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SABINE ARVE-BUTLER has a master's degree in biomedicine, obtained at Lund University (bachelor) and Uppsala University (master), Sweden. Her doctoral research has focused on understanding the underlying immunology of autoimmune rheumatic diseases, and her thesis investigates the role of neutrophils and autoantibodies in the two diseases juvenile idiopathic arthritis (JIA) and systemic lupus erythematosus (SLE).

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Abstract

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This thesis aimed to increase the knowledge of the immunological mechanisms underlying autoimmune rheumatic disease, by investigating neutrophil function and autoantibody profiles. The diseases studied in this thesis are juvenile idiopathic arthritis (JIA), an inflammatory arthritis affecting children, and systemic lupus erythematosus (SLE), a rheumatic multi-organ disease primarily affecting women.

Neutrophils are the most common immune cells of the body, primarily known as pro-inflammatory cells in the first line of defense against infections. Less known, but no less important, is that neutrophils also have anti-inflammatory and regulatory functions. The balance between pro- and anti-inflammatory neutrophil functions needs to be fine-tuned to maintain a healthy immune system. In this thesis I studied how neutrophil function is affected by the local environment in the blood or inflamed joint, and by a genetic variant mediating low production of reactive oxygen species.

Autoantibodies are antibodies targeting molecules and structures present in the own body, hallmarks of autoimmune disease. The autoantibody repertoire is often associated with the disease progression, and autoantibody-testing can therefore be useful in clinical practice, for instance to help clinicians make the correct diagnosis or predict disease outcomes. In this thesis I studied autoantibody profiles in relation to neutrophil function and clinical outcomes.

In the works of this thesis, we discovered that neutrophils in inflamed joints of children with JIA are altered in ways which skews the immunological balance towards a state that facilitates continued inflammation. These neutrophil alterations are therefore likely to propel local inflammation and arthritis in JIA. We also found that a genetically inherited impairment in production of reactive oxygen species by neutrophils was associated with an increased likelihood to have certain autoantibodies and a form of cardiovascular co-morbidity in SLE. Finally, we investigated proteins targeted by autoantibodies in JIA and identified several novel autoantibody-targets, some of which were associated with JIA-related eye disease.

Taken together, the results of this thesis identified immunological alterations in both neutrophil function and autoantibody profiles of patients with JIA and SLE. The results highlight the importance of neutrophils as immunological brakes, necessary to prevent autoimmune reactions, and how autoantibody profiles can be strongly connected to different disease outcomes.

Key words

Neutrophils, autoantibodies, autoimmunity, juvenile idiopathic arthritis, systemic lupus erythematosus

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To understand the world at all,
sometimes you could only focus on a tiny bit of it,
look very hard at what was close at hand
and make it stand in for the whole

Donna Tartt, *The Goldfinch*

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Populärvetenskaplig sammanfattning

Immunförsvaret kan vara förrädiskt. Vid autoimmun reumatisk sjukdom har det skiftat från att vara skyddande till att vara skadligt, med en oprovocerad och helt missriktad attack mot kroppens egna celler och vävnader. Idag kan vi inte bota autoimmuna reumatiska sjukdomar, men symptom kan lindras med läkemedel som påverkar olika aspekter av den felaktiga immunresponsen. En detaljerad kunskap om hur immunförsvaret fungerar, både vid sjukdom och hälsa, är därför viktigt om vi ska ha en chans att i framtiden kunna utveckla bättre behandlingsalternativ för autoimmuna reumatiska sjukdomar.

Denna avhandling syftar till att öka kunskapen om de underliggande immunologiska mekanismerna bakom autoimmun reumatisk sjukdom, med fokus på neutrofiler och autoantikroppar. Sjukdomarna som studerats i avhandlingen är juvenil idiopatisk artrit (JIA), en inflammatorisk artritsjukdom som drabbar barn, och systemisk lupus erythematosus (SLE), en multi-organsjukdom som primärt drabbar kvinnor.

Neutrofiler är kroppens vanligaste immunceller, främst kända för att vara pro-inflammatoriska celler i det akuta försvaret mot infektioner. Mindre känt, men definitivt inte mindre viktigt, är att neutrofiler också är anti-inflammatoriska och regulatoriska. Balansen mellan de pro- och anti-inflammatoriska funktionerna måste vara precis rätt för att bibehålla ett friskt immunförsvar. I min avhandling har jag studerat hur neutrofilers funktion påverkas av miljön de befinner sig i, i blodet eller den inflammerade leden, och av en genetisk variant som minskar deras förmåga att producera reaktiva syreradikaler.

Autoantikroppar är antikroppar som binder till molekyler och strukturer som finns i den egna kroppen. Att ha autoantikroppar är karaktäristiskt för autoimmun sjukdom. Unika kombinationer av autoantikroppar är ofta associerade med hur sjukdomen fortgår, och kan därför fungera som kliniska markörer och användas för att underlätta att sätta rätt diagnos eller förutspå hur sjukdomen kommer utvecklas. I min avhandling har jag studerat hur autoantikroppsprofiler är relaterade både till neutrofilfunktion och sjukdomsbilden i stort.

I avhandlingsprojekten såg vi att neutrofiler i inflammerade leder hos barn med JIA var förändrade på ett sätt som skiftar immunbalansen och skapar gynnsamma förutsättningar för fortsatt inflammation. De här förändringarna i neutrofilfunktion bidrar sannolikt till att driva lokal inflammation och artrit i JIA. Vi såg också att en genetiskt försämrad förmåga hos neutrofiler att bilda reaktiva syreradikaler var

associerat med en ökad sannolikhet att ha vissa autoantikroppar och ökad risk för en typ av kardiovaskulär samsjuklighet vid SLE. Slutligen studerade vi autoantigen, proteiner som binds av autoantikroppar och identifierade flera nya autoantigener i JIA, av vilka några var associerade med JIA-relaterad ögonsjukdom.

Sammantaget har avhandlingsprojekten identifierat flera olika immunologiska förändringar, gällande både neutrofiler och autoantikroppar, hos patienter med JIA och SLE. Resultaten visar på vikten av korrekt fungerande neutrofiler som kan agera som en immunologisk broms och förhindra autoimmuna reaktioner, och hur vissa autoantikroppar är starkt sammanlänkade med olika sjukdomsutfall.

Popular scientific summary

The immune system is not only defending us. In autoimmune rheumatic disease, it has shifted from protecting against to causing disease, and drives an unprovoked and misguided attack against our own cells and tissues. Today, autoimmune rheumatic diseases are incurable, but symptoms can be managed with therapies that regulate different aspects of the misdirected immune response. Therefore, detailed knowledge about the immunological alterations in autoimmune rheumatic disease is essential for understanding of how these conditions can be treated.

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Aims

The studies presented in this thesis aim to increase the understanding of the underlying immunological alterations of autoimmune rheumatic disease, by describing the role of neutrophils and autoantibodies in juvenile idiopathic arthritis (JIA) and systemic lupus erythematosus (SLE).

The specific aims, addressed in the four studies of the present investigation, are:

I. To characterize the phenotype and function of neutrophils in JIA, by analysis of neutrophils at the site of inflammation in the arthritic joints in comparison to neutrophils in the circulation.

II. To investigate the capacity of, and mechanisms for, neutrophil-mediated suppression of T cells, and how this is affected by neutrophil migration and the local environment in inflamed joints in JIA.

III. To investigate how the NCF1-339 polymorphism, that mediates impaired function of the NADPH oxidase II, affects neutrophil function, autoantibody profile, and clinical features in SLE.

IV. To identify JIA autoantigens, evaluate their potential to distinguish between JIA patients and paediatric controls, and explore associations between autoantibodies and JIA-related uveitis.

List of publications and manuscripts

I. **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.

II. **Arve-Butler S,** Mossberg A, Schmidt T, Welinder C, Yan H, Berthold E, Król P & Kahn R. Neutrophils lose the capacity to suppress T cell proliferation upon migration to inflamed joints in juvenile idiopathic arthritis. *Manuscript. Under revision in Frontiers in Immunology.*

III. Linge, P., **Arve, S**., Olsson, L. M., Leonard, D., Sjöwall, C., Frodlund, M., Gunnarsson, I., Svenungsson, E., Tydén, H., Jönsen, A., Kahn, R., Johansson, Å., Rönnblom, L., Holmdahl, R., & Bengtsson, A. (2020). NCF1-339 polymorphism is associated with altered formation of neutrophil extracellular traps, high serum interferon activity and antiphospholipid syndrome in systemic lupus erythematosus. *Annals of the rheumatic diseases*, *79*(2), 254–261.

IV. **Arve-Butler S,** Kahn F, Mossberg A, Berthold E, Król P & Kahn R. Identification of novel autoantigens in juvenile idiopathic arthritis. *Manuscript.*

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

- 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.
- Reid, S., Alexsson, A., Frodlund, M., Morris, D., Sandling, J. K., Bolin, K., Svenungsson, E., Jönsen, A., Bengtsson, C., Gunnarsson, I., Illescas Rodriguez, V., Bengtsson, A., **Arve, S**., Rantapää-Dahlqvist, S., Eloranta, M. L., Syvänen, A. C., Sjöwall, C., Vyse, T. J., Rönnblom, L., & Leonard, D. (2020). High genetic risk score is associated with early disease onset, damage accrual and decreased survival in systemic lupus erythematosus. *Annals of the rheumatic diseases*, *79*(3), 363–369.
- Wirestam, L., **Arve, S**., Linge, P., & Bengtsson, A. A. (2019). Neutrophils - Important Communicators in Systemic Lupus Erythematosus and Antiphospholipid Syndrome. *Frontiers in immunology*, *10*, 2734.

Abbreviations

ACPA – Anti-citrullinated protein antibody

ACR – American College of Rheumatology

ANA – Anti-nuclear antibodies

APC – Antigen presenting cell

APRIL – A proliferation-inducing ligand

APS – Antiphospholipid syndrome

ATP – Adenosine triphosphate

BAFF – B cell activating factor (also known as BLyS, B lymphocyte stimulator)

B2GP1 – β2-glycoprotein 1

CD – Cluster of differentiation

CGD – Chronic granulomatous disease

CL – Cardiolipin

CTL – Cytolytic T lymphocyte

CYBA – Cytochrome b558 subunit α

CYBB – Cytochrome b558 subunit β

DAMP – Damage associated molecular pattern

Fc receptor – Receptor recognizing the antibody Fc-region (fragment crystallizable)

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GTPase – Hydrolase converting guanosine triphosphate (GTP) to diphosphate (GDP)

HLA – Human leukocyte antigen (human version of MHC)

IC – Immune complex

IFN – Interferon

Ig – Immunoglobulin

IL- – Interleukin-

ILAR – International League of Associations for Rheumatology

JIA – Juvenile idiopathic arthritis

LC-MS – Liquid chromatography mass spectrometry

LE cell – Lupus erythematosus cell

LDG – Low density granulocyte

LDN – Low density neutrophil

MAVS – Mitochondrial antiviral-signaling protein

mtROS – Mitochondrial reactive oxygen species

NADPH – Nicotinamide adenine dinucleotide phosphate

NCF1 – Neutrophil cytosolic factor 1 (also known as p47phox)

NCF2 – Neutrophil cytosolic factor 2 (also known as p67phox)

NCF4 – Neutrophil cytosolic factor 4 (also known as p40phox)

NE – Neutrophil elastase

NETs – Neutrophil extracellular traps

NOX2 complex – NADPH oxidase II complex

MAPK – Mitogen activated protein kinase

MHC – Major histocompability complex

MPO – Myeloperoxidase

MØ – Macrophage

PAMP – Pathogen associated molecular pattern

PBMC – Peripheral blood mononuclear cells

-phox – Phagocyte oxidase protein

PKC – Protein kinase C

PMA – Phorbol-myristate-acetate

PRR – Pattern recognition receptor

RA – Rheumatoid arthritis

Rac - Ras-related C3 botulinum toxin substrate

RF – Rheumatoid factor

ROS – Reactive oxygen species

SLE – Systemic lupus erythemoatosus

SNP – Single nucleotide polymorphism

SSA – Sjögren's syndrome antigen A

SSB – Sjögren's syndrome antigen B

TLR – Toll like receptor

Th – Helper T cell

Treg – Regulatory T cell

Introduction to the field

Brief introduction to the immune system

The immune system is fascinating. It has the capacity to both protect from and cause disease, and there's a very fine line between. It is orchestrated by extensive cell communications, and all components of the immune response are connected in this intricate network of cell-cell interactions.

Neutrophils and autoantibodies represent two important aspects of the immunology behind autoimmune rheumatic diseases, from the innate and the adaptive branches of the immune system respectively.

The innate immune system

The innate immune system is the rapid first line of defence, and the evolutionary most conserved portion of the immune system (1,2). It consists of a multitude of cell types, proteins, and processes, as well as physical barriers provided by epithelial cells at mucosal tissues.

Among the immune cells, innate immunity is largely dependent on phagocytes (2), including the granulocytes neutrophils, eosinophils, and basophils, and the mononuclear monocytes, macrophages, and dendritic cells. Neutrophils are the most numerous of our immune cells and have a wide array of immune functions which will be described in detail in the following sections.

Mononuclear phagocytes are not only involved in phagocytosis but are also important communicators via their production of cytokines and their capacity to initiate adaptive immune responses by antigen presentation (2). Monocytes can differentiate into macrophages and certain types of dendritic cells, and there is a large heterogeneity in both phenotype and function of these cells depending on the local microenvironment where they arise (3).

The innate immune defence is aided by circulating proteins, including the complement system; a protein network of more than 30 proteins discovered in the 1980's to "complement" the immune cells in bacterial killing (4). The complement system is involved in multiple immunological functions, including opsonization for phagocytosis, generation of pro-inflammatory and chemotactic molecules, and

microbe or cell lysis (1,4). A functional complement system is crucial for efficient clearance of dead cell remnants and debris.

Sensing danger

The cells of the innate immune system recognize threats to the body by sensing danger signals via pattern recognition receptors (PRRs), such as the toll-like receptors (TLRs), which recognize molecular patterns associated with infections or tissue damage. PRRs are activated by proteins and molecular structures associated with pathogens (pathogen-associated molecular patterns (PAMPs)), or damage (damage-associated molecular patterns (DAMPs)). In contrast to PAMPs, which are molecular motifs found on microbes, DAMPs are molecules normally present in the body. Almost any protein or metabolite can become a danger signal if misplaced or present at abnormal levels (5,6). For example, DNA (both nuclear and mitochondrial), RNA, histones and ATP are recognized as DAMPs and provokes an immune response if not contained within a cell (5,6).

Trained immunity

Due to the relative short-lived nature of most innate immune cells and their "non-specific" means for sensing danger via molecular patterns, the innate immune system cannot form a long-lived and specific immunological memory in the way the adaptive immune system does. Yet, there is evidence of "innate immune memory" or "trained immunity", obtained primarily via epigenetic alterations in the effector immune cells and sometimes also in their hematopoietic stem cell precursors (7). Trained immunity mediates enhanced responsiveness of innate immune cells when they reencounter pathogens, and evidence suggest that this phenomenon could also contribute to the chronic inflammation in autoimmune rheumatic disease (7,8).

Neutrophils

Approximately 60-70 % of all circulating immune cells are neutrophils (9), with a plethora of functions essential for maintaining health. Morphologically, neutrophils are recognized by their lobulated nucleus which facilitates rapid migration to sites of inflammation (10), and their high content of cytoplasmic granules loaded with antimicrobial peptides, enzymes, cytokines and receptors (11), which they can release in response to various stimuli.

Neutrophils are primarily known for their pro-inflammatory effects, and there are overwhelming amounts of evidence that neutrophils are major players in both acute (12–15) and chronic inflammatory conditions (16). It is less well-known, but not of less importance, that neutrophils also have immunoregulatory functions (17–19). Neutrophils thus have dual roles of both propelling and suppressing inflammation,

and the balance between these two functions is key to maintaining a healthy immune system.

In response to damage or infection, via recognition of DAMPs, PAMPs, or chemokines, neutrophils quickly migrate into the affected tissue (5,20–22). The chemotactic signals induce increased expression of adhesion molecules on both neutrophils and the endothelial cells of the blood vessels. Interactions between neutrophil adhesion molecules and endothelial receptors result in reduced speed of the circulating neutrophils, so that they start rolling along the endothelium, followed by firm adhesion and eventually transendothelial migration (20–22). During migration, the actin cytoskeleton actively reshapes the neutrophil so that it can move in an amoeboid fashon (22).

Neutrophils are generally considered to be short-lived, with a lifespan counted in hours (23), but this is challenged by observations that circulating neutrophils can live for several days (24). Furthermore, the neutrophil lifespan can be prolonged or shortened depending on its microenvironment in blood or tissue, ongoing inflammation, infection or presence of immunomodulatory molecules (25,26).

Neutrophil effector functions

The classical neutrophil effector functions are degranulation, phagocytosis, oxidative burst, and release of neutrophil extracellular traps (NETs) (15) (Figure 1), all of which are important in killing and clearing infectious agents.

The non-classical effects of neutrophils as regulators of the adaptive immune response will be described in the following sections.

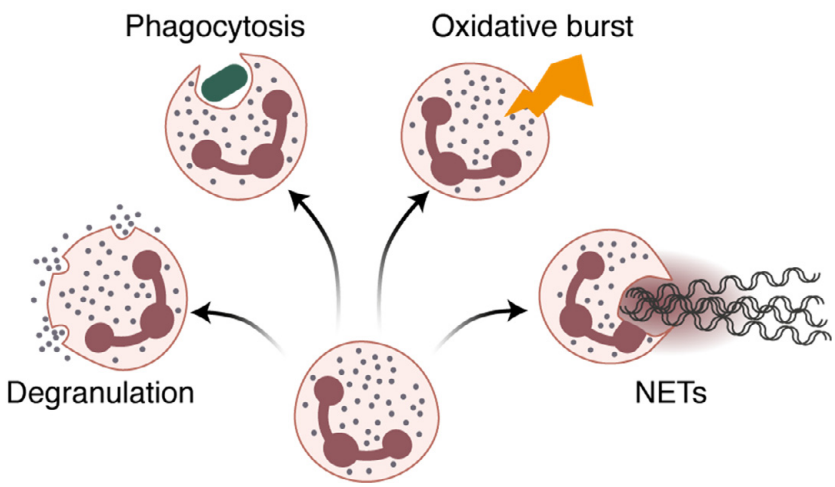

Figure 1. Schematic illustration of classical neutrophil effector functions.

Phagocytosis is the process of engulfment of extracellular particles into a phagosome, a plasma membrane-enclosed vacuole. Neutrophil phagocytosis is often initiated by antibodies or complement proteins on the particle surface, opsonizing ("flagging") it for phagocytosis. The phagocytosed material is degraded within the cell by acidification and proteolytic enzymes of neutrophil granules (27).

Neutrophil granules are of three types: azurophil, specific and gelatinase. Azurophil granules contain high levels of myeloperoxidase (MPO), neutrophil elastase (NE), and acidic hydrolases, specific granules have a high content of antimicrobial proteins, and gelatinase granules contain extracellular matrix-degrading enzymes (14). Additionally, neutrophils have plenty of secretory vesicles, containing a reservoir of membrane proteins including adhesion molecules and receptors, such as PRRs (14). The secretory vesicles and gelatinase granules are the most readily released, the azurophil granules are not released as often but are important for the degradation of phagocytosed material as they can fuse with the phagosome (14,27).

Oxidative burst and the NADPH oxidase II complex

The release of large amounts of reactive oxygen species (ROS) during neutrophil oxidative burst is mediated by the NADPH oxidase II (NOX2) complex, placed in the cell membrane.

ROS produced by the NOX2 complex is crucial for an efficient defence against infections, evidenced by the frequent and severe infections affecting individuals with chronic granulomatous disease (CGD), a rare genetic syndrome of deficient NOX2 complex function. However, the importance of NOX2-derived ROS goes beyond protection of infections. Patients with CGD often suffer from hyperinflammation and are at high risk of developing autoimmune symptoms (28,29). This suggests that there is a close relationship between oxidative burst and protection from autoimmune reactions.

The NOX2 complex is a protein complex consisting of two membrane-bound and three cytosolic proteins (Figure 2). The membrane-bound enzyme is cytochrome b_{558}, or NOX2, with the two subunits α (CYBA) and β (CYBB). It is the β-subunit that has the enzymatic activity, generating ROS by electron transfer from NADPH to oxygen. The cytosolic proteins are neutrophil cytosolic factor 1 (NCF1), NCF2, NCF4, and a Rac GTPase (30).

The production of ROS by the NOX2 complex is strictly regulated. ROS production can only occur after neutrophil stimulation which leads to the phosphorylation and translocation of the cytosolic components to the membrane bound NOX2 (30,31). In absence of stimuli, NOX2 is inactive, and the membrane and cytosolic components are separated. The NCF proteins are phosphorylated by protein kinase C (PKC) and mitogen activated protein kinase (MAPK). Phosphorylated NCF1 and NCF4 have membrane-binding sites, and NCF1 and NCF2 bind to NOX2; NCF1 to CYBA, and NCF2 to CYBB (30,31).

Furthermore, in resting neutrophils only a fraction of the total NOX2 proteins are found in the plasma membrane, and most of NOX2 is found in membranes of specific- and gelatinase granules and secretory vesicles (11,30,32). The intracellularly stored NOX2 can be rapidly released to the plasma membrane in response to neutrophil activation or priming. Upon phagocytosis, intracellular ROS is produced into the phagosome, but intracellular ROS production by NOX2 can occur also in the absence of phagocytosis (32). Activation of the NOX2 complex in the plasma membrane or intracellular membranes is regulated via different signalling pathways (33), and the assembly of the NOX2 complex differs between intracellular and extracellular ROS production, where NCF4 is primarily necessary for phagosomal, but not extracellular, ROS production (34).

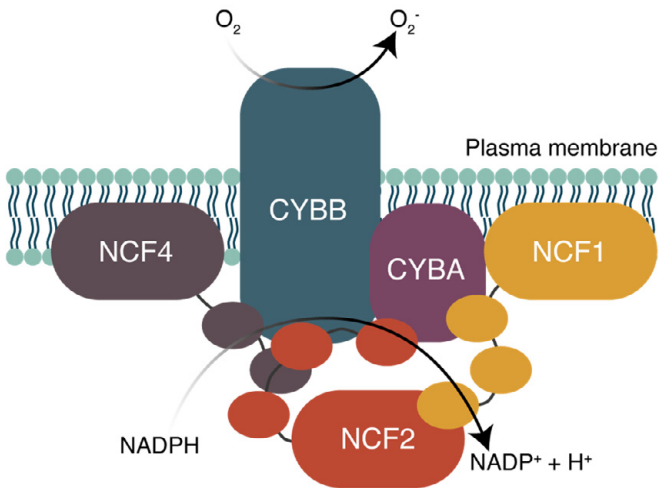

Figure 2. The ROS-producing NOX2 complex.

Neutrophil extracellular traps

NETs are web-like traps of chromatin decorated with granule proteins, extruded by activated neutrophils. They were first discovered in 2004 as an antimicrobial defence with capacity to capture and kill bacteria (35), and have later become recognized for their multiple roles in both health and multiple disease states. NETs can act as DAMPs and are thought to be important in the pathogenesis of many autoimmune rheumatic diseases (36,37), described in more detail in the following chapters.

As NETs contain nuclear material, the release of NETs is most often accompanied by neutrophil cell death (38), in a process called NETosis. During NETosis, the nuclear membrane is disrupted to allow fusion of nuclear and granular contents, followed by extracellular release of NETs as the neutrophil ruptures (38). However, NET release without cell death is also possible. In some cases, neutrophils can

survive for short periods as cytoplasts without a nucleus, and under other conditions certain stimuli can induce NETs made of mitochondrial rather than nuclear DNA, leaving the nucleus intact (39,40).

The most well-described pathway for NETosis is dependent on NOX2-derived ROS and the azurophil granule proteins MPO and NE (41,42). This pathway can be initiated by neutrophil activators including microbial components and chemical stimuli such as the PKC activator phorbol-myristate-acetate (PMA) (38,42). Other stimuli can induce NOX2-independent NET release, commonly dependent on histone citrullination by protein-arginine deiminase type 4 (PAD4) (43). NOX2-independent pathways driven by mitochondrial ROS and calcium influx have also been described (44).

The adaptive immune system

The other branch of the immune system is the adaptive immune system, which mediates long-lasting immunological memory and specific responses targeted to certain antigens. The following description of the cells and functions of the adaptive immune system is a brief overview of the most fundamental aspects of adaptive immunity.

The effector cells of the adaptive immunity are T- and B lymphocytes, mediating cellular and humoral immunity respectively (45). Each clone of T- and B cells recognize a specific antigen, as the T- and B cell receptors are tailored and selected in a process of somatic V(D)J recombination, creating a unique antigen-specific receptor for each cell (1,45).

T cells are classified based on their function and surface marker expression (1,45). Most T cells are $CD4^+$ T helper (Th) cells, which produce cytokines to induce effector functions of other immune cells and activate B cells recognizing a matching antigen (45). $CD8^+$ T cells are cytolytic T lymphocytes (CTLs), specialized in killing host cells harbouring intracellular pathogens(45). A subset of the $CD4^+$ T cells are regulatory (Tregs), and have anti-inflammatory and suppressive functions which can inhibit the responses by effector Th cells and CTLs (46).

B cells mediate humoral immunity, and are classified by surface markers and developmental stages (45). Plasma cells, differentiated from activated B cells, are the specialized antibody-producing cells (1,47). Antibodies are antigen-recognizing immunoglobulins (Ig), with a main purpose of opsonization. In the classical antibody-mediated effects, the opsonizing antibody acts as a connector between a particle (such as a microbial component or dead cell remnant) and an Fc receptor-expressing immune cell (1,48). The opsonizing antibodies primarily target particles for phagocytosis, activation of the complement system, or antibody-dependent cytotoxicity (1).

Prior to encounter of an antigen which matches their receptors, T- and B cells are "naïve". After antigen recognition they become activated, undergo clonal expansion, and may initiate immune responses. Activated T- and B cells can differentiate into memory T- and B cells (and plasma cells in the case of B cells), which survive for years after antigen encounter, making the proportion of naïve cells decrease and memory cells increase with age (1).

Interplay between the innate and adaptive immune system

As the innate immune system is phylogenetically older and evolved long before the adaptive immune system, the functions of the adaptive immune system are both supported by and dependent on the interaction with innate immune cells (2,49).

For instance, most antigens need to be presented to the adaptive immune cells by an antigen presenting cell (APC) to initiate an adaptive immune response. Monocytes, macrophages, and dendritic cells are professional APCs which present foreign (or self-) antigens to T cells. The T cell become activated if the antigen matches the T cell receptor and the T cell receives a co-stimulatory signal by the APC (50). In rare cases, neutrophils can also acquire antigen-presenting capacity in response to certain stimuli (51,52).

Immunomodulation by neutrophils

Neutrophils and their effector molecules shape the adaptive immune response via both activating and suppressive pathways (53–55). The neutrophil interaction with the adaptive immune system is mediated by direct effects on B- and T cells and via modulation of antigen presentation. Neutrophils have an especially important relationship with T cells, with the potential to strongly shape the immune response (55). A summary of neutrophil immunomodulating effects is presented in Table I.

Neutrophils exert their main immunosuppressive effects on T cells. Mechanisms for neutrophil-mediated T cell suppression include extracellular release of ROS and granular proteins, probably via an immunological synapse between the neutrophil and the T cell as this process is contact-dependent (55,56). Neutrophil can also suppress adaptive responses by other mechanisms, such as by inactivation cytokines and cytokine receptors, and capture of antigens to decrease antigen availability for APCs.

The mechanisms by which neutrophils potentiate adaptive responses include cytokine release, including B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) (54,57). However, other neutrophil effects which potentiate adaptive responses are primarily occurring in different disease states. For instance, resting, circulating neutrophils do not express MHC class II, but neutrophils in certain inflammatory environments can (58,59), and antigenicity of

macromolecules and NETs are primarily important in autoimmune conditions (60–63).

In addition to these described mechanisms, neutrophils can affect adaptive responses via interaction with antigen presenting dendritic cells. However, this cell-cell crosstalk is not easily categorized as suppressive or activating, as neutrophils can mediate dendritic cell maturation and activation in ways which can both enhance and decrease their antigen presenting capacity (53).

Table I. Neutrophil immunomodulating mechanisms

Neutrophil mediator	Effect on adaptive immune cells	References
	Suppressive effects	
ROS	Inhibition of T cells, Inhibition of IFN-signaling	(56,63–65)
MPO	Inhibition of T cells	(17,55)
Arginase-1	Inhibition of T cells	(56,66)
Serine proteases	Cleavage of cytokine receptors	(55)
NETs	Cleavage of pro-inflammatory cytokines	(67,68)
Antigen capture in lymph node	Decreased antigen presentation by APCs	(53)
	Activating effects	
MHC II antigen presentation	Th cell activation	(51,69)
NETs	T cell activation, IFN signaling	(60–62)
Oxidation of macromolecules	Increased antigenicity	(63)
BAFF, APRIL	B cell activation, antibody production	(57)

Autoimmunity

What is autoimmunity?

Autoimmunity can roughly be defined as disease caused an inability of the immune system to discriminate between self and non-self (1). Typically, autoimmunity presents together with autoreactive T cells and/or autoantibodies. Autoimmune disease has many faces, and can be organ specific or systemic, depending on the target of the autoimmune reaction.

There is no precise definition of or criteria for what is considered autoimmunity. Attempts have been made to create defining criteria, such as that autoreactive T cells and/or autoantibodies should be present in the affected organ/tissue, and transfer of the autoreactive T cells or autoantibodies should induce disease in healthy individuals or animals (70). However, the usefulness of these criteria is debated, and several established autoimmune diseases fail to fulfil the criteria. For instance, animal models of disease transfer via transfer of autoantibodies or T cells is difficult to study, as human autoantibodies or autoreactive T cells might not recognize the animal homologue of the antigen (71). Therefore, autoimmunity is commonly defined more broadly as a disease caused by an autoreactive immune response towards self-antigens.

Autoimmunity is a consequence of failed immunological tolerance. The V(D)J recombination which creates clonally unique T- and B cell receptors has random elements, and some of the T- and B cells will inevitably get receptors recognizing self-antigens. Immune tolerance is the process of eliminating or neutralizing these autoreactive cells, and works by clonal deletion, induction of anergy (unresponsiveness to antigen), or receptor editing (72,73). Tolerance occurs both during lymphocyte development, prior to their release to the circulation (central tolerance), and in peripheral tissue (peripheral tolerance). Autoreactive cells escaping the central tolerance and can lead to autoimmune reactions if the peripheral tolerance fails (74). Regulatory T cells (Tregs), which have anti-inflammatory and suppressive functions, are of crucial importance in peripheral tolerance by inhibition of autoreactive effector T cells (46). There is no easy answer to why immune tolerance sometimes fails, but autoimmunity is generally believed to occur in genetically susceptible individuals exposed to environmental triggers (72,75).

Most autoimmune diseases have a strong female predominance (75,76). The immune system is different between men and women, where women tend to have greater ability to combat infection, enhanced antibody production, stronger Th1 responses, and better response to vaccinations, which, unfortunately, comes with the prize of an increased risk for developing autoimmune disease compared to men (77). Sex hormones affect the immune cells and are likely contributors to the gender bias in autoimmunity (75,77). Yet, sex hormones cannot explain why autoimmune

disease debuting pre-puberty is more prevalent in girls. Having two X chromosomes, which carries multiple immune related genes, is associated with increased risk of autoimmunity also in XX and XXY males, and an abnormal X chromosome inactivation has been observed in some autoimmune diseases (75,78,79).

The phenomenon of autoimmunity is common due to the large number of diseases it covers. A systematic review from 2012 lists 81 diseases as autoimmune, many of which are rheumatic, and estimates the overall prevalence of autoimmunity to 4.5 % of the population (80).

Autoantibodies

Autoantibodies are perhaps the most classic feature of autoimmunity and broken tolerance, often arising before full blown autoimmune disease develops (81).

Autoantibodies can be harmful and drive pathology via several mechanisms. Among other things, they can induce cell-lysis or tissue inflammation, they can induce or block cell signalling if targeting a receptor, and by forming immune complexes (IC) with their antigens they can activate a wide range of immune cell effector functions (Figure 3) (82,83).

In addition to increasing our understanding of the disease pathogenesis, many autoantibodies can be used as biomarkers in the clinical care of autoimmune disease. Biomarkers are biological and measurable features of disease which can be diagnostic (found in disease but not healthy individuals), prognostic (associated with clinical outcomes), or monitoring (associated with disease activity) (84).

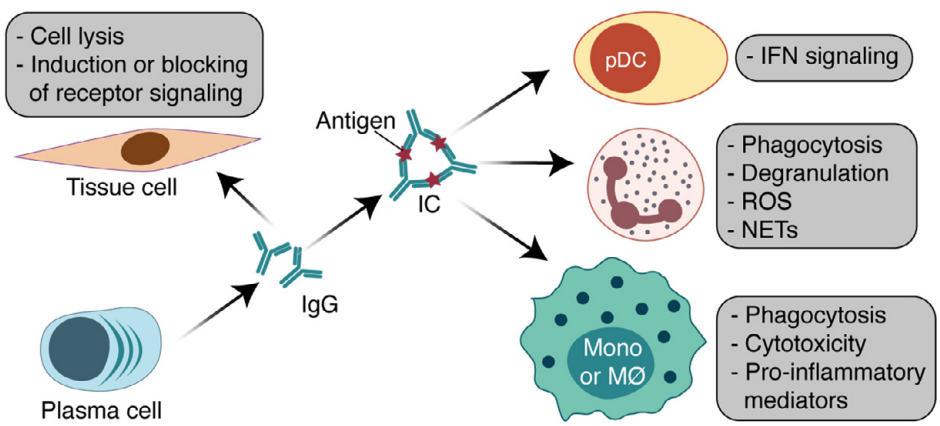

Figure 3. Autoantibody-mediated pathological effects. Antibodies targeting host cells can induce lysis, tissue inflammation or altered receptor signaling. ICs can activate immune cells and cause phagocytosis or inflammatory responses. Mono – monocyte, MØ – macrophage.

Autoimmunity vs autoinflammation

Some inflammatory diseases are caused by unprovoked inflammation in the absence of autoreactive T cells or autoantibodies; these conditions are therefore not considered autoimmune. The term "autoinflammatory" was first used in the late '90s to describe inflammatory diseases such as hereditary fever syndromes, characterized by an overactivated innate immune response (85). As the innate immune cells do not recognize specific antigens but are activated by pattern recognition, autoinflammation is not caused by an inability to discriminate self from non-self, but as a response to various kinds of DAMPs and/or genetic defects in danger-sensing or inflammatory signalling pathways (5,86). The pathogenesis of autoinflammatory diseases is typically driven by IL-1β, but other cytokines including IL-6, TNFα and type I interferon (IFN) are important drivers of certain autoinflammatory diseases (87–90). Neutrophils, monocytes, and macrophages often play a notable role in autoinflammatory disease progression (86,87). In contrast to autoimmunity, which is much more prevalent in women, there is no gender bias in truly autoinflammatory diseases (87,91), indicating how these diseases are driven by different facets of the immune system.

Today we know that the underlying immunological features of both autoimmune and autoinflammatory conditions overlap. Autoimmunity and autoinflammation are not separate concepts but part of the same spectrum where many diseases have features of both autoimmunity and autoinflammation (91–93,87) (Figure 4).

It is a common misunderstanding that autoimmunity is driven by the adaptive immune system and autoinflammation by the innate. Even though the adaptive immune system is primarily engaged in autoimmunity, the innate immune system is important throughout the whole spectrum as it can shape the adaptive immune responses (49,53). Presence of autoantibodies and autoreactive T cells in autoimmune pathology does not at all mean that the innate immune system cannot be defective as well.

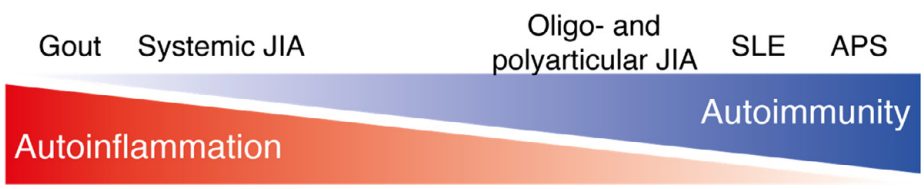

Figure 4. The spectrum of autoimmunity and autoinflammation.

Autoimmune rheumatic diseases

Most rheumatic diseases can be placed somewhere along the spectrum of autoimmune and autoinflammatory diseases (87,93). It is generally considered that rheumatoid arthritis (RA), systemic sclerosis and Sjögren's syndrome are autoimmune, while gout, systemic JIA, and monogenic fever syndromes are autoinflammatory (87). Ankylosing spondylitis and psoriatic arthritis are typically considered mixed-pattern diseases with features of both autoimmunity and autoinflammation (87,94).

The studies in this thesis investigate immunological aspects of patients with oligoarticular juvenile idiopathic arthritis (JIA) and systemic lupus erythematosus (SLE), as well as SLE patients with secondary antiphospholipid syndrome (APS). All these conditions are found in the mixed- to autoimmune end of the spectrum (87,93) (Figure 4).

SLE is often used as an example of a prototypical autoimmune disease, as it has an immunopathogenesis largely driven by autoantibodies towards self-antigens (87,95). Oligoarticular JIA is more difficult to place as its immunopathogenesis is less well understood. There is a female predominance and common to have autoantibodies in oligoarticular JIA (96,97), suggesting autoimmunity, but there are also patients without detectable autoantibodies or autoreactive T cells. Despite being in the autoimmune end of the spectrum, both SLE and oligoarticular JIA have features of inflammation driven by innate immunity (62,98–103).

Introduction to JIA

Clinical definition

JIA is the most common rheumatic disease among children. A JIA diagnosis is given if a child has persistent arthritis during at least six weeks, with a symptom debut before the age of 16 (104). The arthritis has to be "idiopathic", meaning that it is of unknown cause and not a consequence of infection, trauma or other known condition (97,105).

The JIA diagnosis is relatively new, the diagnosis criteria were defined by the International League of Associations for Rheumatology (ILAR) in the 1990s (106). Before that, the disease went under the names of juvenile chronic arthritis (107) and juvenile rheumatoid arthritis (108). Not only the name, but also the disease definitions vary slightly, and it can therefore be difficult to compare results from research studies on patients diagnosed according to different criteria.

JIA can be further divided into seven subcategories, each with different symptoms and disease progression (105) (Figure 5). There is ongoing debate if the current

diagnosis criteria are the best, or if JIA and its subtypes could be better defined by other measures reflecting immunological mechanisms rather than the number of inflamed joints (109,110).

Epidemiology

The incidence rate and prevalence of JIA varies between geographic regions and ethnicities and is more common in Scandinavia than the global average. The annual incidence rate in the Nordic countries (Sweden, Denmark, Norway, Finland and Iceland) is estimated to 15/100 000 children (111). In Skåne, southern Sweden, where JIA patients in the thesis projects are recruited, the annual incidence rate is 12.8/100 000 children (112).

Approximately two of three JIA patients are female, although the female-to-male ratio differs between the different subtypes (76,104,113,114). The only JIA subtype with a male predominance is enthesitis related JIA (96,104).

Description of the subtypes

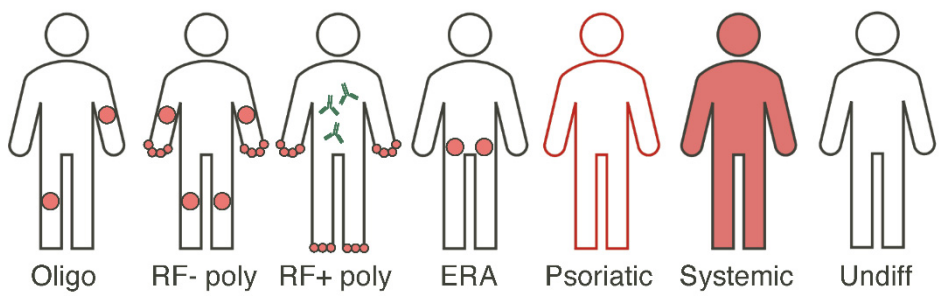

Figure 5. The JIA subtypes. Red areas indicate site of inflammation.
Abbreviations: oligo – oligoarticular, poly – polyarticular, RF – rheumatoid factor, ERA – enthesitis related arthritis, undiff – undifferentiated.

Oligoarticular JIA

The most common JIA subtype is oligoarticular JIA, representing approximately 30-60 % of all JIA cases (115), and the main JIA subtype studied this thesis. Children with oligoarticular JIA have a disease onset where one to four joints are affected during the first six months of disease, often asymmetrically in large joints such as the knees. Oligoarticular JIA can be further classified as persistent or extended, depending on whether the disease continues to affect four or fewer joints (persistent) or develops to include five or more joints after the first six months of

disease (extended) (104). The majority of patients with oligoarticular JIA have antinuclear autoantibodies (ANA).

Oligoarticular JIA typically debuts in early childhood, with a peak in incidence by the age of two (112). The symptoms in oligoarticular JIA are often mild but can be painful, limit mobility, and in some cases result in local growth disturbances. The inflammation in oligoarticular JIA is typically limited to the joint, but 10-25 % of the patients develop chronic uveitis, an eye inflammation which might cause permanent visual impairment if not detected and treated early (116–118).

Polyarticular JIA, rheumatoid factor negative

Children with inflammation in five or more joints, in the absence of rheumatoid factor (RF), will be diagnosed with RF negative polyarticular JIA (104). Some patients with RF negative polyarticular JIA have symptoms very similar to those with extended oligoarticular JIA, while others have symmetric inflammation in both large and small joints such as knees, wrists, ancles, hands and feet (96). Similar to oligoarticular JIA, chronic uveitis affects approximately 20 % of the children with RF negative polyarticular JIA (117,118).

Polyarticular JIA, rheumatoid factor positive

Children with polyarticular JIA and a positive RF test have a very similar pathology to adult rheumatoid arthritis (RA), and the disease typically debuts in adolescence (96,104). The small joints in hands and feet are most affected, but larger joints can also be involved. This JIA subgroup is not at risk of developing uveitis (117,118).

Systemic JIA

Systemic JIA is autoinflammatory and very different from the other forms of JIA, with the major symptom not being arthritis but systemic inflammation (104). Typical symptoms include high fever, skin rash, enlargement of liver and spleen, and inflammation in serous tissue. When joints are affected, it is typically symmetric polyarticular arthritis (96). Systemic JIA has no gender bias, and symptom debut can be at any age. The symptoms are very similar to adult onset Still's disease (96,97).

Enthesitis related JIA

Enthesitis related JIA is the only JIA diagnosis more common in boys than girls, with a female-to-male ratio of 1:4 (104). Typical symptoms are inflammation in the connective tissue between the bone and tendons or ligaments. Arthritis mostly affects the lower extremities and hip, and can progress to affect spinal and/or sacroiliac joints (96), making it resemble ankylosing spondylitis. Common comorbidities are inflammatory bowel disease and acute uveitis (96,97).

Psoriatic JIA

Psoriatic JIA resembles oligoarticular JIA regarding type of arthritis and age of disease onset, but with simultaneous psoriasis. The main difference between oligoarticular JIA and psoriatic JIA, except for the presence of psoriasis, is the increased presence of dactylitis (swelling of fingers and/or toes) and small joint involvement in psoriatic JIA (96).

Undifferentiated JIA

This subgroup of JIA is not characterized by a set of symptoms but includes all patients who either do not fulfill criteria for any other subgroup or meet the criteria for more than one (104).

JIA immunopathogenesis

The immunopathogenesis underlying JIA has thus far not been fully characterized and differs largely between the different subtypes. Especially systemic JIA, as an auto-inflammatory disease, is immunologically distinct from the other subtypes. The immunological features of oligoarticular JIA primarily overlap with RF negative polyarticular JIA. As the patients studied in this thesis belong to these subtypes, the following description is based on studies of oligo- and RF negative polyarticular JIA, as well as some studies including JIA patients of all non-systemic subtypes. The immunological alterations in oligo- and RF negative polyarticular JIA are summarized in Figure 6.

Genetics

JIA is a complex, polygenic disease with a genetic landscape overlapping with other autoimmune diseases. About one third of JIA patients has a first-degree relative with autoimmune disease (119). HLA genes are strongly associated with JIA and is estimated to account for 8-13 % of JIA susceptibility (115,120). Gene variants in cytokine and immune signalling genes and genomic regions associated with neutrophils and $CD4^+$ T cells are also associated with JIA (115,120–122).

Immunological alterations

Previous research has emphasized the role of adaptive immune responses, as activated T cells are enriched in synovial fluid and synovial tissue contains lymphoid follicular structures of aggregated T-, B-, and plasma cells (123–126). Presence of lymphoid follicles is associated with ANA and plasma cell infiltration (126), suggesting that these lymphoid structures might drive local autoimmunity. An increased amount of memory B cells and IgG secreting plasma blasts have been observed in synovial fluid of inflamed joints (127).

In inflamed joints, Th17 cells are expanded and suppressive Tregs found in decreased numbers (128,129), an imbalance thought to be important in the JIA pathogenesis (130). However, this was contradicted in a recent study that found extensive Th1 skewing of synovial T cells, where also the synovial Tregs had features of Th1 polarization, while Th17 cells were not enriched (131). Patients with extended oligoarticular JIA have Tregs in lower numbers and with decreased suppressive capacity compared to patients with the persistent form (132), indicating that Tregs are important for determination of disease severity (133).

Autoantibodies are common in oligoarticular JIA, especially ANA (96,97,110). Typically, two of three patients or more with oligoarticular JIA are ANA positive, and ANA is detected in approximately 50 % of JIA patients across all subtypes (112,134). It is unclear whether the antibodies have direct pathogenic effects, but ANA positivity is associated with clinical phenotype and development of uveitis (110,117,135–138).

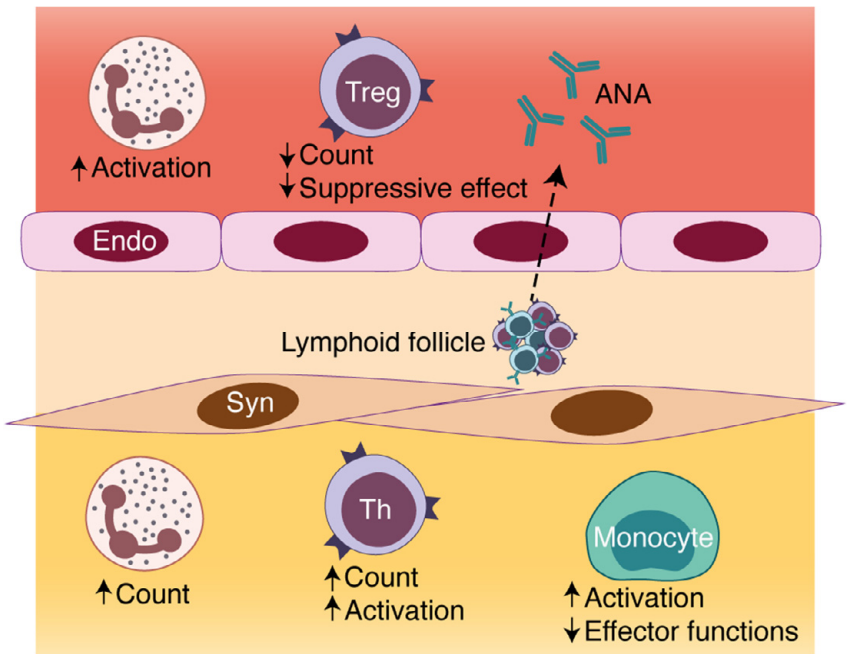

Figure 6. Immune cell alterations in blood (red) and synovial fluid (yellow) in JIA. Endo – endothelium, Syn – synoviocytes.

Contribution of the innate immune system to the JIA pathogenesis has gotten less attention. However, JIA neutrophils show signs of systemic activation and are the most common immune cells in inflamed joints (101,139–141). The evidence for the

role of neutrophils in JIA will be described in detail in the next chapter. Monocytes in inflamed joints are activated, polarized and have impaired phagocytic and ROS-producing capacity compared to monocytes in the blood (142).

Synovial fluid contains high levels of cytokines typical of innate immune system activation (103,143). Cytokines and chemokines which are increased in synovial fluid during arthritis include, but are not limited to, IL-6, IL-8, IL-10, IL-1β, TNFα, IL-1RA, IL-12, IFNγ, IL-17, GM-CSF and chemokines (129,130,142–144). Some of the cytokines are also increased in blood, and their levels are associated with disease activity and JIA subtype (130,143).

Introduction to SLE

Clinical definition

SLE is a systemic, multi-organ disease which can manifest in almost any organ system. SLE primarily affects women and usually debuts during reproductive age (95,145).

SLE symptoms can include manifestations in the skin (rashes and photosensitivity), oral mucosa (ulcers), joints (arthritis and arthralgia), serous tissues (pericarditis), kidneys (nephritis), central nervous system (seizures and psychosis), blood (anaemia, leukopenia, and thrombocytopenia), and immune system (ANA and antibodies towards DNA, Sm, and phospholipids) (146–148).

Currently there are no diagnostic criteria for SLE, and due to the heterogeneity of the disease it can be difficult to diagnose (149).To ensure that SLE patients in research and clinical studies share a similar disease, classification criteria have been established, representing different SLE symptoms and immunological alterations (146,150,151). Patients are generally considered to have SLE if four or more of the eleven classification criteria are fulfilled, with manifestations in at least two organ systems and at least one immunological alteration, which cannot be explained by any other known condition (146,147). The most recent update of classification criteria from 2019 uses ANA as an entry criterion and has weighted the criteria, so that a combined weight of 10 classifies as SLE, independent of if the sum is obtained from few criteria with high weight or many with low (151,152).

Epidemiology

The incidence and prevalence of SLE varies greatly between countries and ethnicities, with a lower incidence in Europe compared to Asia, the Americas and Oceania (153–155). In Sweden, the prevalence has been estimated to 46-85/100 000 individuals (156), and the annual incidence in Skåne, southern Sweden, is estimated to 4.8/100 000 individuals (157).

The female to male ratio in SLE is approximately 9:1 (145,155). There is less of a female predominance in SLE patients with disease onset either pre puberty or post menopause, indicating an important role of sex hormones (145).

SLE heterogeneity

Since SLE can manifest in various ways and affect almost any organ, there are large heterogeneities among patients regarding symptoms, disease progression, and clinical outcomes, despite sharing the same diagnosis (95,158). A mild version of SLE can present with persistent low disease activity and skin rashes or ulcers as main symptoms, while severe SLE is characterized by high disease activity, nephritis, neuropsychiatric involvement, and cardiovascular disease. Many patients will have a disease course characterized by recurrences of similar manifestations over time, while other patients can change clinical phenotype over the years.

The heterogeneity among SLE patients is also reflected in the immune cells, where transcriptomic and epigenetic profiles are associated with disease activity, clinical phenotypes and autoantibody profiles (159–161).

Antiphospholipid syndrome (APS)

APS is a syndrome characterized by thrombosis and pregnancy complications, such as recurrent miscarriages and preterm delivery, in the presence of antibodies towards phospholipids or phospholipid associated proteins (antiphospholipid antibodies, aPL) (162,163). The aPLs analysed for diagnosis of APS are anti-cardiolipin (CL) and anti-β2 glycoprotein I (B2GPI) antibodies, and the lupus anticoagulant test (162,164). In addition to thrombosis and obstetric complications, symptoms also include thrombocytopenia, anaemia, cognitive impairment and skin ulcers (162).

APS can occur as a primary syndrome, without underlying disease, but it is estimated that more than a third of all APS cases are associated with SLE (165,166). Around 40 % of SLE patients have at least one type of aPL, and many of these patients will develop clinical APS (165). The APS pathogenesis is partly shared with SLE (98,167), a possible explanation to why the two diseases so often occur together.

SLE immunopathogenesis

The immunology underlying SLE is much more well-characterized than the JIA immunopathogenesis. Important immunological mechanisms and pathways in SLE are summarized in Figure 7.

Genetics

SLE is partly genetic, among SLE patients with monozygotic twins the concordance rate is 24 %, 10-fold higher than in dizygotic twins (168). As in other autoimmune

diseases, HLA genes are associated with SLE (169,170). Genetic deficiencies in proteins important for clearance, including complement proteins (169,171), Fcγ receptors, and nucleases, are strongly associated with SLE (169,170,172). Genetic deficiencies in ROS production are also predisposing for SLE (170,173–175). In rare cases SLE can be monogenic, as is the case with loss of function in complement proteins, C1q, C2 and C4 (171,172), but in most cases the risk for SLE increases with the cumulative number of genetic variants (176).

Clearance

SLE is characterized by an imbalance between generation and disposal of apoptotic material, resulting in an increased autoantigenic burden (172). The complement system, phagocytosis/efferocytosis, and extracellular nucleases are vital for efficient clearance of apoptotic material and abnormalities in all of these functions are strongly associated with SLE (171,177–180).

Apoptotic material can act as DAMPs and trigger inflammatory responses. If recognized by TLR7-9 or cytosolic sensors, nucleic acids will initiate production of type I IFNs, IL-6, IL-1β and TNF (172,177,178).

Autoantibodies and immune complexes

Almost all SLE patients have autoantibodies towards nuclear components, and multiple other self-antigens. The high presence of autoreactive B cells in SLE is associated with overexpression of BAFF, a protein contributing to failed tolerance by promoting B cell activation and preventing deletion of autoreactive B cells (181,182).

Apoptotic material targeted by autoantibodies will form ICs, found in elevated levels in the circulation in SLE and deposited in kidney glomeruli in lupus nephritis (172,183,184). The autoantibodies are pathogenic as IC containing nuclear material will activate TLR7-9 and initiate a pro-inflammatory response and production of type I IFNs (158,178,185). ICs are also thought to mediate tissue inflammation, as IC deposits are found in, and thought to be able to induce, glomerulonephritis in SLE (184,186).

Type I IFNs

The interferons are cytokines named for their capacity to interfere with viral replication, classified into three families. The type I IFNs, including IFNα, -β, -ε, -κ, and -ω, are elevated in the circulation of many patients with SLE (187,188). Ongoing IFN signaling in SLE is typically detected as an “IFN signature” with expression of IFN-stimulated genes (189,190). Epigenetic studies have identified demethylation of IFN-regulated genes in SLE (191,192), suggesting a trained immunity and enhanced IFN-responsiveness.

Plasmacytoid dendritic cells (pDCs) are the main producers of type I IFN, and they release large amounts of IFN upon activation by IC containing nucleic acids. Type I IFNs contribute to autoimmunity as they mediate enhanced antigen presentation by dendritic cells, impair the effect of Tregs, increase plasma cell differentiation and antibody production, increase survival of T cells, and is sustained in a positive feedback loop by enhancing further IFN production by pDC (187).

Neutrophils

Neutrophils contribute to the SLE pathogenesis in various ways, as described in the next chapter. They are primarily described to contribute to the autoantigenic burden via increased apoptosis and release of nuclear content in NETs (193,194).

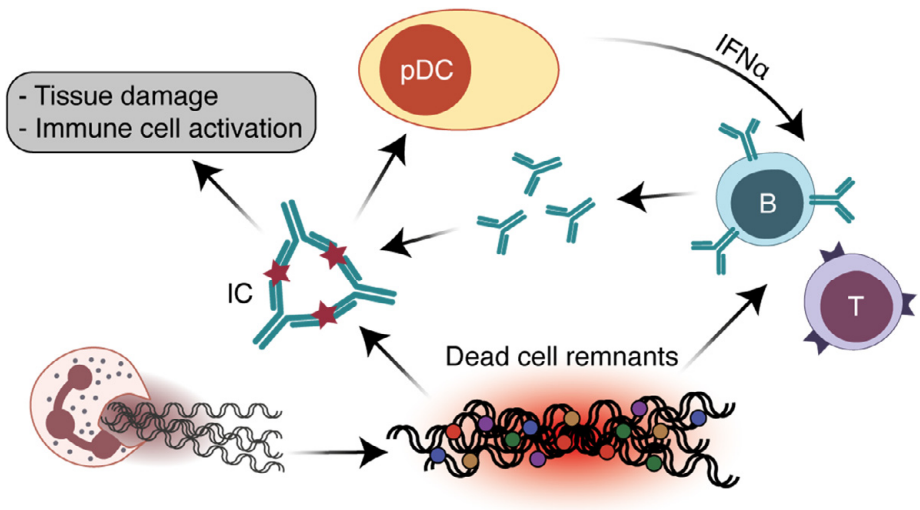

Figure 7. Immunopathogenesis of SLE.

Neutrophils in JIA and SLE

Neutrophils are highly important in the pathogenesis of many autoimmune rheumatic diseases (193), and in SLE there is a large body of evidence for the contribution of neutrophils to pathology (98,193,194). In oligo- and polyarticular JIA on the other hand, a limited number of studies have investigated the role of neutrophils and much remains to be unravelled.

Even though neutrophils are terminally differentiated cells, they still have some plasticity and depending on maturation state and presence in circulation or tissue, their phenotypes are quite heterogeneous even in health (26,195). In autoimmune diseases like JIA and SLE, neutrophils can be very atypical with abnormal functions, which impacts the whole immune system.

Neutrophils in JIA

Like in the general immunopathogenesis of JIA, the role of neutrophils is not the same in all JIA subtypes. The following summary is primarily based on studies on oligo- and polyarticular JIA.

Neutrophil transcriptomic alterations

Even though oligo- and polyarticular JIA are usually not considered to be systemic diseases, there is evidence for systemic neutrophil activation. On the transcriptomic level, neutrophil transcriptomic profiles in RF negative polyarticular JIA are altered compared to controls, and remain so also during periods of inactive disease (101,196,197). By gene expression analysis of JIA peripheral blood mononuclear cells (PBMC), a neutrophil signature was observed, demonstrating a presence of low-density neutrophils, which co-purified with the PBMC, with increased transcription of granular proteins (102). These transcriptional alterations of neutrophils in JIA suggest a primed state of JIA neutrophils.

Calgranulins and neutrophil proteins

Several studies have found elevated levels of calgranulins in JIA blood compared to healthy controls (198–201). Calgranulins, S100A8, S100A9, and S100A12, are calcium-binding proteins with pro-inflammatory and chemotactic properties, associated with inflammatory arthritis (202). The S100 proteins are primarily expressed and released by phagocytes. Neutrophils are a primary source of calgranulins, due to the high relative numbers of neutrophils compared to other phagocytes and that S100A8 and S100A9 are the most abundant proteins in neutrophil cytoplasm (202). Calgranulins are secreted by activated neutrophils (203,204), and elevated levels of S100 proteins could therefore be considered as surrogate markers for neutrophil activation. Calgranulin levels in JIA increases with

disease activity (198–201), and decreases in response to therapy (199,201). Other neutrophil proteins, including neutrophil lipocalin and MPO, are also increased in JIA blood compared to healthy controls (201).

Neutrophils in inflamed joints

The role of neutrophils in joint inflammation in JIA is less studied. Neutrophils are the most common immune cells in synovial fluid of inflamed joints in JIA (141), and JIA synovial fluid contains high levels of S100A8/A9 and different neutrophil proteases (200,205). A recent study reported JIA synovial fluid neutrophils to have a hyperactivated phenotype and express atypical markers, such as HLA-DR (144), which has also been observed on synovial fluid neutrophils in RA (58,59,206).

Much remains to be explored regarding the role of neutrophils in JIA, but the available studies agree that aberrant neutrophil activation is important for JIA pathogenesis.

Neutrophils in SLE

Clearance

Neutrophils have long been known to contribute to SLE pathogenesis. Early studies of neutrophils in SLE identified the lupus erythematosus cell (LE cell), a neutrophil which has phagocytosed, but not degraded, large amounts of autoantibody-opsonized nuclear remnants and apoptotic bodies, pushing the neutrophil nucleus become toward the cell edges (207,208). For a while, the LE cell phenomenon was the most specific test for SLE available (209). Neutrophils in SLE are generally less efficient at phagocytosis and degradation of ingested particles compared to healthy control neutrophils (210,211).

Neutrophil extracellular traps

Despite participating in clearance, neutrophils contribute to the autoantigenic burden. SLE neutrophils in have an increased rate of apoptosis (179,212) and are prone to undergo NETosis (37,99), often accompanied by deficiencies in degradation and clearance of NETs (213,214).

Since the discovery of NETs, they have gained a lot of attention as culprits in SLE (Figure 8) (37,193). NETs can contribute to autoimmunity by exposure of nuclear material, often targeted by autoantibodies in SLE (215–217), and NET components and NET-containing IC are potent inducers of IFN by pDC (61,62,99,218). Mitochondrial and oxidized DNA, sometimes present in NETs, is especially interferogenic (61,219,220). NETs can also induce tissue damage (99) and contribute to the increased cardiovascular morbidity in SLE by acting as a pro-thrombotic surface and cleaving anti-thrombotic proteins (221,222). Aberrant NET

production in SLE quickly becomes a vicious circle, as NETs and NET-containing IC can induce further NET release from other neutrophils (223–225).

SLE patients often have a subset of neutrophils with lower density than normal mature neutrophils (226,227). These cells, called low-density granulocytes (LDGs) or low-density neutrophils (LDN), spontaneously release NETs (61,99), produce type I IFN and other pro-inflammatory cytokines (228), and are thought to account for a large amount of the neutrophil- and NET-mediated pathology in SLE.

However, as with everything, the picture has more dimensions to it. NETs have actually been found to participate in resolution of inflammation by degradation of pro-inflammatory cytokines (67). Additionally, in mouse models with genetic inability to form NETs, SLE features are more severe or unaltered (229,230), indicating that NETs might not be as harmful as often described.

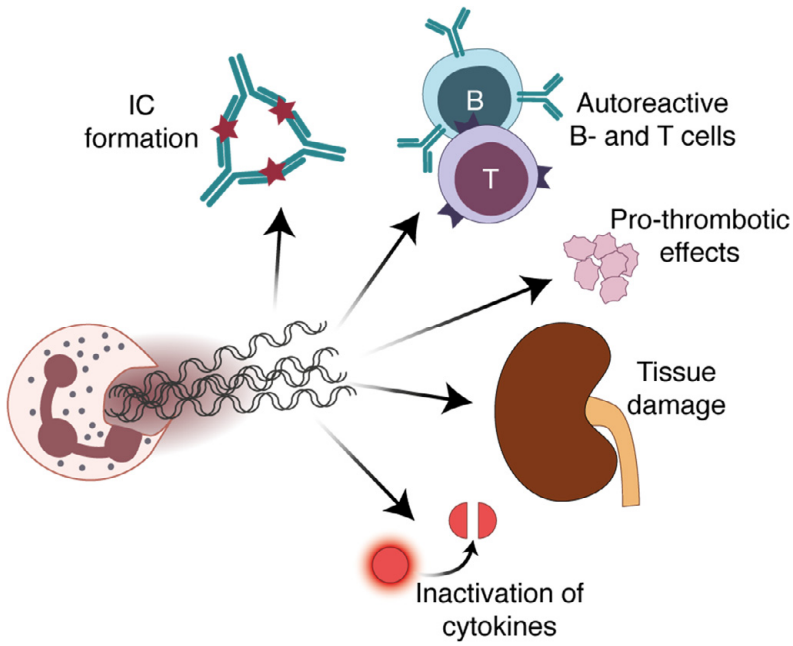

Figure 8. NETs in the pathogenesis of SLE.

Reactive oxygen species

ROS produced by the neutrophil NOX2 complex during oxidative burst are infamous for their potential to induce tissue damage, but in many ways they protect us from autoimmunity and SLE (Figure 9) (231).

SLE neutrophils have impaired oxidative burst compared to healthy controls, associated with organ damage (232). Genetic variants mediating impaired function

of the NOX2 complex are associated with both SLE and other autoimmune rheumatic diseases (174,233–235), and in mouse models of deficient NOX2 function, SLE like symptoms either develop spontaneously or become exacerbated (100,229,236,237).

NOX2-derived ROS have several effects on the immune system which might be involved in the protection from autoimmunity. In phagocytes, NOX2-derived ROS are necessary for quiescent clearance of dead cell remnants by facilitating phagosomal pH regulation and degradation of phagocytosed material (238,239). Extracellular ROS are important messenger molecules which affects other immune cells. For instance, ROS can inhibit IFN production (236,240,241), and neutrophils can suppress T cell proliferation and activation via release of ROS (242–245) (Figure 9).

The second source of ROS, present in all cell types, is the mitochondrion, producing ROS during cellular respiration (246). Unlike NOX2-derived ROS, released by tightly controlled mechanisms, mitochondrial ROS (mtROS) is more of a loose cannon. There is a tight relationship between dysfunctional mitochondria, mtROS, and production of pro-inflammatory cytokines in many chronic diseases (247). In SLE, there is evidence for mitochondrial dysfunction and subsequent oxidative stress (248,249). The mitochondria contain DNA, which is not enclosed in a nucleus and thereby highly sensitive to oxidative damage by mtROS. Oxidized mitochondrial DNA, which can be released extracellularly in NETs or via extrusion of mitochondria or mitochondrial components, is highly interferogenic and found extracellularly in elevated levels in SLE (61,220). Another contribution of mtROS to IFN signaling is that excessive mtROS in SLE can induce virus-independent oligomerization of mitochondrial antiviral-signaling protein (MAVS), which initiates production of IFN (250).

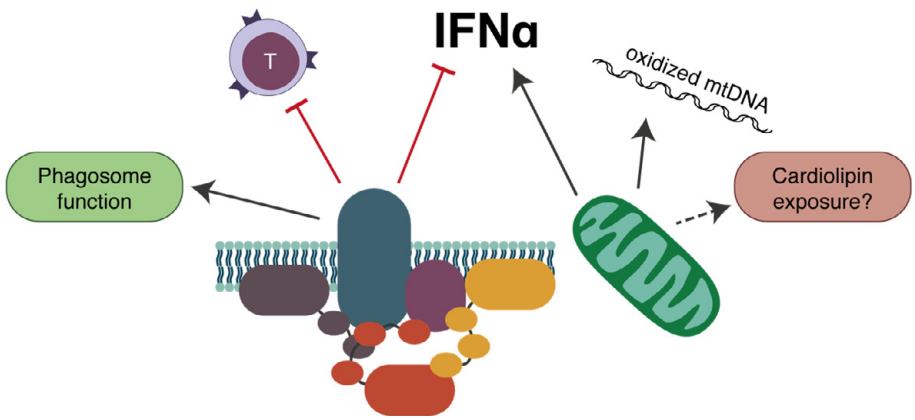

Figure 9. The effects of ROS produced by the NOX2 complex and mitochondria in SLE. Modified from (98).

Autoantibodies in JIA and SLE

Autoantibodies in JIA

ANA

Approximately half of JIA patients across all subtypes have ANA, and in oligoarticular JIA the numbers are as high as 60-80 % (112,134). Presence of ANA in JIA is more common in girls than boys and associated with early disease onset and an increased risk of developing uveitis (136,138,251). As ANA are associated with a certain clinical phenotype in JIA, it has been suggested as a classification criterion for redefined JIA subtypes (109,110).

The nucleus contains a multitude of potential autoantigens, both proteins and nucleic acids. ANA are commonly detected by immunostaining of permeabilized cells and can therefore be studied without knowledge of the specific antigen that they target. The antigen repertoire targeted by ANA in JIA is not fully characterized, but identified nuclear autoantigens in JIA include histones (138,252,253), high mobility group box 1 and 2 (254,255), and heat shock proteins (256,257). ANA positivity in JIA is more common than specific antibodies towards these identified antigens, and it is therefore likely that ANA target also other, yet undescribed, autoantigens.

The limited knowledge of autoantigens in JIA makes it difficult to use autoantibodies as biomarkers in JIA. As ANA are so common but uveitis quite rare (occurring in approximately 10-25 % of patients with oligo- or RF negative polyarticular JIA (116–118)), there is a need for a better biomarker for identification of patients at risk for developing uveitis.

Other autoantibodies

Based on the association between autoantibodies and uveitis, some studies have investigated potential ocular autoantigens in JIA (258,259). However, these studies either found proteins expressed in many tissues or detected autoantibodies by staining whole ocular tissue without determination of the specific proteins, so it is still unclear if antigens with restricted expression in the eyes could be involved in the pathogenesis of uveitis.

Antibodies towards extracellular matrix proteins have been detected in JIA (260,261), and were in these studies associated with joint inflammation or a more aggressive disease.

An important diagnostic autoantibody in JIA is RF, an antibody targeting the Fc-portion of IgG, which separates patients with RF positive and negative polyarticular JIA (96,134). Among patients with RF positive polyarticular JIA, but not any other

JIA subtype, it is also common to have antibodies towards citrullinated proteins (ACPA) (262). Both ACPA and RF are common in adult RA, and there are many similarities in the clinical features of RA and RF positive polyarticular JIA.

Except for ACPA, antibodies targeting other types of post-translationally modified proteins are present in many JIA subtypes (134).

Autoantibodies in SLE

SLE is a disease of many autoantibodies, with more than hundred described autoantigens (263).

Autoantibodies in SLE are clearly contributing to the disease progression and clinical symptoms in SLE (263), especially when they form IC. As described in the immunopathogenesis of SLE, IC made of ANA and nuclear material can contribute to disease by inducing IFN production, NET release, or inflammation in tissues with IC-deposition.

ANA

The most common type of autoantibodies in SLE is ANA. ANA are present in almost all patients, often used as diagnostic biomarkers, and suggested as an entry criterion for SLE classification (151,186). In contrast to ANA in JIA, many ANA-targets in SLE are identified. The most commonly targeted nuclear antigens in SLE are DNA, histones, and RNA binding proteins (186,263), and in addition to these there is a long list of other nuclear components sometimes targeted by ANA.

Antiphospholipid antibodies

APS represent a typical case of where an antibody profile is associated with a clinical outcome. It is estimated that approximately 40 % of SLE patients have at least one aPL, and that 50-70 % of the patients with aPL will develop clinical APS with thrombotic and/or obstetric events (165). The clinical features of APS are largely driven by the aPL, which mediate pro-coagulant effects via platelet activation, endothelial cell perturbations, disruption of anticoagulation processes, and placental cell disturbances (164).

The aPL included in the diagnosis for APS are targeting cardiolipin, B2GPI and lupus anticoagulant. The aPL nomenclature is confusing as it is unclear if aPL target the phospholipids directly or if the binding is indirect, mediated by phospholipid-binding proteins (167). For instance, B2GPI is not a phospholipid but one of these phospholipid-binding proteins.

Anti-B2GPI has the most clinical significance of the aPL, and these antibodies are the most well-described mediators of pro-thrombotic effects (164,167). B2GPI has five domains, where domain V is the phospholipid-binding domain, and domain I is

recognized by the pathogenic aPL. B2GPI is cationic and binds to negatively charged phospholipids on the surface of apoptotic cells, and when doing so it exposes the antigenic domain I (264).

Cardiolipin is a phospholipid of the inner mitochondrial membrane, named after its first identification in bovine heart. CL can be exposed on the surface of apoptotic cells (265), and other sources of possible CL exposure include mitochondrial components present in NETs or extruded from neutrophils (61,220).

The lupus anticoagulant antibody is another misnomer. It is named for the increased clotting time it mediates in vitro, but contrary to the name its presence in disease is associated with thrombotic events (266). Unlike CL and B2GPI, lupus anticoagulant is detected by its in vitro effect, and which specific phospholipid or phospholipid-binding protein it targets is not fully known (266).

Antibody associations with clinical outcomes

Not only aPL are associated with clinical outcomes in SLE. Anti-DNA antibodies are associated with both disease activity and nephritis (186,267–269), but it is unclear whether their presence can be used for disease monitoring or prediction of disease flares. Other autoantibodies associated with certain clinical phenotypes in SLE are antibodies towards ribonucleoproteins which are more common in patients with Raynauds phenomenon (whitening of fingers due to temporary vessel spasms), anti-SSA and -SSB (Sjögren's syndrome antigen A and B) which are associated with skin manifestations, and anti-ribosomal P protein which is associated with neuropsychiatric lupus (269–271).

The present investigation

The four studies of this thesis have investigated several aspects of the immunology of JIA and SLE.

Study I is a descriptive study of neutrophils in inflamed joints compared to blood in children with JIA. Neutrophils were studied both regarding phenotype and function.

Study II investigated the influence of neutrophils, from blood or inflamed joints of JIA patients, on T cell proliferation. This study also investigates mechanisms for neutrophil-mediated suppression of T cells and how this is affected by neutrophil migration.

Study III investigated the effect of a genetic variant in NCF1, mediating impaired NOX2-mediated ROS production, on multiple aspects of SLE pathology. The parameters studied included neutrophil function, autoantibody profile, and clinical outcomes.

Study IV explored autoantibodies and autoantigens in JIA, and their potential clinical use as biomarkers. The study aimed to identify novel JIA autoantigens, using both exploratory and targeted methods.

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

Background and aim

Neutrophil alterations are evident in many autoimmune rheumatic diseases. In JIA, studies suggest that circulating neutrophils are activated, especially during arhtritis flares. However, in oligoarticular JIA, symptoms are generally not systemic but localized to the affected joints. Inflamed joints are infiltrated by high numbers of neutrophils, yet little is known about neutrophils at the site of inflammation in JIA.

This study aimed to characterize neutrophils regarding both phenotype and function in blood and synovial fluid of children with oligoarticular JIA.

Method

Neutrophils were investigated in paired samples of blood and synovial fluid from 17 patients with active oligoarticular JIA. In six of the patients, blood neutrophils were also studied during inactive disease.

Neutrophil phenotype was investigated by flow cytometry by staining JIA blood and synovial fluid for surface markers related to neutrophil activation, maturation, migration, and polarization. Neutrophil phenotype was also investigated in healthy blood neutrophils after in vitro exposure to oligoarticular JIA synovial fluid. Healthy oral cavity neutrophils, which have migrated towards a non-inflammatory site, were studied as tissue migration controls.

Neutrophil effector functions phagocytosis and oxidative burst were both investigated by flow cytometry. Neutrophils in paired blood and synovial fluid were allowed to phagocytose fluorescently labelled and opsonized bacteria, quantified by flow cytometry. Oxidative burst was quantified by DHR-123, a component which is nonfluorescent until oxidized by ROS, in stimulated blood and synovial neutrophils.

Key findings

The neutrophil phenotypes were distinctly different between blood and synovial fluid. Synovial fluid neutrophils had increased activation markers and a phenotype associated with neutrophil maturity and age compared to neutrophils in the blood. The synovial fluid neutrophils had gained expression of CD206, a mannose receptor

commonly not found on neutrophils but on monocytes, macrophages, and dendritic cells.

Blood neutrophils had a similar phenotype both during flares and inactive disease, suggesting that local rather than systemic alterations are important in the arthritic inflammation.

It was not possible to induce the phenotype of synovial fluid neutrophils simply by exposing healthy blood neutrophils to synovial fluid in vitro, nor was the phenotype of oral cavity neutrophils shared with synovial fluid neutrophils. Thus, the phenotype of synovial fluid neutrophils was not mediated by synovial fluid or tissue migration alone.

Neutrophil phagocytosis, and to some extent also the oxidative burst, was impaired in synovial fluid neutrophils compared to blood neutrophils. The impairment in effector functions had an inverse correlation with expression of CD206, where neutrophils in the synovial fluids with the highest proportion of $CD206^+$ neutrophils had the lowest phagocytic and ROS producing capacities.

Conclusions

This study demonstrated that neutrophils in synovial fluid of inflamed joints in children with oligoarticular JIA are altered both regarding phenotype and function.

Synovial fluid neutrophils have a distinct phenotype compared to circulating neutrophils in the same patient. The synovial fluid neutrophil phenotype is characterized by markers related to neutrophil activation and age, and an acquired expression of CD206. The synovial fluid neutrophils also have impaired effector functions, which is associated with the phenotype shift.

These neutrophil alterations induced at the site of inflammation might contribute to the sustained joint inflammation.

Study II. Neutrophils lose the capacity to suppress T cell proliferation upon migration towards inflamed joints in juvenile idiopathic arthritis

Background and aim

Neutrophils are important immunoregulators with the capacity to both potentiate and suppress inflammatory reactions. A central immunosuppressive feature of neutrophils is their ability to suppress T cell proliferation and cytokine production.

In inflamed joints in JIA, neutrophils are altered in both phenotype and function, compared to circulating neutrophils. However, the immunosuppressive capacity of neutrophils at the site of inflammation in JIA has not previously been investigated.

This study aimed at characterizing the interplay between neutrophils and T cells in oligoarticular JIA, and how it is affected by neutrophil migration from the blood towards the inflamed joint.

Method

Neutrophils were isolated from blood (n=9) and synovial fluid (n=11) from children with oligoarticular JIA. Suppression of T cells was investigated by co-culture of isolated JIA neutrophils with healthy donor T cells, activated by CD3 and CD28 stimulation, followed by analysis of T cell proliferation. T cells were labelled with the fluorescent dye CellTrace, the concentration of which is halved by each cell division, and T cell proliferation could be analysed by flow cytometry.

An artificial synovial membrane was made using transwell inserts with endothelial cells on the inside and knee synoviocytes on the underside of the membrane, placed in culture wells containing JIA synovial fluid. Healthy donor neutrophils were allowed to migrate over the artificial synovial membrane followed by analysis of T cell suppression, oxidative burst, and proteomic alterations.

Oxidative burst was quantified by DHR-123, as in study I.

Proteomic alterations of in vitro migrated neutrophils were investigated by liquid chromatography mass spectrometry (LC-MS), identifying and quantifying the abundances of proteins present in the neutrophils based on peptide mass fingerprinting.

Key findings

Neutrophils isolated from JIA blood suppressed T cell proliferation to a similar extent as blood neutrophils from healthy controls. Neutrophils isolated from synovial fluid on the other hand clustered into two groups, where synovial neutrophils from six of the patients were highly suppressive and synovial fluid neutrophils from five patients were completely non-suppressive.

Healthy donor neutrophils which were allowed to migrate towards JIA synovial fluid in the artificial synovial membrane lost their capacity to suppress T cell proliferation. However, neutrophils incubated in the same synovial fluid retained an inhibitory effect, demonstrating that the migration process rather than exposure to synovial fluid mediates the loss of T cell suppressive capacity.

In vitro-migrated healthy neutrophils had impaired oxidative burst compared to neutrophils incubated in synovial fluid. On the proteomic level, in vitro migration induced alterations in proteins involved in immunoregulation, cell-cell contact, and synapses (both immune and neuronal), suggesting that these mechanisms might be important for neutrophil suppression of T cells.

Conclusions

This study showed that synovial fluid neutrophils in many oligoarticular JIA patients are unable to suppress the proliferation of activated T cells.

The loss of suppressive capacity could be replicated in healthy neutrophils which migrated towards JIA synovial fluid in a model system of a synovial membrane. Results from the migration model suggest that the loss of T cell suppression may be mediated by decreased oxidative burst, alterations in immunomodulating proteins and impaired cell-cell contact.

Loss of suppressive capacity of neutrophils in the inflamed joint could hinder resolution of inflammation or contribute to development of local autoimmune reactions.

Study III. NCF1-339 polymorphism is associated with altered formation of neutrophil extracellular traps, high serum interferon activity and antiphospholipid syndrome in systemic lupus erythematosus

Background and aim

ROS, produced by the NOX2 complex in phagocytes are important negative regulators of the immune response. A recently discovered missense single nucleotide polymorphism (SNP) in the *NCF1* gene, encoding the NOX2 complex subunit NCF1 (also known as p47phox), affects the ROS producing capacity. The SNP is denoted NCF1-339 and the minor NCF1-339 T allele mediates impaired ROS production compared to the major C allele. Additionally, the *NCF1* gene can vary in copy number, and the function of the NOX2 complex is impacted by both *NCF1* copy number and NCF1-339 genotype.

Previous studies have demonstrated that the NCF1-339 T allele is strongly associated with SLE, but the underlying mechanisms and possible associations with clinical outcomes are unknown. This study aimed to characterize the effects of

NCF1-339 on different aspects important in the SLE pathology: neutrophil release of NETs, serum IFN, presence of autoantibodies and clinical phenotype.

Method

Neutrophil NET release was studied in 31 SLE patients, all with two copies of the *NCF1* gene but different NCF1-339 genotypes. NETs were induced by stimuli activating pathways dependent on or independent of NOX2-derived ROS. To determine the importance of different sources of ROS, inhibitors blocking either NOX2-derived or mitochondrial ROS were used. NET release was measured by extracellular DNA quantification.

Serum IFN levels were quantified with an indirect method, where a responder cell line was incubated with serum from 141 SLE patients followed by analysis of IFN inducible gene expression.

Presence of autoantibodies, analysed by accredited clinical laboratories at Lund University hospital, were studied cross-sectionally in 305 SLE patients. Clinical parameters of antiphospholipid syndrome (APS) were investigated in 1087 SLE patients from four Swedish university hospitals.

Patients were divided into genotype groups to reflect the ROS producing capacity based on NCF1-339 genotype and *NCF1* copy number. A “normal ROS” genotype has two or more *NCF1* copies with C at NCF1-339, other genotypes are “low ROS”. For analyses of NETs, IFN, and autoantibodies, patients were categorized based on number of *NCF1* copies with C at NCF1-339; as 0C, 1C and ≥2 C genotypes. In the clinical analysis of APS patients were divided into two groups: C genotypes (≥2 C) and T genotypes (0C and 1C).

Key findings

Neutrophils from SLE patients with NCF1-339 0C and 1C genotypes had significantly impaired capacity to release NOX2 dependent NETs compared to neutrophils from patients with NCF1-339 2C genotype. NET release from neutrophils of NCF1-339 0C genotype patients was more dependent on mitochondrial ROS than neutrophils from patients with NCF1-339 2C genotype. NET release via pathways independent NOX2 derived ROS was unaffected by NCF1-339 genotype.

Four out of five SLE patients with NCF1-339 0C genotype had high serum IFN levels, despite inactive disease or low disease activity at the time of blood sampling. This was significantly higher than the proportion of ca 20 % of SLE patients with NCF1-339 2C genotype which had high serum IFN levels.

A significantly increased proportion of SLE patients with NCF1-339 0C genotype had antibodies towards B2GP1 and CL compared to patients with NCF1-339 1C and 2C genotypes. No other antibodies investigated were significantly associated with NCF1-339 genotype.

Antibodies towards B2GP1 and CL are hallmarks of the cardiovascular syndrome APS, common among SLE patients. In an analysis of 1087 SLE patients from four university hospitals, SLE patients with NCF1-339 T genotypes to a larger extent had a clinical diagnosis of APS.

Conclusions

This study showed that NCF1-339 genotype affects many aspects of the immune system and is associated with alterations in both cellular, systemic, and clinical features of SLE.

On a cellular level, a NCF1-339 0C genotype with impaired NOX2 function, affects the capacity of neutrophils to release NETs and skews NET release towards a pathway dependent on mitochondrial ROS. Systemically, the NCF1-339 0C genotype is associated with high levels of circulating IFN and presence of antiphospholipid autoantibodies. On a clinical level, SLE patients with low ROS NCF1-339 T genotypes to a larger extent have secondary antiphospholipid syndrome.

Together the results demonstrate how phagocyte ROS production can shape many aspects of the immune system, from neutrophil function to autoantibody profile and clinical outcomes.

Study IV. Identification of novel autoantigens in juvenile idiopathic arthritis

Background and aim

Many patients with JIA are positive for autoantibodies, but the specific antigens targeted by autoantibodies in JIA are largely unknown. ANA are common, and as ANA are detected by immunofluorescence staining of cells presence of these autoantibodies can be detected without knowledge of the specific antigens. In JIA, ANA are associated with early disease onset and an increased risk of developing uveitis.

However, as ANA are very common, they are of limited use as predictive biomarkers to identify children with risk to develop uveitis. Knowledge of specific

autoantigens in JIA could both increase understanding of the pathogenesis and be helpful in the search for biomarkers with diagnostic, prognostic, or monitoring potential.

This study aimed to identify JIA autoantigens with the potential to distinguish between JIA patients and healthy controls, and autoantigens associated with JIA related uveitis.

Method

Two large-scale exploratory methods were used to identify potential autoantigens in JIA; an autoimmune profiling planar array and immunoprecipitations.

In the planar array, 42 100 peptides from 18 000 unique proteins, representing approximately 94 % of the human proteome, were spotted on glass slides. The slides were incubated with JIA plasma pools, stained for anti-human IgG, and detected by immunofluorescence scanning.

In the immunoprecipitations, JIA plasma was incubated with whole cell protein extracts. IgG were captured by Protein G-coupled magnetic beads, bound proteins were eluted and identified by LC-MS which identifies proteins based on peptide mass fingerprinting.

Based on hits from the planar array, immunoprecipitations, and previous literature on autoantigens in JIA and uveitis, a selection of 335 peptides were investigated in a targeted array, coupled to colour coded magnetic beads. Serum from 57 patients with oligoarticular or RF negative polyarticular JIA and 22 paediatric healthy controls were analysed in this array.

Briefly, JIA autoantigens were defined as antigens with reactivity in <10 % of the paediatric controls and >10 % of the JIA patients.

Key findings

Reactivity towards a total of 332 peptides were detected in the planar array, and 131 proteins in the immunoprecipitations. There was very little overlap between the two methods, with only two proteins being detected by both methods.

In the targeted array, 73 peptides were excluded based on high reactivity in the controls. Of the remaining 262 peptides, 20 had reactivity in at least 10 % of the JIA patients.

Of the 20 potential JIA autoantigens, 16 were completely novel, to our knowledge not previously described in JIA. The identification of JIA autoantibodies towards histones, Sjögren's syndrome antigen B (SSB) and GAPDH, previously described autoantigens in JIA and autoimmune eye disease, verified the assay.

JIA patients had a higher number of reactivities towards the identified potential autoantigens compared to the controls. As many as 86 % of the patients had antibodies towards at least two of the peptides, compared to 23 % of the controls. Antibodies towards three or more of the peptides was present in 47 % of the JIA patients and 4.5 % of the controls.

Three peptides, from the proteins KDM7A, KDM3B and ZBTB22, were strongly associated with uveitis. All patients with uveitis in the cohort had antibodies towards at least one of these peptides, compared to 19 % of the patients without uveitis.

Conclusions

This study used a combination of exploratory and targeted methods for identification of JIA autoantigens. A total of 20 potential JIA autoantigens were identified, 16 of which have not previously been described as autoantigens in JIA. Three antigens were strongly associated with uveitis.

Discussion

The entire immune system is affected in autoimmune rheumatic diseases, where abnormalities in one aspect can disrupt the balance of the whole immune system. This thesis has focused on the role of neutrophils and autoantibodies, connected via intricate immune cell communications, in autoimmune rheumatic disease. Figure 10 represents a schematic overview of the interactions between neutrophils and the adaptive immune system studied in this thesis.

The underlying immunopathology of JIA and SLE have several common features, including the presence of autoantibodies and a neutrophil activation signature. The fact that the autoantibodies in both JIA and SLE are primarily targeting nuclear material, albeit with different specific autoantigens, is indicative of a shared underlying mechanism.

Possibly, impaired clearance of dead cell remnants by neutrophils can contribute to the autoimmunity towards nuclear material. Neutrophils in SLE are described to have impaired phagocytic capacity (210), and this was also observed in neutrophils of inflamed joints in JIA patients in this thesis. Impaired production of NOX2-derived ROS could also be a common denominator between JIA and SLE. ROS are important immunoregulators and low capacity of oxidative burst was associated with disease in studies I-III of this thesis. In this thesis, neutrophil alterations were detected in the blood in SLE but only locally in the inflamed joint in JIA, indicative of how SLE is a systemic and JIA an organ-specific autoimmune disease.

The main difference between the immunopathogenesis of JIA and SLE is the IFN-signature, central in the SLE pathogenesis but not in JIA. IFNα-containing cells are found in JIA synovial tissue and -fluid, but their presence is not associated with elevated IFNα in blood or synovial fluid (272). However, the IFNα-expressing cells were localized adjacent to lymphoid follicular structures in the synovial tissue (272), indicating that it could have a role in local stimulation of T- and B cells in inflamed joints.

The partially shared immunological alterations between JIA and SLE are expected as the diseases are part of the same disease spectrum. Considering this, it is likely that the immunological mechanisms and processes studied in this thesis are involved also in the pathogenesis of other autoimmune rheumatic diseases, in addition to JIA and SLE.

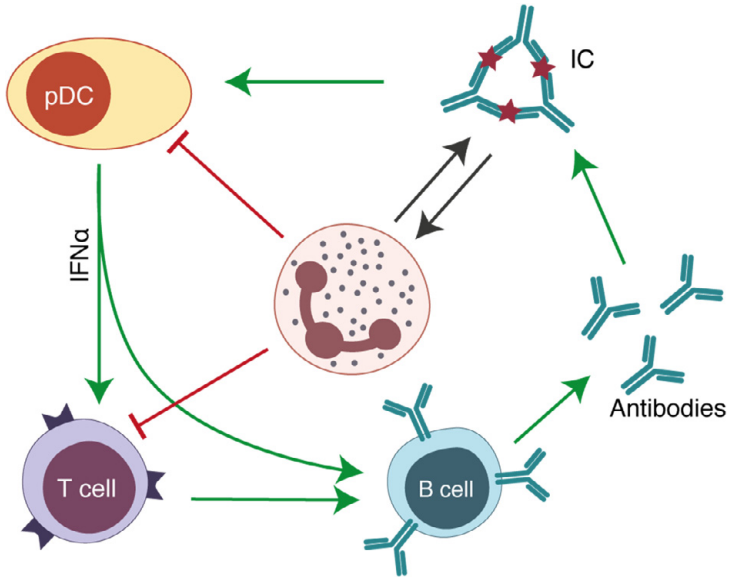

Figure 10. Summary of neutrophil- and antibody-driven immunology in JIA and SLE. Green arrows indicate activating signals, red lines indicate inhibition, grey arrows indicate interaction. Neutrophils can suppress T cell activation and IFNα production by pDCs, and have a dual relationship with IC as they can both contribute with autoantigens, clear IC via phagocytosis and become activated by IC-stimulation. B cells are activated by T cells and IFNα signaling, and can differentiate into antibody-producing plasma cells. ICs can induce further IFNα release from pDCs.

In study I, neutrophils in synovial fluid were found to be activated and functionally impaired. The synovial neutrophil phenotype was polarized, with expression of surface markers related to monocytes and macrophages. Although being of different cell types, neutrophils and monocytes are closely related and developed from the same myeloid lineage. Recent research suggest that immune cells are developed in a phenotypic continuum (273), and neutrophils expressing APC-markers and even neutrophils "transdifferentiating" into monocytes have been demonstrated in vitro (51,274,275), showing that neutrophils can polarize towards other cell phenotypes despite being terminally differentiated.

The impaired phagocytic capacity seen in synovial fluid compared to circulating neutrophils could mediate impaired clearance in the inflamed joint, and thus contribute to increased autoantigenic burden or IC-formation (Figure 10). Both phagocytosis and oxidative burst were related to the proportion of CD206-expressing neutrophils in synovial fluid, demonstrating that the phenotype and function is closely connected. Monocytes tend to be slower at phagocytosis and have lower capacity for oxidative burst compared to neutrophils, so possibly the

alteration in neutrophil function in synovial fluid is another feature of the neutrophil polarization.

In study II, both JIA synovial fluid neutrophils and neutrophils which had migrated towards synovial fluid in vitro displayed impaired capacity to suppress Th cells. A lack of Th cell suppression is likely to contribute both to sustained inflammation and development of local autoimmune reactions in the joint, as activated Th cells potentiate further adaptive immune responses (Figure 10). There are T cell imbalances in the JIA immunopathogenesis, and it is possible that some of these T cell imbalances might be a consequence of impaired immunoregulation by neutrophils.

The mechanism for lack of immunosuppression seems to be at least partially driven by decreased NOX2-derived ROS, indicating that the functional impairment of synovial neutrophils detected in study I can have negative consequences on the local immunoregulation. Immunosuppressive delivery of ROS from neutrophils to T cells is thought to occur via an immunological synapse, as T cell suppression is dependent on both ROS and cell-cell contact (56). In the proteomic analysis of migrated neutrophils, many proteins involved in cell-cell contact were decreased, supporting this hypothesis.

In study III, a the NCF1-339 SNP, mediating impaired NOX2 function, was associated with altered NET formation, a type I IFN-signature and presence of aPL. This study is adding on to previous results demonstrating that NCF1-339 T genotypes are strongly associated with SLE and an early disease onset (175). The importance of the polymorphism is supported by a recent study investigating NCF1-339 (NCF1 R90H) in a mouse model. They found that mice homozygous for the polymorphism spontaneously developed features of autoimmunity, including elevated type I IFN levels (276). When challenged with pristane, the homozygous mice developed SLE-symptoms including nephritis and autoantibodies (276).

Contradictory to many studies demonstrating how NETs contribute to SLE pathology, neutrophils from the patients with the low-ROS NCF1-339 T-genotypes had impaired capacity for canonical NOX2-dependent NETosis. However, NET release by NOX2-independent pathways was not affected by the SNP, and evidence suggest that SLE-NETs are primarily generated by pathways independent of NOX2-derived ROS (277). The decreased capacity for NOX2-dependent NETosis in patients with NCF1-339 T-genotypes might therefore be of limited biological relevance. More probably, it is the impairment in ROS-mediated suppression of excessive immune responses, including inhibition of IFNα signaling and T cell activation (Figure 10), which is responsible for the disease association of the NCF1-339 T-genotype.

Little is known about the role of NOX2 in APS, but the two conditions share several immunological mechanisms (98,167). Potentially, the low levels of NOX2-derived

ROS cause a compensatory increase in mtROS, which could lead to mitochondrial dysfunction and extrusion, exposing cardiolipin.

In study IV, JIA autoantigens and their relation to uveitis were investigated. 20 potential JIA autoantigens were detected, three of which were significantly enriched among patients with uveitis. Among the potential JIA autoantigens, four have previously been described as autoantigens in JIA and uveitis; three of them (two histone peptides and SSB) in JIA (137,252,261), and one (GAPDH) in autoimmune eye disease (278). The detection of previously known antigens validates our method and increases the trustworthiness of the novel antigens detected.

No antigen could singlehandedly distinguish between JIA patients and paediatric controls, but the higher cumulative number of reactivities in patients than controls suggest that an analysis of antibody combinations could be useful to identify JIA. Three of the antigens were significantly associated with uveitis, and all patients with uveitis included in the study had antibodies towards at least one of these antigens, compared to less than 20 % of the patients without uveitis. This indicates that these antigens could be much more useful than ANA-status when predicting risk for uveitis.

Conclusion

The studies of this thesis identified alterations in, and a connection between, neutrophil function and autoantibody profiles in JIA and SLE. In these works, the neutrophils seem to mediate disease primarily by being less efficient at immunoregulatory processes such as clearance and immunosuppression, rather than by actively potentiating inflammation. The lack of neutrophils acting as an immunological brake increases the risk of autoimmune reactions, and thereby affects the autoantibody profile. The autoantibody profile is in turn associated with the clinical outcomes. Everything is connected.

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