed to contribute to increased vascularization (311). In addition, synovial monocytes display impaired gp130/IL-6R signalling (315). Functionally, they have recently been shown to promote T-cell proliferation (314). Regarding SF, concentrations of several monocyte derived cytokines differ between JIA subgroups and RA patients, potentially reflecting different disease mechanisms (316). Additionally, synovial monocytes in oJIA have different expression levels of surface markers compared to other diseases (299). Finally, there are also differences in the activation pattern present when comparing synovial monocytes of oJIA patients to patients with septic arthritis (312).

Thus, both circulating and synovial monocytes display signs of alterations and activation at the epigenetic- and phenotype level in oJIA.

Neutrophils in JIA

Neutrophils are the most prevalent cells in the SF but are scarce within the tissue (273, 317). Circulating neutrophils show signs of an immature phenotype, and also form aggregates with platelets, which together are believed to drive inflammation (e.g., via degranulation and cytokine production) (318). Products derived from neutrophils, such as MPO, can also be measured in the circulation (318, 319). Additionally, an elevated neutrophil-to-lymphocyte ratio is observed in patients with active disease (320). Furthermore, low-density neutrophils (neutrophils described to be more inflammatory) are also elevated in JIA patients (321). Finally, studies at the gene- and transcriptomic level suggest there is an ongoing degranulation, in addition to other neutrophil activation related processes occurring

in patients with JIA (321-324). Thus, circulating neutrophils show signs of activation in JIA.

Additionally, synovial neutrophils display increased markers of activation (e.g., CD66b and CD11b), signs of degranulation and transcriptomic changes related to IFN, IL-6 and hypoxia signatures (278, 313, 325). Moreover, synovial neutrophils also express markers atypical to neutrophils, such as HLA-DR, suggesting a role in antigen processing and presentation (325). Interestingly, patients with high synovial neutrophil counts and subsequently low lymphocyte counts commonly have elevated concentrations of cytokines (313). Taken together, these studies suggest there is activation of both circulating and synovial neutrophils within JIA.

General methodology

Patient material

Patients used in the different studies of this thesis were admitted/examined at the Department of Paediatrics, Section for paediatric Rheumatology, Skåne University Hospital between 2016-2023. All four studies have focused on oJIA and have utilised synovial fluid (SF) and blood samples. Cells (monocytes, neutrophils, and S-Fib) from blood or SF have been used throughout to study the function and phenotype of the aforementioned cells, whilst cell-free SF and serum/plasma have been used to study the impact of the surrounding environment by using these fluids to stimulate healthy cells *in vitro*. In paper I and III, synovial biopsies were collected and used for immunofluorescence and immunohistochemistry staining.

Blood and SF samples were collected as part of routine therapeutic joint aspiration. Patients were either newly diagnosed or in relapse following long-term remission of medication at inclusion. Patients in paper I, II and III were untreated or had only received NSAIDs, with no patient receiving steroids or DMARDs for at least six months prior to inclusion. However, in paper IV, a number of patients used for S-Fib donation had received DMARDs, such as methotrexate.

Cell isolation

Cells were isolated in different ways for use in this thesis (**Figure 19**). For paper I and II, monocytes were isolated from either blood or SF from patients. These were subsequently used in several downstream applications, such as mRNA isolation or T-cell activation (described more below). In parallel, and also for paper IV, monocytes were isolated from healthy controls, which were utilised to investigate several parameters *in vitro*, such as the effect of the surrounding environment (patient derived SF).

For patients, the monocytes were isolated from blood or SF in two main steps. First, mononuclear cells were separated from both red blood cells and granulocytes through density centrifugation. Alternatively, SF samples were first centrifuged, and cell-free SF was then collected. Secondly, monocytes were further isolated using antibodies targeting CD14, and then labelled with magnetic beads. By using a magnet, the CD14 labelled monocytes could be separated from non-labelled cells, allowing for an enrichment of monocytes. Following several washes, monocytes

could be removed from the magnet, yielding a pure monocyte population. Disadvantages of targeting CD14 for isolation (termed positive selection) compared to removing all other cells (negative selection) include the risk of unintentionally activating cells. However, we did not observe any spontaneous activation of the monocytes, such as spontaneous cytokine production. Alternative ways of isolating monocytes, such as fluorescence activated cell sorting (FACS) also exist. However, these methods are more time consuming and more difficult to perform on a daily basis.

For paper III and IV, neutrophils primarily isolated from blood of both patients and healthy individuals were used. As with monocyte isolation, a density centrifugation step was performed, but the lower fraction containing red blood cells and granulocytes (primarily neutrophils but also eosinophils) was used instead. Red blood cells were removed by sedimentation using dextran, leaving only granulocytes in the solution. These were subsequently collected, with any remaining red blood cells lysed using a short water incubation. As with monocytes, neutrophils could have also been isolated using magnets instead, removing eosinophils and yielding a pure neutrophil population. However, as neutrophils are the predominate cell type compared to eosinophils, in addition to being notoriously easy to activate, we opted to not perform additional steps.

For paper IV, S-Fib were isolated from SF by passaging the synovial cells. Freshly isolated SF cells are heterogenous in nature, containing a mixed population of cells. As such, most of these cells do not adhere to surfaces or die shortly after isolation, leaving primarily MØs and S-Fib following medium replacement or after the first passage (326). After 2-3+ passages, S-Fib represent the main cell population (326, 327).

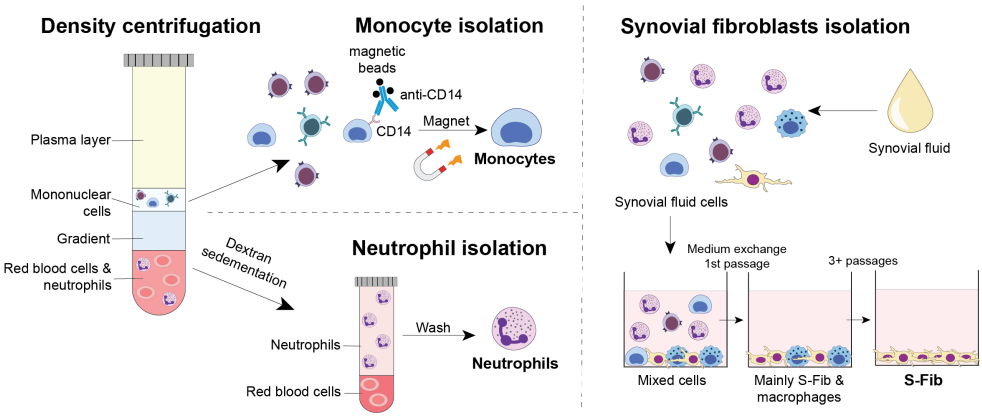


Figure 19. Isolation of monocytes, neutrophils, and synovial fibroblasts. An overview of the different methods utilised to isolate cells to be used in the four different studies. Monocytes and neutrophils were isolated from blood or synovial fluid samples, whilst synovial fibroblasts (S-Fib) were isolated from synovial fluid.

Flow cytometry

Flow cytometry is the main analysis technique used throughout the four studies of this thesis. It has a broad range of applications, ranging from the analysis of phosphorylation patterns to phenotype determination. The power of flow cytometry as an analytical tool comes from its ability to analyse multiple parameters at the single cell level in a liquid sample. In a nutshell, flow cytometry is based on two main principles: fluorescence and light scattering.

The technique builds upon the labelling of cells with fluorescent molecules (called fluorophores). Fluorophore-conjugated antibodies who are directed against a marker of interest, such as a surface receptor, are often used, but other types of fluorescent probes or dyes are also available. The use of antibodies originates from their nature of being highly specific for their antigen, thus enabling the identification and quantification of your marker of interest. Labelled cells are injected into the flow cytometer and then combined with sheath fluid in the fluidics system to allow the alignment of cells into a single file through a narrow stream (see **Figure 20**). The cells subsequently enter a system called the flow cell, where the sample is exposed to a light source, most commonly a laser.

A flow cytometer is equipped with one or several lasers. These lasers emit light at specific wavelengths and passes through an excitation filter, which makes sure that only the desired wavelength hits the cells. As the laser excites the fluorophores, they absorb the light and reach an excited state, from which the fluorophores soon re-emit the absorbed light at a longer wavelength. This light subsequently passes through dichroic mirrors that separate the light into different detector channels based on wavelength. Finally, the light passes through another filter (emission filter) which is subsequently captured by a detector. The detector converts the light (photon signal) into an electrical signal. This data can then be visualised using various softwares, but is often portrayed in a two-dimensional graph, where each dot in the graph corresponds to an event (or cell). Given that different fluorophores have different excitation and emission spectra, flow cytometry allows for the simultaneous analysis of multiple fluorophores. Modern flow cytometers can easily capture 10+ different types of fluorophores. Besides fluorophores, the emission of light based on the cell size and granularity is also used for analysis. Forward scatter (FSC) is used to detect the size of the cells based on the light scattered in the forward direction. Larger cells will scatter more of the light. Side scatter (SSC) measures the granularity of the cells and is detected at an angle to the laser. Thus, FSC combined with SSC provides information about cell size and granularity, which can be used to separate different types of cells from each other.

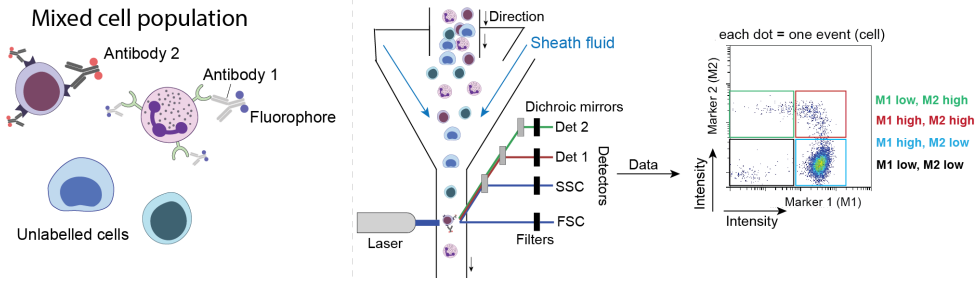


Figure 20. Overview of the principles of flow cytometry. Cells are labelled with antibodies conjugated to fluorophores. As they are acquired by the flow cytometer, they pass through the flow cell and are exposed to a laser. As the fluorophores are excited, they emit light which is then divided by dichroic mirrors and collected by detectors, which subsequently converts the light to data points. Based on the intensity of the emitted light, cells can be separated into different populations.

The most common application of flow cytometry is immunophenotyping, which is the analysis and subsequent identification of the cells of interest in a mixed population of cells. Flow cytometry can also be used for sorting cells of interest for downstream use, called FACS. Here, we used flow cytometry for multiple purposes, both analysing the phenotype of cells isolated from patients, and as a readout for functional assays.

For phenotyping, several activation markers were used to study the state of monocytes and neutrophils. These included receptors related to migration, phagocytosis, and co-stimulation to mention a few, which are used as surrogate markers to determine the activation state of cells. In addition, the actual function of the cells was assessed as described below.

Functional assays

Various *in vitro* assays have been used to study the functional properties of monocytes and neutrophils, both in patients and in healthy cells using *in vitro* assays (**Figure 21**). The assays were chosen as they are believed to reflect key functions of the immune cells, providing information of the functional state of the given cells. This section will highlight a few of the most used assays in this thesis.

Phagocytosis

Phagocytosis was assessed in both monocytes and neutrophils using two different methods. In paper I and III, phagocytosis was assessed in blood or SF using a commercial kit termed PhagoTest™. Fluorescein (FITC)-labelled *E.coli* were used as bait cells to be phagocytosed, and bound (but not internalized) *E.coli* were quenched. Finally, red blood cells were lysed to reduce noise, and cells were fixated before acquisition and analysis using flow cytometry. In paper II, as the PhagoTest™ was designed for whole blood and was also discontinued,

phagocytosis in monocytes was instead assessed using opsonized, FITC-labelled beads. Extracellularly bound beads were quenched with trypan blue before acquisition. In both conditions, unspecific binding was also assessed in experiments where phagocytosis was performed on ice (instead of at 37°C).

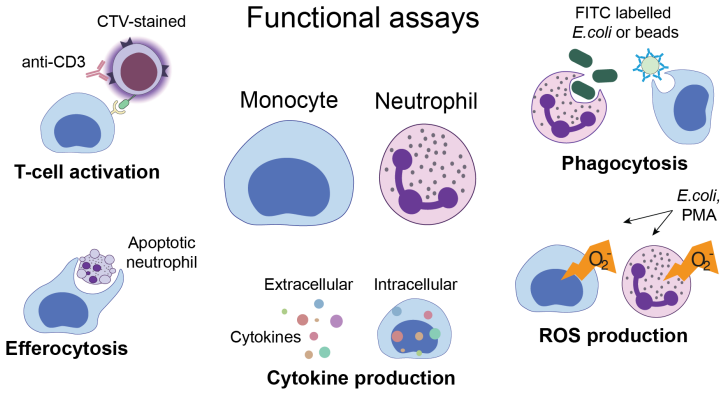


Figure 21. General assays used throughout this thesis. Monocytes and neutrophils, either from healthy controls or patients, were studied using several functional assays. Highlighted above is an overview of five assays that are included within several of the studies. *CTV – CellTrace violet, ROS – Reactive oxygen species, PMA – Phorbol 12-myristate 13-acetate, FITC - Fluorescein.*

ROS

As with phagocytosis, ROS was assessed in both monocytes and neutrophils using two different ways. In paper I and III, a test called PhagoBurst™ was used. In this test, cells were activated either with *E.coli* or PMA, resulting in ROS production. ROS is measured using Dihydrorhodamine (DHR)-123. DHR-123 is nonfluorescent and can diffuse across the cell membrane, but is oxidized by ROS to rhodamine-123, which is fluorescent and remains within the cell. As with PhagoTest™, red blood cells are lysed, with samples being fixated before acquisition and analysis by flow cytometry. In paper II and IV, ROS was assessed in purified cells using H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate). This builds on the same principle as DHR-123, as H_2DCFDA can freely diffuse into the cell, where it is cleaved and oxidized into fluorescent 2',7'-dichlorofluorescein (DCF). However, in contrast to PhagoBurst™, ROS generated in this assay was analysed using a plate reader. As with phagocytosis, H_2DCFDA was also implemented due to the discontinuation of PhagoBurst™ and the use of purified cells.

T-cell activation

An important function of monocytes is their induction of T-cell activation. Thus, this function was studied as a measurement in paper II and IV. In this assay, T-cells were isolated from healthy donors and stained with CellTrace violet (CTV). CTV is a dye which passively diffuses into the cell where it is converted by esterases into a fluorescent product which covalently binds to amines. This allows the tracking of

cell proliferation, as dividing cells become less and less fluorescent upon each division (see **Figure 22**). Following staining with CTV, T-cells were activated with anti-CD3, and co-cultured with monocytes, which provides the co-stimulatory aspect necessary for prominent T-cell activation. Thus, we measured the ability of monocytes to activate T-cells through co-stimulation (and potentially the skewing of T-cell activation by cytokines produced by monocytes). T-cells and monocytes were co-cultured for 72hrs before analysis by flow cytometry. Besides proliferation which was measured as a percentage of CTV positive T-cells, the T-cells were also stained with surface markers, as an additional method to study activation. In paper IV, intracellular cytokine production in T-cells was also studied, using a method described below.

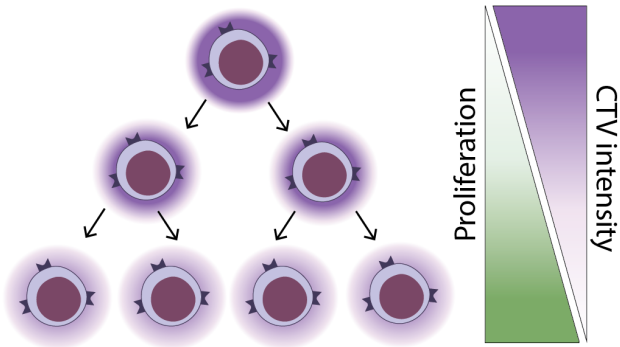


Figure 22. T-cell proliferation assay. T-cells were isolated from healthy controls and stained with CTV. As they proliferate, the dye loses intensity. *CTV – CellTrace violet.*

Efferocytosis

Efferocytosis by monocytes was studied in paper II and IV. In this assay, bait cells (cells to be efferocytosed) were generated from healthy neutrophils. These were then stained with CTV and incubated overnight in a serum-poor environment to induce apoptosis. Apoptosis was confirmed through flow cytometry, with bait cells being subsequently co-cultured with monocytes. To assess the degree of bait cell uptake, monocytes were analysed by flow cytometry for CTV positivity. In order to exclude bound, but not internalized bait cells, samples were additionally stained with anti-CD14 and CD66b. As such, cells positive for both markers were considered bound cells and excluded. Monocytes that had internalized bait cells were $CD14^{+}CTV^{+}$.

Cytokine production

The production of cytokines in monocytes was assessed in paper II and IV. In paper II, cytokines were analysed by flow cytometry through intracellular staining. This was achieved by blocking the Golgi apparatus of cells by an inhibitor called Brefeldin A. This prevents the release of cytokines out of the cell. Monocytes were subsequently activated for a period of time, before they were stained with surface antibodies against CD14, to be able to discriminate them from other cells. After

fixation and permeabilization, cells were further stained with anti-cytokine antibodies (specifically IL-1, IL-6, IL-8 and TNF). Finally, the samples were analysed by flow cytometry. The advantage of this process over traditional methods of cytokine measurement in supernatants is that it allows for the identification of cytokine producing cells in a mixed cell population. However, as it is measured intracellularly, the question remains on whether cytokines are actually released from monocytes (or just synthesised), which means that the absolute cytokine concentration cannot be determined.

In paper IV, we instead measured cytokine concentrations in the supernatants of co-cultures between monocytes and S-Fib. These were analysed using a technique from MesoScale. The basic principle of MesoScale is that of a sandwich immunoassay technique. It utilises capture antibodies, to which the antigen binds to, followed by a secondary antibody that also binds to the antigen. This secondary antibody is then labelled with a molecule that expels light when voltage is applied. The use of electrochemiluminescence, as opposed to absorbance, allows for multiplexing, as the plate is analysed from the bottom at predefined spots within each well, where individual capture antibodies can be placed. However, the disadvantage of using this method in co-culture settings between monocytes and S-Fib is that we cannot ascertain the cytokine source. However, the MesoScale method allowed for an easier workflow as supernatants could be collected, thus allowing for an easy analysis of many samples (as necessary in paper IV).

Other methods

Besides the aforementioned assays, other methods have occasionally been used throughout the thesis. In paper I, we utilised reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to study mRNA expression of several immunological markers in synovial- vs circulating monocytes. In short, RT-qPCR first involves the reverse transcriptase phase, which describes the isolation of RNA from cells, followed by the conversion into cyclic DNA (cDNA). qPCR analysis involves a thermal cycler, that employs a series of temperature cycles, during which cDNA is amplified exponentially. This technique also utilises a fluorescent probe or dye that binds to the amplified cDNA, emitting a signal that is detected by a fluorescence detector. The signal is measured at each cycle, with the number of cycles inversely related to the initial amount of mRNA, allowing for the quantification of mRNA. By comparing the cycle threshold to a reference gene, it is possible to calculate the relative expression of mRNA. By extension, by comparing the relative expression of these samples to a control, the fold change between different samples (also called the $\Delta\Delta$Ct method) can also be calculated.

In paper II and IV, we utilised liquid-chromatography mass spectrometry (LC-MS) to study changes at the proteomic level. LC-MS combines liquid-chromatography, which separates molecules based on their physical properties; with tandem mass

spectrometry, which provides structural information about the separated molecules. Following LC, the sample is injected into the MS. Inside the MS, molecules are ionized and fragmented into smaller ions. These ions are then analysed based on their mass-to-charge ratios, allowing for the identification and quantification of target molecules in the sample. In paper II, we investigated the proteomics of monocytes following SF activation; and in paper IV, the proteomics of S-Fib.

Cytokine concentrations within plasma and SF samples were measured in paper I and II. In paper I, a bead-based assay (Cytometric bead assay, CBA) was used, which allowed for multiplexing. In CBA, multiple bead populations are used, which vary in size and are labelled with fluorescent dyes. In addition, each bead population corresponds to a different analyte of interest. Samples are incubated with a mix of beads, allowing the cytokines to bind to their respective beads. The mixture is then analysed using flow cytometry. Each bead population is identified, with fluorescence intensity corresponding to the amount of analyte present in the sample, thus enabling the quantitative measurement of multiple analytes in parallel. In paper II, MesoScale (as described above) was used for the same purpose. These two methods offer similar advantages with multiplexing, but MesoScale was implemented here as it offers a more extensive dynamic range and could easily be performed in a 96-well plate (compared to the use of FACS tubes in the CBA method).

Ethical considerations

Working with children always poses ethical challenges and dilemmas. On one hand, it can be considered unethical to expose children to sample collection at all if there is no clinical purpose. On the other hand, it can be considered unethical not to perform research and increase our understanding of both the diseases affecting children and potentially the discovery of new therapeutics. Another dilemma on paediatric research is that a young child might not be able to fully understand the concept of the research, or what it means to participate.

In these projects, samples were primarily collected as part of routine therapeutic joint aspirations. Hence, samples were collected in parallel with blood samples needed for clinical purposes, limiting additional discomfort to the patient. Additionally, any collected synovial fluid would have been discarded if not used for research purposes. Any biopsies collected were done using ultrasound guidance on patients under anaesthesia. Finally, informed written consent was obtained from patients and/or their legal guardians.

The present investigation

At the time of initiation for the projects presented in this thesis, there was limited knowledge on the role of monocytes and neutrophils in oJIA. Given the proposed role of these cells in adults, in addition to the fundamental roles these cells have in driving and regulating inflammation, the aims of this thesis was to investigate how monocytes and neutrophils may contribute to disease in oJIA. The specific aim for each paper was:

I. To describe the polarization pattern of synovial monocytes in oJIA, through surface- and intracellular expression analyses, comparing synovial monocytes to circulating monocytes.

II. To investigate possible functional alterations of synovial monocytes in oJIA; and to explore the mechanisms of how monocytes obtain these alterations by *in vitro* studies using healthy cells.

III. To study the function and phenotype of synovial neutrophils in oJIA, through comparing synovial- to circulating neutrophils using surface markers and functional assays.

IV. To investigate the ability of S-Fib from oJIA patients to induce activation in healthy monocytes and neutrophils, with or without prior activation with synovial fluid.

Paper I. Children with oligoarticular juvenile idiopathic arthritis have skewed synovial monocyte polarization pattern with functional impairment – a distinct inflammatory pattern for oligoarticular juvenile arthritis

Rationale

At the beginning of this study, there was remarkably little known about the synovial monocyte phenotype, and no study had investigated the polarization pattern of synovial monocytes in oJIA. Polarization highly influences the effector functions of monocytes, which have the ability to execute both inflammatory- and regulatory functions. For example, monocytes can produce cytokines that both promote and inhibit inflammatory responses. Moreover, in adults, it had been shown that monocytic polarization differed between arthritic diseases, promoting an interest in investigating the situation in oJIA patients. This polarization state could give an initial indication as to the role of the monocytes. Thus, the aim of this study was to thus map the polarization pattern of synovial monocytes in oJIA.

Method

In this paper, monocytes from paired blood- and SF samples from n=13 patients with oJIA were characterized by flow cytometry using several markers of polarization. In brief, cells were stained for polarization markers such as CD86, PDL1, CD206 and CD163, with monocytes being also negatively selected through staining for CD3, CD19 and CD56. In addition, the functional properties of synovial- vs circulating monocytes were further investigated through phagocytosis and ROS production using PhagoTest™ and PhagoBurst™ respectively, according to the manufacturer's instructions.

Monocytes were also isolated from SF and blood using an anti-CD14 biotin antibody and streptavidin conjugated dynabeads, followed by magnetic isolation. mRNA expression of 28 polarization related genes was subsequently analysed by qPCR. These markers were selected to complement the surface marker expression on monocytes. To investigate the impact of the surrounding environment (i.e., inflammatory SF), healthy monocytes were also isolated by magnetic beads (using the Miltenyi isolation technique, an alternative to dynabeads) and stimulated with SF from oJIA patients and subsequently analysed for polarization markers as described above. Any cytokine expression in plasma and cell-free SF was then investigated by CBA, targeting 9 different cytokines. Finally, biopsies from n=3 patients were also isolated. These were stained for polarization markers (CD163, CD206 and CD40) by immunofluorescence. Biopsies were also stained for CD163

through immunohistochemistry, and mRNA expression of IL-10 and TNF using *in situ* hybridization.

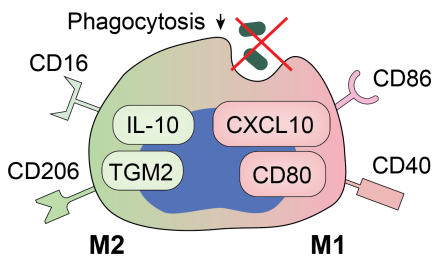
Results

Synovial monocytes were primarily an "intermediate"-like phenotype, being $CD14^{+}CD16^{+}$, compared to $CD14^{+}CD16^{-}$ circulating monocytes. Furthermore, synovial monocytes displayed a mixed polarization with both M1 (pro-inflammatory)- and M2 (regulatory) features, compared to circulating monocytes. This was evident both at the surface level with expression of markers such as CD40, CD86 and CD206, but also at the mRNA level, were cells further expressed IL-10, transglutaminase 2 (TGM2) and CD80. Moreover, synovial monocytes showed signs of functional alterations, as they had impaired phagocytosis and ROS production compared to circulating monocytes. Notably, synovial monocytes did not express increased levels of CD163.

SF induced expression of M2 markers (such as CD16 and CD206) but not M1 (such as CD40 and PDL1) markers in healthy monocytes. Furthermore, the SF contained several cytokines associated to M2 polarization, such as IL-10 and IL-6, but also some M1 related cytokines, such as IL-8 and IL-1β. Finally, monocytes/macrophages in the tissue expressed both M1 (CD40 and TNF) and M2 markers (CD206 and IL-10), both at the surface- and mRNA level.

Conclusion

Patients with oJIA have a distinct polarization pattern of synovial monocytes with mixed M1- and M2 features. In addition, the examined monocytes displayed functional impairment, highlighted by a reduced ability to phagocytose and produce ROS. SF may be responsible for the M2 features, whilst monocytes may obtain their M1 features within the tissue. This paper therefore highlights the activation and functional alterations in synovial monocytes from patients with oJIA.



Paper II. Synovial monocytes contribute to chronic inflammation in childhood-onset arthritis via IL-6/STAT signaling and cell-cell interactions

Rationale

In adult arthritic diseases, it is believed that synovial monocytes and MØs contribute to inflammatory processes through interactions with other cells and the production of inflammatory cytokines. However, it is becoming increasingly acknowledged that regulatory monocytes can contribute to, and sustain, chronic inflammation. Understanding the role of monocytes and their impact on arthritis pathogenesis is crucial for developing potential treatment strategies. From paper I, we know that synovial monocytes in oJIA are activated, and display some signs of functional alterations. We also have some indications that the inflamed SF and interactions in the tissue might be important for development of the synovial monocyte phenotype. Still, we did not explore this further, and little is known from the literature of how the synovial monocytes obtain their activated phenotype, the mechanisms involved and their contribution to disease. Thus, the aim of this paper was to characterize how monocytes are functionally affected in oJIA, primarily through how cells obtain their specific features and the underlying associated mechanisms.

Method

In total, n=33 oJIA patients were included in this study. The function and phenotype of synovial monocytes were analysed using flow cytometry. At the surface level, monocytes were stained with several markers of polarization such as CD16, MerTK, CD86 and HLA-DR. Functionally, synovial monocytes were compared to circulating monocytes using several different functional assays. Throughout, monocytes were assessed for their ability to: induce proliferation and surface markers in healthy T-cells, take up apoptotic neutrophils through efferocytosis, being primed to specific signalling pathways using defined cytokines and produce intracellular cytokines following LPS activation.

Influence of SF (compared to serum) was studied using healthy monocytes and the assays specified above. Furthermore, to investigate changes at the proteomic level, monocyte proteomics in SF- vs serum polarized monocytes were analysed by LC-MS. Signalling pathways induced by SF were studied using a broad-spectrum phosphorylation assay, targeting 37 kinases, as well as flow cytometry and specific inhibitors (tocilizumab, an IL-6 inhibitor, and tofacitinib, a JAK/STAT inhibitor). Additionally, six cytokines were measured in plasma and SF samples using

MesoScale. Finally, any further effects on monocytes were studied in co-culture systems with healthy fibroblast-like synoviocytes (FLS).

Results

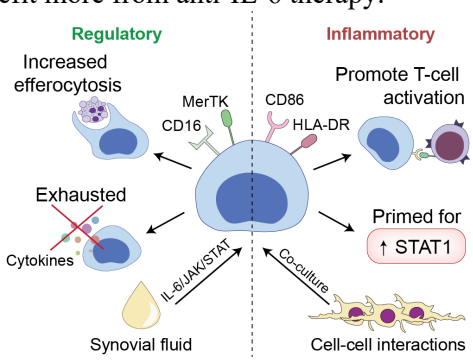
We observed that synovial monocytes from oJIA patients exhibited altered functions that have implications in both inflammatory- and regulatory processes. In line with Paper I, synovial monocytes, compared to circulating monocytes, had a mixed inflammatory and regulatory phenotype, expressing markers such as MerTK, CD16, CD86 and HLA-DR. Accordingly, synovial monocytes displayed an increased ability to induce T cell activation (measured as proliferation and expression of surface markers). In addition, monocytes were primed for STAT1 phosphorylation upon activation. Monocytes also had an increased efferocytosis and showed resistance to cytokine production (IL-1β, IL-6, IL-8, and TNF) following activation.

Regulatory effects, such as an increased efferocytosis and resistance to cytokine production, were replicated by polarizing healthy monocytes using SF (compared to serum). SF primarily induced the phosphorylation of STAT3, and inhibition assays revealed that this activation was mainly driven by an IL-6/JAK/STAT mechanism. Interestingly, the magnitude of IL-6/JAK/STAT activation was also reflected in circulating markers of inflammation, such as IFNα2a and IL-6.

Finally, we found that inflammatory aspects, such as the expression of antigen presentation markers and the increased ability to induce T-cell activation, were induced by interactions with FLS in co-cultures.

Conclusion

Synovial monocytes contribute to chronic inflammation in oJIA. These cells acquire their functional alterations through inflammatory SF *via* IL-6/STAT signalling, and through interactions with FLS. Finally, our data suggest a proportion of patients that could potentially benefit more from anti-IL-6 therapy.



Paper III. Synovial fluid neutrophils in oligoarticular juvenile idiopathic arthritis have an altered phenotype and impaired effector functions

Rationale

Neutrophils are the most prevalent immune cell in inflammatory SF. Still, at the beginning of this study, there were no more than a handful studies looking into these cells in oJIA. Neutrophils could contribute to the pathogenesis of arthritis in multiple ways. For example, an impaired clearance of debris and immune complexes could amplify autoimmune reactions through prolonged exposure of autoantigens. In addition, an imbalance in ROS production could impact the inflammatory environment. Finally, an extended survival of neutrophils could prolong their effector functions, contributing to a sustained inflammatory environment.

Given these points, it is surprising that the phenotype and function of the synovial neutrophils had not been characterized. Thus, the aim of this study was to characterize the phenotype and functional alterations of synovial neutrophils in oJIA.

Method

Paired blood- and SF samples from n=17 patients with oJIA were used to examine neutrophil phenotype and function. Additionally, neutrophils within blood from six of these patients were also used to study neutrophils during inactive disease.

The neutrophil phenotype was investigated using flow cytometry and surface markers related to activation (such as CD66b and CD11b), maturation (such as CD10) and migration (such as CD62L). The functional properties of neutrophils were investigated by examining their phagocytosis ability and ROS production through flow cytometry using PhagoTest™ and PhagoBurst™, respectively.

To investigate the effect of the inflammatory environment (SF), neutrophils were isolated from healthy donors using density centrifugation and dextran sedimentation, followed by stimulation using SF. Additionally, the effect of transmigration on the neutrophil phenotype was studied by comparing blood neutrophils to neutrophils isolated from the oral cavity from healthy donors. Finally, neutrophils in synovial biopsies (n=3) from oJIA patients were stained using immunofluorescence.

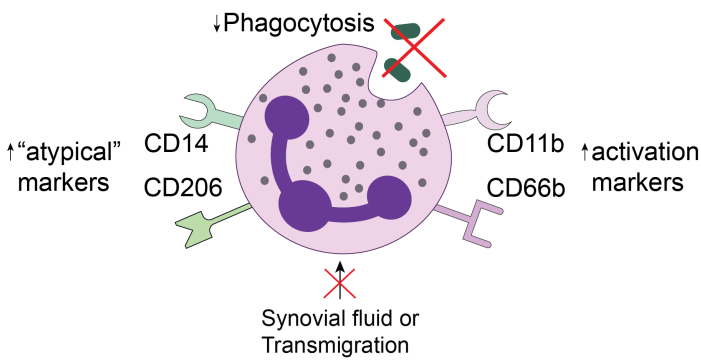
Results

There was a clear difference in the SF neutrophil phenotype compared to paired circulating neutrophils. For example, SF neutrophils expressed higher levels of CD66b, CD11b, CD10, indicative of an activated phenotype. SF neutrophils also expressed higher levels of atypical markers, specifically CD14 and CD206, which is more commonly found on monocyte-lineage cells. Functionally, SF neutrophils had an impaired phagocytosis and a trend of reduced ROS production compared to circulating neutrophils. Interestingly, the amount of $CD206^+$ neutrophils correlated with the impaired phagocytosis and ROS production. $CD206^+$ neutrophils could be identified in synovial biopsies, suggesting that they might acquire this marker in the tissue.

Only minor differences were noted between the neutrophil phenotype during active and inactive disease, suggesting a pronounced role of local- compared to systemic inflammation in driving the neutrophil phenotype. Additionally, the SF neutrophil phenotype could not be replicated *in vitro* by stimulating healthy neutrophils with inflammatory SF. Finally, the phenotype of oral cavity neutrophils was clearly distinct from that of SF neutrophils.

Conclusion

Here, we showed that synovial neutrophils have an activated phenotype and impaired effector functions. Importantly, synovial neutrophils did not obtain this phenotype through the inflammatory environment (SF) or transmigration alone. This suggests that cell-cell interactions and/or complex interactions within the synovial tissue are responsible for driving the neutrophil phenotype. Interestingly, the impairment of effector functions was associated with a monocyte-like phenotype. Thus, we speculate that these neutrophil alterations are important in the pathogenesis of oJIA.



Paper IV. Synovial fluid potentiates local fibroblasts to drive inflammatory monocytes in childhood-onset arthritis

Rationale

Synovial fibroblasts (S-Fib) have a crucial role in maintaining local homeostasis. However, it is becoming increasingly established that S-Fib are important drivers of synovial inflammation. Studies in adults with arthritis suggest that S-Fib are heterogenous, being both destructive and inflammatory, with them also acquiring a more potent inflammatory phenotype following previous priming. Studies in oJIA are scarcer but suggest that fibroblasts display an activated, but less destructive, phenotype and produce pro-inflammatory cytokines. One study has shown that following previous activation with cytokines, S-Fib mediate leukocyte adhesion. However, no study has thus far investigated if S-Fib from oJIA patients drive activation in monocytes and neutrophils, nor has any study investigated the impact of priming S-Fib with inflammatory SF, mimicking a disease relapse. As aforementioned, our previous studies suggest that cell-cell contact could be important in driving activation in these cells. Thus, we set out to investigate the contribution of S-Fib from oJIA patients, with or without prior SF-priming, in driving activation in healthy monocytes and neutrophils.

Method

S-Fib were isolated from the SF of patients with oJIA through passaging cells. Following isolation, S-Fib cells were either primed with 20% SF or unprimed. The effect of SF-priming on S-Fib was investigated through production measurements of IL-6 and IL-8 using MesoScale. In addition, the ability of S-Fib supernatants to induce monocyte- and neutrophil migration was studied in transwell systems. Finally, proteomic differences following priming were studied using LC-MS.

The effect of S-Fib on monocytes and neutrophils was studied in co-cultures using healthy monocytes or neutrophils. Co-cultures were both assessed for changes in phenotype and viability using surface markers and flow cytometry. Functional alterations in monocytes were studied by investigating their ability to induce T-cell activation (measured as proliferation, surface marker expression and intracellular cytokine production) following co-culture with S-Fib. Furthermore, monocytes were also analysed for cytokine production (IL-1β, IL-6, IL-8 and TNF), which were measured in supernatants using MesoScale. Neutrophils were assessed for MPO release through analysis of supernatants using ELISA. Supernatants were also analysed for elastase activity. Finally, ROS production was measured using

H_2DCFDA staining following PMA activation. To investigate the contribution of soluble factors, cells were stimulated with supernatants from S-Fib cultures. Additionally, the degree of adhesion by monocytes to S-Fib was studied in co-cultures placed on an orbital shaker.

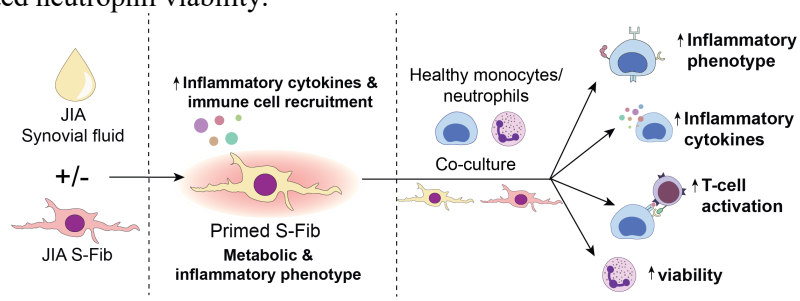
Results

SF-priming induced the production of IL-6 and IL-8 in S-Fib. Accordingly, supernatants from both S-Fib- and SF-primed S-Fib induced migration in monocytes and neutrophils, which was more pronounced in SF-primed S-Fib supernatants. Priming also resulted in a metabolic- and inflammatory shift at the protein level.

S-Fib with or without SF-priming were co-cultured either with healthy monocytes or neutrophils. For monocytes, a co-culture with S-Fib induced an inflammatory phenotype and an increased ability to induce T-cell activation. Additionally, there was a major increase in pro-inflammatory cytokines in these co-cultures. Interestingly, these effects were more pronounced following SF-priming of the S-Fib. As the influence of S-Fib on monocytes was not due to soluble factors, and monocytes had increased adhesion to SF-primed S-Fib, suggests a role of cell-cell contact between S-Fib and monocytes. Furthermore, there were only minor effects of a S-Fib co-culture observed on neutrophil phenotype and function. However, neutrophils co-cultured with SF-primed S-Fib in contrast were more viable.

Conclusion

Our data highlight the crucial role of S-Fib in driving synovial inflammation through the induction of inflammatory monocytes, which was further enhanced through prior priming of S-Fib with SF, emphasising the role of SF in driving S-Fib activation. However, these effects were not due to soluble factors alone, supporting the need for therapies targeting cell-cell interactions in the treatment of arthritis. Finally, S-Fib had only minor effects on neutrophils, suggesting that other mechanisms drive neutrophil activation in the joint, although SF-primed S-Fib promoted neutrophil viability.



Discussion

Monocytes and neutrophils both have crucial roles as effector cells in the innate immune system, serving not only as pathogen eliminators but also as vital regulators of inflammation and homeostasis maintenance. Still, their role in the inflamed joint of patients with oJIA has long been neglected. In the four studies of this thesis, we have: 1) characterized the phenotype of these cells within the joint, 2) highlighted functional alterations that could be crucial in driving and sustaining chronic inflammation, and 3) emphasised mechanisms and pathways that drive their activation. Consequently, these studies have laid a foundation for further research into how monocytes and neutrophils can be manipulated for therapeutic purposes.

For monocytes and MØs, the research focus for almost two decades has centred around the concept of polarization. The initial concept was to try and categorize the monocytes and MØs into pro-inflammatory (M1) or anti-inflammatory (M2), as this was believed to reflect their function and their role in disease (mirroring the Th1 and Th2 T-cell categorization). However, the dynamic nature of these cells quickly gained attention, revealing that a simple two-sided categorization does not adequately fit their plasticity. Thus, it is becoming clear that it is crucial to study the function of these cells in a disease context, rather than to solely characterize their polarization and make assumptions of their functional role. Indeed, we found throughout our studies that synovial monocytes of patients with oJIA have a mixed polarization pattern, expressing markers of both M1 (e.g., CD40 and CD86) and M2 (e.g., CD16 and MerTK). Interestingly, in paper I, we did not find an increased expression of CD163, a marker that has repeatedly been identified in patients with arthritis, such as in RA, SpA, ERA and sJIA (26, 270, 328, 329). Even though we did not make direct comparisons to other disease groups, we did however exclude two patients with ERA and sJIA, that initially were included due to an oligoarticular disease course, with us detecting increased CD163 expression in both these patients. Interestingly, this suggests that differences may exist between arthritic diseases in terms of monocyte activation. In support of this, MØs were found to respond differently to SF from RA compared to SpA patients (271). Still, we have found several similarities between MØs, such as an increased expression of CD40 and a high prevalence of an “intermediate-like” phenotype (256, 330). Thus unsurprisingly, some features of monocyte activation might be retained across diseases, whilst other features are more unique. Future studies directly comparing

monocyte activation between arthritic diseases is therefore warranted. This is especially true in regard to the function of these cells.

The main results of our studies related to monocyte function and their implications in the joint are summarised in **Figure 23**. Overall, we found that synovial monocytes are functionally distinct compared to circulating monocytes. Notably, their functional alterations coincided with a mixed polarization pattern, encompassing both pro-inflammatory and regulatory aspects. Firstly, we found that synovial monocytes promote both T-cell proliferation and activation. As T-cells have a well-established role as drivers of inflammation in both oJIA and arthritis in general, the activation of these cells by monocytes highlights the monocytes' capability to promote inflammation (331). Furthermore, our results are supported by other studies, both in JIA and in adults (256, 314). Secondly, we observed that synovial monocytes were primed for STAT1 phosphorylation. STAT1 is induced by cytokines such as IFNγ, and is considered to drive pro-inflammatory responses, as its induction results in, for example, the production of inflammatory cytokines (332). Therefore, a sensitivity to STAT1 signalling in synovial monocytes suggests that cells are more responsive to pro-inflammatory activation. Thirdly, we showed that synovial monocytes have an impaired phagocytosis and ROS production. The phagocytosis assay was performed using opsonized *E. coli*, suggesting an impaired Fc/complement- mediated clearance. A possible consequence of this in the joint could be a failed clearance of immune complexes (ICs). ICs have a well-established role in other diseases, with a failed clearance of these complexes resulting in a continuous inflammatory response, further exacerbating the progression of arthritis through, amongst others, the activation of immune cells (333-335).

On the other hand, synovial monocytes also displayed various functional alterations that can be associated with a regulatory role. Firstly, synovial monocytes had an increased uptake of apoptotic cells compared to circulating monocytes. Thus, synovial monocytes may have an impaired clearance of certain pathways (as discussed above), whilst remaining functional in others. Accordingly, the maintained efferocytosis may be a factor contributing to the limited presence of specific autoantibodies, in contrast to other autoimmune diseases such as systemic lupus erythematosus (336). Secondly, we showed that synovial monocytes produced less pro-inflammatory cytokines upon activation. Possible mechanisms to explain this phenomenon may include exhaustion due to previous activation, or differences in receptor expression of TLRs. Collectively, these findings suggest that monocytes may have a compensatory role, trying to regulate and inhibit any excess inflammation.

Still, a regulatory phenotype may not be beneficial in chronic inflammation. For example, in response to IC stimulation and TLR activation, regulatory MØs are potent producers of pro-inflammatory cytokines, which are prevalent in the inflamed joint (337). Additionally, a regulatory phenotype is associated with fibrosis and angiogenesis, two processes that can sustain inflammation (274, 275).

Indeed, the synovial tissue of JIA patients have prominent vascularization (273, 338). However, the contribution of synovial monocytes to these specific processes were not tested by us. Thus, further studies are needed to determine whether the regulatory phenotype of synovial monocytes is beneficial or detrimental in oJIA.

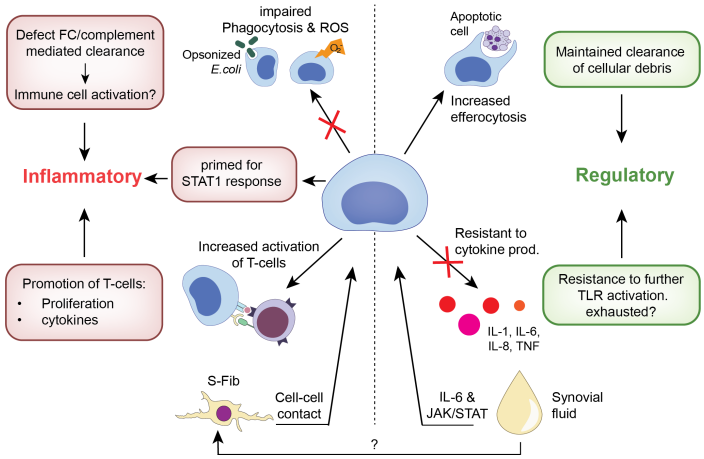


Figure 23. Summary of the main results regarding monocytes. Shows the main findings from our studies (paper I, II and IV) regarding synovial monocytes, and highlights the implications of these results. *TLR – Toll-like receptor, ROS – Reactive oxygen species, S-Fib – Synovial fibroblasts, TNF – Tumor necrosis factor, IL – Interleukin, JAK – Janus Kinase, STAT – Signal transducer and activator of transcription.*

In paper I and II, we found that the inflammatory SF, and primarily the IL-6/JAK/STAT pathway, are responsible for the regulatory features of the synovial monocyes. As with the concept and presence of regulatory monocytes, it seems contradictory that IL-6 induces a regulatory phenotype in monocytes. This is due to most in the field regarding IL-6 as a classic pro-inflammatory cytokine with multiple roles in driving inflammation. For example, it drives antibody production in B-cells and the differentiation of Th17 cells (339, 340). Still, in line with our results, others have also shown that the effect of IL-6 on monocytes and MØs seems to be regulatory (341, 342). For example, IL-6 has been shown to limit M1 responses (341). Accordingly, blocking of IL-6 signalling could have different implications depending on the cell type. Thus, in the setting of arthritis, with anti-IL-6 inhibitors approved (such as tocilizumab) and JAK inhibitors on the rising (such as tofacitinib), it is crucial to continue to explore the impact of these drugs on immunological mechanisms. Indeed, in paper II, we observed that patients could be separated into two groups based on their synovial IL-6 activity, and that patients with high activity also have increased levels of circulating markers of inflammation. Although exploratory and preliminary, these findings suggest a potential group more likely to benefit from anti-IL-6 therapy.

Interestingly, we found that S-Fib are potent inducers of inflammatory monocytes. In paper II and IV, we observed that monocytes co-cultured with S-Fib are more

prominent T-cell inducers and produce a higher degree of inflammatory cytokines. Furthermore, monocytes produced less ROS and had decreased phagocytosis following co-culture, reminiscent of the *in vivo* phenotype of patients' monocytes. Hence, it is suggestive that co-culture with S-Fib mimics most inflammatory aspects observed in the patients' synovial monocytes.

Notably, as observed in paper IV, prior activation of S-Fib with inflammatory SF potentiates the S-Fib to induce an even more noticeable monocyte activation. This activation could be due to several factors within the inflammatory SF, such as IL-1β and TNF (343). Other potential candidates include TLR ligands, which are known to induce activation in S-Fib (223). Furthermore, even though soluble factors in the SF induce activation of S-Fib, our results indicate that direct cell contact is necessary for the induction of inflammatory monocytes, as supernatants from S-Fib do not induce the observed monocyte phenotype. Additionally, we found in paper I that monocytes/ MØs in the synovial tissue express inflammatory markers, supporting that cells may acquire this activation pattern in the synovial tissue. Still, the precise mechanism of this activation remains undetermined. Previous studies however suggest that VCAM-1 expression by S-Fib could be a possible candidate, as it mediates leukocyte retainment (237). In support of this, we found increased adhesion of monocytes to SF-primed S-Fib in paper IV. Other candidates could include ICAM-1, which has also been shown to be important in leukocyte adhesion to S-Fib (344). Therefore, determining the factors responsible for the induction of inflammatory monocytes by S-Fib represent a promising strategy to identify new ways to target inflammation in arthritis.

Given that neutrophils are abundant in both the circulation and inflammatory SF, surprisingly few studies have investigated these cells in JIA. The main results from our studies regarding neutrophils are summarised in **Figure 24**. At the initiation of paper III, there was limited information on even the most basic concepts, such as the neutrophil phenotype. Thus, in this paper, we looked into the activation state of synovial neutrophils, and found that they displayed markers of activation, such as CD66b and CD11b. This has also been confirmed by a separate study, which found that, excluding activation markers, synovial neutrophils have a hyper segmented nucleus (325). Hence, but maybe not surprisingly, these results suggest that synovial neutrophils have an activated phenotype compared to their circulating counterparts and imply that cells may have undergone degranulation. There are several ways that neutrophil degranulation can drive pathogenesis in arthritis. For example, neutrophilic granules containing elastase and collagenases can cleave various proteins upon release, such as collagens and elastin, in a process which is believed to contribute to cartilage damage in RA (345, 346). In animal models, mice with impaired activation of proteases are protected from arthritis, accompanied by a diminished local production of TNF and IL-1β (347). Notably, SF from RA contain more neutrophil derived proteins compared to SF from SpA patients, suggesting a difference in the role of neutrophils between arthritis types (296). Finally,

degranulation results in the release of cytokines and chemokines that promote activation and attraction of immune cells. In short, neutrophils in oJIA display signs of activation, and thus further studies determining the degree of activation (e.g., degranulation) compared to other diseases are warranted.

Remarkably, we also found that neutrophils expressed markers more often related to monocytes, such as CD14 and CD206. Accordingly, *Metzemaekers* et. al. also found increased expression of HLA-DR on synovial neutrophils (325). Similar expression patterns have additionally been emphasised in RA, were synovial neutrophils show antigen presentation capabilities (348). Furthermore, this observation of a close relationship between neutrophils and monocytes has been observed before, with the suggestion that neutrophils can "transdifferentiate" during inflammatory conditions *in vitro*, acquiring monocyte-like properties (349-351). Moreover, these both these cells also share a common myeloid ancestor, emphasising their similarities. Hence, even though neutrophils are considered terminally differentiated, they are surprisingly plastic and may take up atypical functions, such as the APC capacity of monocyte lineage. This is important as it highlights the neutrophil's capacity to promote activation of the adaptive immunity during inflammation.

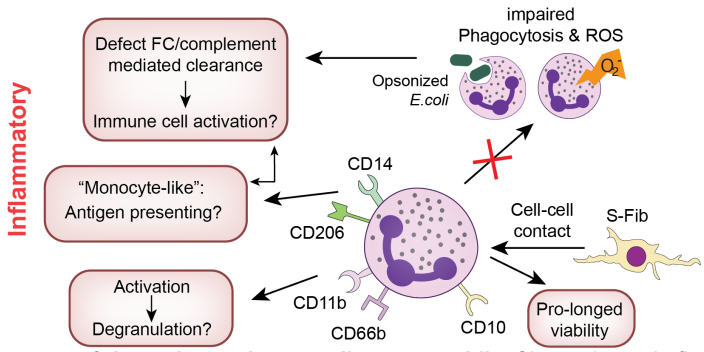


Figure 24. Summary of the main results regarding neutrophils. Shows the main findings from our studies (paper III and IV) into synovial neutrophils, and highlights implications of these results. *ROS – Reactive oxygen species, S-Fib – Synovial fibroblasts.*

Furthermore, we found that synovial neutrophils have an aged-like phenotype. This is supported by studies in RA, which show that neutrophils have a prolonged survival (352). This prolonged survival is speculated to contribute to inflammation, as the neutrophils have an extended time to execute their effector functions. Interestingly, we found in paper IV that co-culture between healthy neutrophils and SF-primed S-Fib prolonged neutrophil survival. Consequently, this is potentially an additional mechanism to how S-Fib can drive synovial inflammation.

Functionally, the synovial neutrophils had an impaired phagocytosis response and a trend towards an impaired ROS production. As with monocytes, an impaired phagocytosis in neutrophils can further autoimmunity through the failure to clear

debris, resulting in an increased autoantigen burden and generation of ICs. ICs, in turn, can drive the activation of neutrophils via Fc receptors, resulting in degranulation (353, 354). Notably, this is not due to the environment alone, as the addition of inflammatory SF to healthy neutrophils did not result in the impairment of phagocytosis. Early studies into RA also support the notion of a reduced phagocytosis by synovial neutrophils (355). Even though excess ROS has been linked to several processes in autoimmunity, such as DNA damage and oxidative stress, an impaired ROS production can potentially also contribute to disease. For example, a defect in a component of the NADPH oxidase resulted in a less oxidative burst and the promotion of arthritogenic T-cells, leading to more severe arthritis in an animal model (356). However, this might be primarily due to MØs and not neutrophils. Regardless, we have recently reported that synovial neutrophils fail to supress T-cell proliferation, a phenomenon, at least partly, dependent on reduced ROS production (357). However, the results of an impaired ROS production are in contrast to adult diseases, where an increase in ROS production has been observed, and theorised to drive inflammation by contributing to DNA damage and inflammatory activation (258). Therefore, although our results need to be confirmed by other researchers, it suggests that functional differences between oJIA and RA may exist in terms of neutrophils.

Interestingly, these functional discrepancies of an impaired phagocytosis and ROS production were linked to the presence of CD206-expressing neutrophils. As monocytes in general are less phagocytic than neutrophils, it could provide further support that synovial neutrophils have acquired a monocyte-like phenotype. Another explanation for the decreased phagocytosis and ROS production could be exhaustion, as the neutrophils have likely already undergone these processes, highlighted by the high expression of activation markers. Indeed, a state of exhaustion is not uncommon in neutrophils following intense activation, such as in sepsis (358).

Nevertheless, the mechanisms of how synovial neutrophils acquire their phenotype and functional properties are still unknown. In paper I, we observed that stimulation of healthy neutrophils with inflammatory SF did not induce the same phenotype observed in patients. Furthermore, we analysed oral cavity neutrophils as a model for neutrophil migration to a different site other than the joint. Again, these neutrophils were not similar to that of synovial neutrophils. In paper IV, we co-cultured healthy neutrophils with S-Fib from oJIA patients, and we did not see an induction of the phenotype observed in synovial neutrophils. Thus, the process in which neutrophils acquire their phenotype remains unknown. Possible factors not investigated here include hypoxia, time, and immune cell-cell interactions. For example, hypoxia has been shown to inhibit apoptosis in neutrophils (359). In addition, time and ageing have been suggested to be key players in driving the synovial neutrophil phenotype (278). Finally, in a separate study, we found that the *in vitro* migration of healthy neutrophils, in a transwell system lined with both

endothelial cells and S-Fib, resulted in a phenotype with reduced ROS and a reduced ability to promote T-cell proliferation, reminiscent of the oJIA patients' neutrophils (357). However, the migration did not alter surface marker expression of the neutrophils, thus still suggesting that other mechanisms are important for the synovial neutrophil phenotype. In short, the mechanisms driving the neutrophil phenotype in oJIA are still unknown.

Monocytes and neutrophils may interact in several ways to promote synovial inflammation. For example, we showed that synovial monocytes promote T-cell proliferation, and in a separate study, that synovial neutrophils fail to suppress T-cell proliferation (357). Furthermore, the impaired phagocytosis and ROS production in both cell types indicate a substantial decrease in Fc-mediated clearance in the joint, which could have implications as discussed above. Finally, the results from paper IV, displaying that co-culture with S-Fib drive a strong inflammatory activation in monocytes and not neutrophils, are interesting. Indeed, the only effect on neutrophils was a prolonged viability. Thus, the mechanisms of how these cells become activated in the joint are probably distinct.

On the basis of this thesis, we propose a hypothesis of the pathogenesis of oJIA with a focus on innate immunity (summarised in **Figure 25**). A genetic predisposition and an unknown trigger (likely environmental factors, or an elevated state of reactivity) drives activation of S-Fib. In support of this theory, S-Fib can be activated independent of the immune system, e.g., by TLR ligands, they are also capable of driving arthritis in animal models, and they are speculated to be involved in flares (231, 360, 361). These studies highlight the independence of S-Fib on the immune system for activation. Their enhanced capacity to produce cytokines and chemokines, in combination with our results from paper IV, signify their potential in the recruitment of immune cells. As the immune cells enter the joint, they are exposed to a progressively inflammatory environment and undergo activation. Neutrophils, partly due to migration and partly due to unknown factors, become functionally impaired, evidenced by a decreased phagocytosis and ROS production, resulting in remanent debris, such as ICs, and thus DAMP exposure. Monocytes interact with S-Fib through unknown cell-cell mechanisms and acquire an inflammatory phenotype. In turn, they become activated to support the activation of autoreactive T- and B-cells, that in turn form tertiary lymphoid organs (TLOs). Synovial neutrophils also lose their T-cell inhibitory capacity, further favouring the proliferation of lymphocytes. Subsequently, B-cells differentiate into plasma cells and produce ANAs. These cells potentially form ICs that drive immune cell activation, which is not properly cleared due to the impaired phagocytosis. This chain of events and activation drive more cytokines, activation, and cell-cell crosstalk, resulting in a sustained inflammation, as suppressors of inflammation (such as Tregs) are impaired (196). Hence, a vicious circle of inflammation, autoreactivity and a failure to supress either drives the chronicity and arthritis associated with oJIA.

Clearly, there are flaws in this hypothesis, particularly the order of events, and substantial more research is needed to further clarify the pathogenesis. Other possible mechanisms involve a direct targeting of the synovial autoantigen by the lymphocytes represents another mechanism (186). However, this represents a more classic view of the pathogenesis. Furthermore, the activation of autoreactive lymphocytes at sites other than the joint that then subsequently triggers synovial inflammation represents another mechanism. Activation of autoreactive lymphocytes could be due to either cross reactivity or activation elsewhere, e.g., following exposure to an infection or changes in gut environment (362-365). Additionally, our theory is based only on *in vitro* and *ex vivo* data, and thus, the situation in the joint may be completely different. Still, regardless of the initial trigger and responsible mechanism, it is clear that once initiated, an imbalance in the ability to control inflammation from both an innate- and adaptive point of view, drives chronicity. Hence, to not only block inflammation, but to restore homeostasis, represents an attractive approach going forward for the treatment, and potential cure, of oJIA.

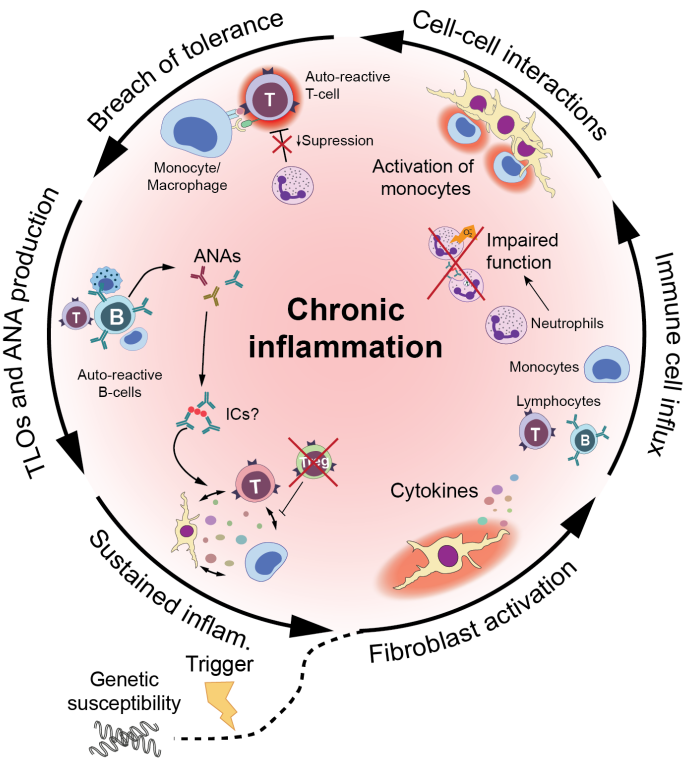


Figure 25. Proposed role of monocytes and neutrophils in the pathogenesis of oJIA. Displays an overview of the innate-immunity centered theory of the pathogenesis of oJIA based on the four studies of this thesis. *ICs – Immune complexes , ANA – Antinuclear antibodies, TLOs – Tertiary lymphoid organs.*

Conclusion

In the studies of this thesis into the role of myeloid cells in oJIA, we found that: 1) synovial monocytes show a mixed polarization pattern with both pro- and regulatory features, 2) synovial monocytes are functionally affected and acquire their phenotype through synovial fluid and cell-cell interactions, 3) synovial neutrophils display an activated phenotype and functional abnormalities and 4) synovial fluid from oJIA patients induces activation of synovial fibroblasts which, in turn, drives inflammatory monocytes. These results highlight an underappreciated role of myeloid cells in the pathogenesis of oJIA and propose several new mechanisms for interfering with their activation.

Future perspectives

As discussed above, there are several important aspects identified during the studies of this thesis that should be examined further. Here, I will highlight a few key points:

- Most studies, as well as the ones in this thesis, do not use disease controls, but focus on a single disease. Consequently, potential mechanisms that these diseases share, or mechanisms that are distinct, are easily overlooked. As a result, is difficult to compare processes across disease states. Thus, it would be beneficial to, in future studies, compare phenotypes, functions and mechanisms across various disease types to better characterize the pathogenesis and potential drug targets of a given disease.
- The precise mechanism of what drives the activation of monocytes in co-cultures with S-Fib should be determined. Identifying potential cell-cell contact molecules could provide interesting new treatment options to block the inflammatory activation of synovial monocytes.
- Determining what drives the neutrophil phenotype within oJIA would be important to further characterise, as it could represent a new target for intervention. In addition, the functional properties of neutrophils, specifically degranulation and NETosis, should be explored, as well as differences to other arthritic diseases.
- The notion made in paper II, that oJIA patients can be separated into two groups based on synovial IL-6/STAT signalling, is interesting and should be further explored. The use of circulating cytokines to categorize JIA patients has been previously attempted with mixed results. Still, no one has focused on oJIA in particular, a disease that is generally not linked to systemic inflammation. Thus, whether oJIA contains a subgroup of patients with a "high" inflammatory pattern, and if this is related to a positive response to therapies such as IL-6 inhibitors, should be explored.
- Regulatory properties of the synovial monocytes should also be further determined, to specify if they are beneficial or detrimental. Specifically, regarding their role in chronic inflammation (i.e., do they contribute to fibrosis and angiogenesis) or as inhibitors of inflammation (e.g., production of anti-inflammatory cytokines and inhibitory interaction with other cell types), should be investigated.

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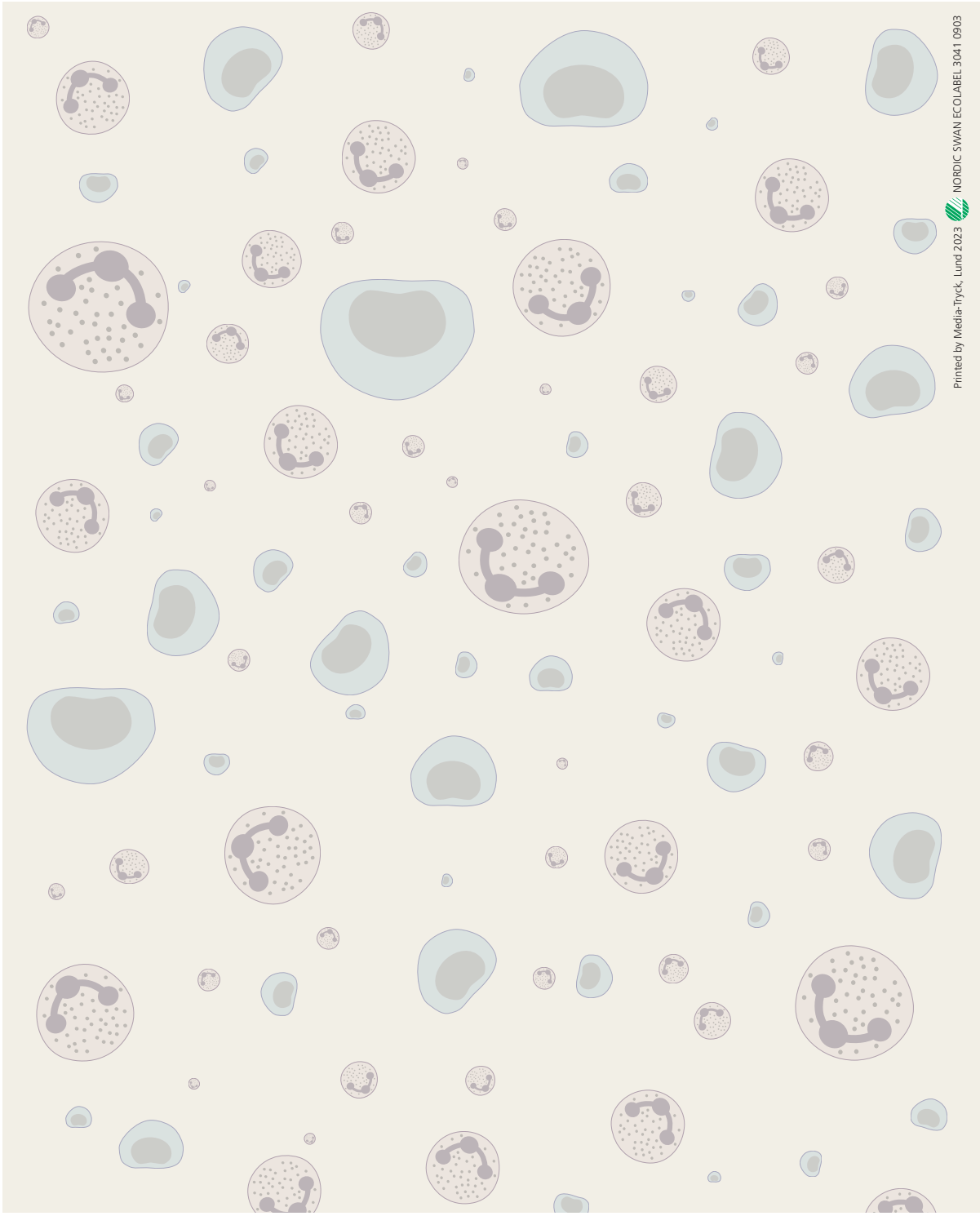

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