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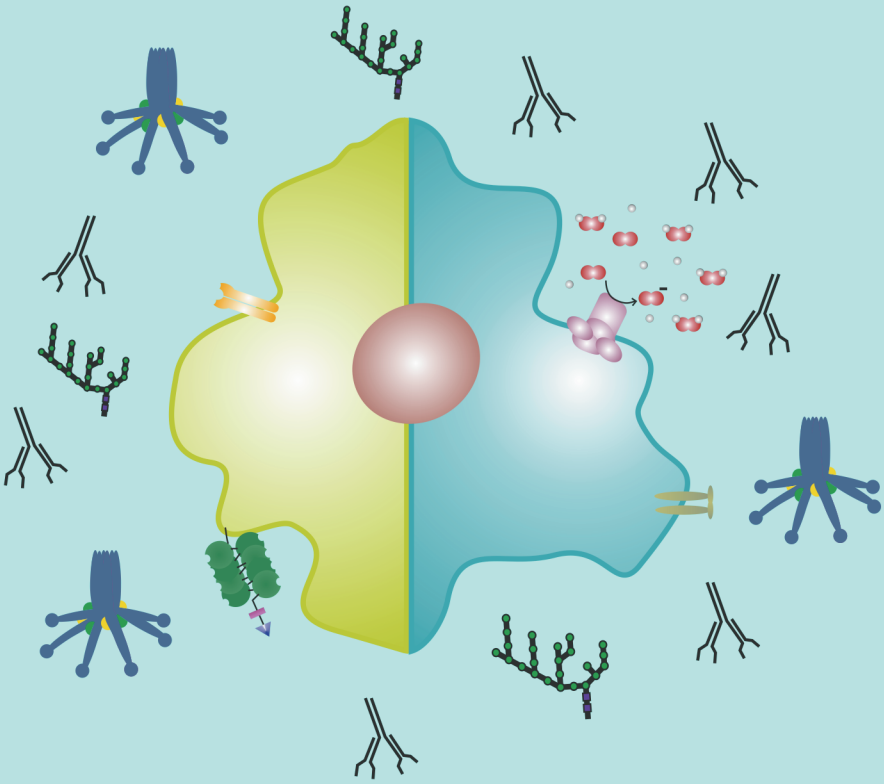

THE INNATE IMMUNE SYSTEM AS A DRIVER OF RHEUMATOID ARTHRITIS

Cecilia Hagert

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Till Britt Elving &
Britt Lena Hagert

You were given this life because you are strong enough to live it.
Unknown

Aut viam inveniam aut faciam
Hannibal

ABSTRACT

Cecilia Hagert
Innate immune system as the driver of Rheumatoid Arthritis

University of Turku, Faculty of Medicine, Medical Microbiology and Immunology, Turku Doctoral Programme of Molecular Medicine (TuDMM), The National Doctoral Programme in Informational and Structural Biology (ISB)

Turku, 2017

Rheumatoid arthritis (RA) is a chronic, inflammatory, autoimmune disease traditionally believed to be driven primarily by the adaptive immune cells. However, a large infiltrate of innate cells can be seen in the inflamed joints of patients. Furthermore, macrophages and the complement system, important parts of the innate immune system, have been implicated to have a role in chronic RA. To investigate the role of the innate immune system in RA a new model, independent of the adaptive immunity was created; called mannan-induced collagen antibody induced arthritis (mCAIA) in which the role of macrophages and complement system was confirmed as enough to drive a chronic arthritis in a reactive oxygen species (ROS) deficient milieu. ROS has previously been implicated to affect the development of arthritis. To further investigate this a transgenic ROS inducible mouse was created to investigate the role of ROS at different stages of the disease; this mouse is deficient of ROS until induced. ROS was shown important for regulating both the primary and the effector phase of the disease, thus postulating a broad role for ROS as regulators of autoimmune disease. Interestingly, the collagen specific T cells were of importance primarily in the beginning of disease but no difference could be seen between sick and healthy mice in later disease, while the macrophages seemed of importance throughout the disease. Another regulator of the autoimmune disease was found to be the macrophage mannose receptor (MR). Loss of MR caused both higher severity in mCAIA and in the psoriatic arthritis model mannan-induced psoriasis (MIP). In conclusion, a hypothesis is presented where the adaptive immunity (e.g. T cells and autoantibodies) is of importance primarily in the initiation of arthritis, but the chronic arthritis is primarily driven by the innate immunity (e.g. macrophages, complement cascade and neutrophils). Further studies are required to confirm this, but if true it could affect the manner we treat RA patients or a subgroup of them, focusing it more towards medicine targeting the innate immunity.

Keywords: Rheumatoid arthritis, reactive oxygen species (ROS), chronic mice model, macrophage mannose receptor (MR), innate immunity

SAMMANDRAG

Cecilia Hagert
Det medfödda immunförsvaret driver reumatism

Åbo Universitet, Medicinska fakulteten, Medicinsk Mikrobiologi och Immunologi, Åbo Doktorandprogram i Molekylär Medicin (TuDMM), Nationella Doktorandprogrammet i Informations- och Strukturellbiologi (ISB)

Åbo, 2017

Reumatisk artrit (RA) är en kronisk, inflammatorisk, autoimmun sjukdom traditionellt definierad som huvudsakligen adaptiv immuncellsdriven. Ett stort infiltrat av medfödda immunceller kan dock ses i patienters inflammerade leder. Både makrofager och komplement systemet, viktiga delar av det medfödda immunsystemet, har kopplats till kronisk RA. För att undersöka det medfödda immunsystemet vidare, utvecklades en ny kronisk artritmodell oberoende av det adaptiva immunförsvaret; kallad mannan inducerad collagen antikropps inducerad artrit (mCAIA). mCAIA drivs av makrofager och komplement systemet i möss med bristfällig produktion av syreradikaler (ROS). ROS har en tidigare påvisad roll i reglering av artrit. För att vidare studera detta utvecklades en mus som saknar full ROS produktion innan det induceras; detta för att kunna undersöka ROS roll i olika faser av sjukdomsförloppet. ROS visades viktigt i regleringen av både den primära och kroniska fasen av RA. Intressant var att collagen specifika T celler visade sig ha en roll i den primära fasen men ingen skillnad sågs mellan sjuka och frisk individer i den kroniska fasen, medan makrofager verkar vara viktiga i bägge sjukdomsfaser. En annan viktig regleringsmekanism av autoimmun sjukdom visade sig vara makrofag mannos receptor (MR). Förlust av MR leder till en allvarligare sjukdom i både mCAIA och i en psoriasis artrit modell. Som konklusion har en hypotes lagts fram där det adaptiva immunsystemet (till exempel T celler och autoantikroppar) är viktigt främst i initieringen av artrit, medan den kroniska artriten främst drivs av det medfödda immunsystemet (till exempel makrofager, komplementsystemet och neutrofiler). Vidare studier behövs, men om det stämmer kan det påverka hur vi medicinerar RA patienter eller en subgrupp av dem. Den nya medicineringen skulle då fokusera mer mot att påverka det medfödda immunförsvaret.

Nyckelord: Reumatisk artrit, syreradikaler, kronisk mus modell, makrofag mannos receptor (MR), medfödda immunsystemet

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ABBREVIATIONS

aCII	anti-CII
ACIA	Antigen- and Collagen Induced Arthritis
ACPAs	Autoantibodies against Citrullinated Peptides
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
APC	Antigen Presenting Cells
BCR	B Cell Receptor
C5	Complement Factor 5
C5aR	C5a receptor
CAIA	Collagen Antibody Induced Arthritis
CD	Cluster of Differentiation
CFA	Complete Freud's Adjuvant
CGD	Chronic Granulomatous Disease
CIA	Collagen Induced Arthritis
CII	Collagen type II
COMPIA	COMP Induced Arthritis
CPA	Chronic Proliferative Dermatitis
DDA	Dimethyl-Dioctadecylammonium Bromide
EAE	Experimental Autoimmune Encephalomyelitis
FcR	Fc Receptor
FcγR	Fcγ Receptor
H	Heavy
HLA	Human Leukocyte Antigen
IFA	Incomplete Freud's Adjuvant
Ig	Imunoglobulin
IFN	Interferon
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
IMQ	Imiquimod
INV-AR	Involucrin Enhancer/Promoter-Dependent Expression of Human Amphiregulin
L	Light
MASPs	MBL-Associated Serine Proteases

mCAIA	Mannan-Induced CAIA
MBL	Mannan Binding Lectin
MHC	Major Histocompability Complex
MIP	Mannan-Induced Arthritis
MS	Multiple Sclerosis
MR	Macrophage Mannose Receptor
Ncf1	Neutrophilic Cytosolic Factor 1
NETs	Neutrophil Extracellular Traps
NK	Natural Killer
NOX	NADPH Oxidase
PADs	Peptidyl Arginine Deiminases
PAMPs	Pathogen-Associated Molecular Patterns
PGIA	Proteoglycan-Induced Arthritis
PTPN22	Protein Tyrosine Phosphatase, Non-Receptor type 22
PIA	Pristine-induced arthritis
Ps	Psoriasis
PsA	Psoriatic Arthritis
PSORS	Psoriasis Susceptibility
RA	Rheumatoid Arthritis
RF	Rheumatic Factor
ROS	Reactive Oxygen Species
SCID	Severe Combined Immunodeficient
SLE	Systemic Lupus Erythematosus (SLE)
TAM	Tamoxifen
TCR	T Cell Receptor
Tfh	T follicular helper
Th	T helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	T regulatory

LIST OF ORIGINAL PUBLICATIONS

The following original publications, which will be referred to in the text by Roman numbers, constitute the basis of this thesis. The original publications are reprinted with permission of the copyright holders.

Paper I. **Chronic active arthritis driven by macrophages without involvement of T cells** Hagert C, Sareila O, Kelkka T, Nandakumar KS, Collin M, Xu B, Guerard S, Bäcklund J, Jalkanen S, Holmdahl R. *Submitted*

Paper II. **The macrophage mannose receptor has a protective role in both mannan-induced psoriasis and rheumatoid-like arthritis.** Hagert C, Sareila O, Kelkka T, Jalkanen S, Homdahl R. *Submitted*

Paper III. **Reactive Oxygen Species Regulate both Priming and Established Arthritis, but with Different Mechanisms.** Sareila O, Hagert C, Kelkka T, Linja M, Xu B, Kihlberg J, Holmdahl R. Antioxid Redox Signal. 2017 DOI: 10.1089/ars.2016.6981

Paper IV. **Comparison of a Natural Loss-Of-Function Single Nucleotide Polymorphism with a Targeted Deletion in the Ncf1 Gene Reveals Different Phenotypes**. Sareila O, Hagert C, Rantakari P, Poutanen M, Holmdahl R. Direct PLoS One. 2015 10(11): e0141974 DOI: 10.1371/journal.pone.0141974

1 INTRODUCTION

The innate immune system is the body's first defense against pathogens, it is fast and imperfect, but it saves us from many pathogen-driven diseases. It is also the inducer of the more specific, but slower, adaptive immune system responsible for long term protection against diseases through memory. Autoimmunity is when these systems become defective, reacting on self-antigens and causing an over active immune system. The body has many methods of regulating the immune system. One of the more recently described regulators is reactive oxygen species (ROS). First presented as an important part of the defense against pathogens, they are now also acknowledged as regulators of signal pathways. ROS are produced in innate cells such as macrophages and neutrophils and, they play an important role in protection against diseases, such as the autoimmune diseases rheumatoid arthritis (RA) and psoriatic arthritis (PsA).

RA is a disease affecting about 1 % of the population, causing joint destruction and pain as well as other comorbidities. Generally, the disease is believed to be initiated by an unknown environmental factor that, because of genetic predisposes, develops into the autoimmune disease in the patient. The recognition of the disease is traditionally thought to be done by cells of the innate immune system that then, just like when trying to protect the body from pathogens, initiates the adaptive immune system. Unfortunately, in autoimmune disease the immune system primes them towards attacking the body's own cells and not a foreign pathogen. For unknown reasons the body fails to block this unwanted effect and the attack on self is becoming chronic. The attack on its own cells is what is causing the pain.

PsA is a form of psoriasis (Ps). Psoriasis affects 2-3 % of humans while 25 % of these develop PsA. Psoriasis affects mainly the skin, causing itchy lesions not only affecting the skin's normal barrier towards pathogens but also causing much irritation for the patient. PsA patients has the lesions of Ps and the joint damage of arthritis. The initiation of Ps and PsA are similarly thought to be instantiated by the innate cells, after an induction of a combination of environmental and genetic factors, initiating a disease driven by an uncontrolled adaptive immune response.

Recently, pathogens have been implicated as initiators of autoimmune diseases in humans. It is therefore interesting that two of the models used in this study are initiated by the injection of mannan, a carbohydrate extracted from the cell wall of *S. cerevisiae*. One of the receptors binding to mannan is the mannose receptor (MR), a C-type lectin receptor present on macrophages and lymphatics. MR is also used as a marker for the macrophages dedicated towards down-regulating an immune response (type II macrophages). Macrophages have been shown to be present in arthritic joints in humans but also to play a role in the PsA mouse model of

mannan-induced arthritis (MIP) and the RA model of mannan-induced collagen antibody induced arthritis (mCAIA). Furthermore, elevated levels of MR have been observed in both PsA and RA patients.

This thesis presents the importance of the innate pathways in chronic arthritis. To this end a new mouse model was designed called mCAIA (paper I). The role of MR in both in the new arthritis model and in a model for PsA was investigated in paper II, also taking the time to investigate the role of ROS in the regulation of this pathway. In paper III, the role of ROS at different stages of the disease were investigated by creating an inducible knock-in mouse. Furthermore, the genetic effects at these stages were studied, taking care that the effects seen are not artificially induced by our genetic modifications as can be seen in paper IV.

2 REVIEW OF LITERATURE

Both innate and adaptive immunity have been implicated multiple times in the initiation of RA and PsA. A general belief is that a combination of genetic and environmental factors triggers the innate cells to become activated against a self-antigen and that these subsequently will activate the adaptive immunity, mostly B and T cells. The B cell will produce autoantibodies, regularly seen years before onset of RA, which binds to protein structures such as collagen. The T cells will attack the antibody marked cells and this will cause damage. The damage will expose more proteins that can be marked and the process can then be repeated. The process is similar for PsA. However, important innate cells such as macrophages and neutrophils have been found elevated in the joints of chronic RA patients hinting that they might play a more important role in the pathogenesis of the disease than previously thought. This has already prompted some investigation into the innate immunity's role in chronic RA. The dogma, however, is still that the adaptive immune system is the main driver of the disease. (Yau, Holmdahl 2016, Khmaladze, Nandakumar et al. 2015, Khmaladze, Kelkka et al. 2014, Krueger, Bowcock 2005, Firestein, McInnes 2017, Kinne, Schmidt-Weber et al. 1995, Kinne, Brauer et al. 2000, Barrera, Blom et al. 2000)

2.1 Innate immunity

The innate immune system is the first response to pathogens. It is divided in immediate immunity (occurring 0-4 hours after an infection by pathogens) and early induced innate response (active 4-96 hours after infection). After this the adaptive immune system becomes the most important actor to clear the disease; if the pathogens have breached the first defense lines. There are five groups of agents causing diseases: virus, bacteria, fungi, protozoa and helminths. The innate system can also recognize stressed or injured cells; by their expression of molecules not commonly expressed in large amounts in healthy cells, for example heat shock proteins. This ability is also used when finding and clearing virus infected cells. Common traits of receptors in the innate immune system is that they are; (a) specifically inherited in the genome, (b) expressed by all cells of a particular type (for example neutrophils), (c) triggering an immediate response of the immune system to combat the pathogen, (d) recognizing a broad spectrum of pathogens and (e) interacting with a range of different molecular structures of a given type. (Murphy, Travers et al. 2008, Abbas, Lichtman et al. 2007)

2.1.1 Macrophages

Macrophages are found in large numbers throughout the body especially in lung, peritoneum, along the blood vessels in the liver and throughout the spleen. They are efficient at phagocytosing and subsequently eliminating pathogens. They also secrete cytokines to stimulate the rest of the immune system, recruiting more cells to the site of infection. To some extent they can also activate the adaptive immunity by peptide presentation. (Murphy et al. 2008, Abbas et al. 2007)

Macrophages can be divided into two main types called the classical or type I macrophages and the alternative or type II macrophages. Type I macrophages are grouped due to their ability to induce prototypic inflammatory responses and express markers such as inducible nitric oxide synthase (iNOS), major histocompability complex (MHC) II and tumor necrosis factor (TNF)-α. Activation of these cells will perform phagocytosis and elimination of pathogens and induce the adaptive immune system towards a Th1 response. The type I macrophages are induced after interferon (IFN)-γ, lipopolysaccharide (LPS) or TNF stimulation. (Gordon 2003, Martinez, Gordon 2014)

Type II macrophages are grouped together mainly due to their ability to antagonize a prototypic inflammatory response. To some extent they can be subdivided into type IIa, IIb and IIc. The three subtypes of type II macrophages have been said to be induced by different stimuli and to induce slightly different responses; (IIa) is induced by interleukin (IL) 4 and IL13, start expressing MR, Arginase I, IL10 and MHC II that will lead to a Th2 response in the adaptive immunity and are involved in the development of allergies, they can also kill or encapsulate parasites, (IIb) induced by Toll-like receptors (TLRs) and IL1 receptor ligands, leading to expression of IL10 and MHC II (but no MR or Arg) causing immune-regulation and Th2 activation, (IIc) is induced by IL10 to express MR and IL10 causing deactivation of the immune system, matrix deposition and tissue remodeling. (Martinez, Gordon 2014) Utilizing an *in vitro* model, (Y. Zhang, Choksi et al. 2013) postulate that ROS play an essential part in differentiating macrophages to a type II phenotype, but not a type I phenotype: a theory that needs further confirmation.

2.1.2 NOX2 and ROS

The NADPH oxidases (NOX) 2 complex is part of a larger family of NOX complexes (in total seven have been identified, e.g. NOX1-5, DUOX1-2) that have been conserved during evolution and are thus identified in plants, fungi, invertebrates and higher animals. General for all NOX complexes are that they have; (a)

six transmembrane spanning domains (DUOX have seven), (b) two haem-binding sites, and (c) one cytoplasmic NADPH binding site. NOX2 is the isoform found in phagocytes such as eosinophils, macrophages, dendritic cells and neutrophils. The NOX2 complex is important for antimicrobial defense and for regulation of inflammation. A disorder of NOX2, called chronic granulomatous disease (CGD), were the phagocytes are defective in generation of ROS, suffer from life-threatening bacterial and fungal infections. Their mortality rates are in direct relation to the number of neutrophils still expressing NOX2. When NOX2 gets activated a translocation of cytoplasmic subunits $p40^{phox}$ (alias NCF4), Ncf1 (alias $p47^{phox}$) and NCF2 (alias $p67^{phox}$) and Rac to the membrane-bound heterodimer cytochrome encompassing $gp91^{phox}$ (alias NOX2/CYBB) and $p22^{phox}$ (alias CYBA) is required. An activated NOX2 produces O_2^-, which quickly transforms to the more long lived H_2O_2. (Sareila, Kelkka et al. 2011, Singel, Segal 2016)

ROS, such as hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-), have been shown to have a physiological role in various biological processes, working either as an independent or cooperative regulator for cellular signaling in response to environmental cues. Due to its relatively long-lived nature and ability to pass easily through cellular membranes, H_2O_2 works as an important secondary messenger maintaining the cellular homeostasis. Furthermore, ROS has been implicated as an important actor in e.g. gene expression, protein translation, posttranslational modification, protein interactions, antigen presentation and cross-signaling to T- and B-cells; showing that ROS can regulate the adaptive immunity at multiple levels. (Tan, Wang et al. 2016, Singel, Segal 2016)

RA is associated with Ncf1 copy number in humans. Also, polymorphism is common in the human Ncf1 gene. However, it plays a different role in different autoimmune disease. The $Ncf1^{m1J}$ loss-of-function mutation is a naturally occurring point mutation, first seen in the C57BL/6J-m db/db mouse. Since, it has been backcrossed to cleaner genetic backgrounds such as C57BL/6 or B10.Q. In these backgrounds a deficiency in Ncf1 remarkably increased susceptibility to the autoimmune symptoms of the animal models of collagen induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE). The $Ncf1^{m1J}$ mice have also been described to develop spontanous lupus-like symptoms when on the Balb/c background. However, the exact role of the ROS in autoimmunity is not yet clarified. (Olsson, Johansson et al. 2017, Olsson, Lindqvist et al. 2007, Hultqvist, Sareila et al. 2011, Olofsson, Holmberg et al. 2003, van der Veen, Dietlin et al. 2000, Huang, Zhan et al. 2000, Hultqvist, Olofsson et al. 2004, Kelkka, Kienhöfer et al. 2014)

In this thesis, the MN mouse is utilized to address the role of macrophage produced ROS. It is a specialized mouse that is overall ROS deficient by a point mutation in

the Ncf1 gene, but is expressing wild type Ncf1 under a human CD68 promotor, hence only in macrophages. (Gelderman, Hultqvist et al. 2007)

2.1.3 Macrophage mannose receptor (MR)

Cluster of Differentiation (CD) 206, alias MR, is a C-type lectin type I transmembrane receptor, structurally consisting of an extracellular region containing an amino terminal cysteine rich domain, a fibronectin type II repeat domain, eight C-type lectin-like domains (CTLDs), a transmembrane region and a short cytoplasmic tail. It exists in at least two conformations; straight and bent. (Kerrigan, Brown 2009) It is present both on the cell membrane and in vesicles in the cytoplasm, it can thus bind both internalized and external antigens. It is designed to bind to certain sugar structures of many different viruses and bacteria to facilitate endocytosis of glycoproteins primarily by macrophages. It binds to high-mannose containing structures and collagen, among other structures, on the surface of potentially pathogenic bacteria, viruses, and fungi. This binding is traditionally thought to facilitate neutralization by phagocytic engulfment. MR has been proven to be present on myeloid cells and lymphatics. It has been shown that MR deficient mice have a decreased adhesion of lymphocytes to the lymphatics compared to wild type mice. Importantly for this thesis, mannan has been described to bind to MR. Of note is that human and mouse MR are homologs. (National Center for Biotechnology Information, U.S. National Library of Medicine 2016b, Barreto-Bergter, Figueiredo 2014, National Center for Biotechnology Information, U.S. National Library of Medicine 2016a, Marttila-Ichihara, Turja et al. 2008, Murphy et al. 2008, Gauglitz, Callenberg et al. 2012)

By investigation of human biopsies an increase of the MR in psoriasis (Ps) and dermatitis patients compared to normal skin was shown. (Wollenberg, Mommaas et al. 2002, de Koning, Rodijk-Olthuis et al. 2010) Human MR signaling was indicated to induce IL-17 production (van de Veerdonk, Marijnissen et al. 2009) a known driver of Ps. (Khmaladze et al. 2015) Also, as recently reported (Heftdal, Stengaard-Pedersen et al. 2017), patients with early arthritis have elevated levels of soluble MR and a decrease in these levels was seen after successful treatment of these patients with anti-TNFα and disease-modifying antirheumatic drugs (DMARDs).

2.1.4 Complement pathway

The complement pathway is made up of a large number of different plasma proteins interacting with each other to opsonize pathogens and act as chemoattractants and activators of macrophages and neutrophils. Complement can be activated by pathogens and large numbers of dying cells as for example in ischemic injury. There are three pathways activating the cascade; classical, lectin and alternate pathway. The **classical pathway** is initiated by C1q protein binding to the pathogens surface in one of three ways; (a) directly binding to components on the bacterial surface such as for example proteins and polyanionic surface structures, (b) binding to C-reactive proteins which binds to bacterial polysaccharides, and (c) binding antibody:antigen complexes. C1q then recruits and binds C1s and C1r, both proteases. This complex will cleave the complement factor (C) 4 and C2 into C4a/C4b and C2a/C2b respectively. (Murphy et al. 2008, Abbas et al. 2007)

Carbohydrate-binding proteins, such as mannan binding lectin (MBL) or ficolins, initiate the **lectin pathway.** MBLs are structurally similar to C1q. They bind mannose residues and MBL-associated serine proteases (MASPs) forming a complex. The complex subsequently cleaves the C4 and the C2 as in the classical pathway. (Murphy et al. 2008, Abbas et al. 2007)

Complement component 3 (C3) is always present in the plasma and is cleaved in a slow pace called the tickover. Hence, a small number of C3b will be present to bind to the surface of a pathogen and can thus in a spontaneous manner activate the **alternate pathway** of the complement pathway. When C3 is cleaved into C3a/C3b and C3b becomes bound to the microbial surface and a structure called factor B gets exposed on the C3b molecule. Bound factor B will in turn be cleaved by factor D, releasing Ba and Bb structures. The Bb structure will bind to C3b, creating the alternative pathways C3 convertase (C3bBb). (Murphy et al. 2008, Abbas et al. 2007)

All three activation pathways will lead to the same outcome; generation of the protease C3 convertase (C4b:C2b in classical and lectin pathway and C3bBb in alternate pathway) which will covalently bind to the pathogen surface. This part of the pathway is regarded as the ‘early part’ of the complement cascade where small pro-enzymes called zymogens are cleaved into a smaller and a larger part. The larger part is staying at the pathogen surface to be part of cleaving in the next step of the cascade while the smaller one leaves and works as a chemoattractant to the rest of the immune system. Initially C3 convertase will cleave C3 into C3a and C3b, the former being a mediator of the immune system, the latter being the main effector molecule of the cascade. C3b can perform two tasks, (a) bind the surface of the pathogen to mark it for destruction by cells expressing the C3b receptor such

as phagocytic cells or (b) bind to the C3 convertase to form the C5 convertase, that will produce the most potent inflammatory peptide (C5a) that will recruit even more phagocytes, as well as C5b that will initiate the late phase of the complement cascade which will eventually lead to the formation of the membrane attack complex that can lyse cells. (Murphy et al. 2008, Abbas et al. 2007)

The immune system has receptors binding to the C1q, C3b, C5a and C3a products of the complement cascade; the complement receptors (CR) and specific receptors C3aR and C5aR. Activation of these receptors stimulates phagocytosis and erythrocyte transport of immune complexes. Too much of the smaller fragments C3a, C4a and C5a can cause anaphylactic shock. In fact, there are decoy receptors such as C5aR2, that bind C5a but no signal is carried forward. Interestingly, increased gene expression of C5aR1 in macrophages is associated with LPS, while IL-4 downregulates C5aR1 expression. In fact, novel roles for components of the complement system in the resolution of inflammation and protection from autoimmune and inflammatory diseases have emerged. In particular, polarization of macrophages can be directed by C1q leading to an increase in pro-resolving macrophages. This will in turn promote clearance of apoptotic cells, diminish pro-inflammatory cytokine production and increased anti-inflammatory cytokine production. C1q thus has a dual role; activating the immune system by initiating the complement cascade and down-regulating the immune system by polarization of macrophages towards a type 2 response. (Murphy et al. 2008, Bohlson, O'Conner et al. 2014)

2.1.5 Toll-like receptors (TLRs)

There are thirteen different TLRs recognized to date. The TLRs all recognize different ligands and their subcellular localization is correlating to the nature of those ligands. Thus, TLR1, -2, -4, -5 and -6 are present on the cell surface membrane and are mainly sensors of bacterial carbohydrate structures. While intracellularly expressed in endosomal compartments are TLR3, -7, -8, -9, -11, -12 and -13, these are sensors of nucleic acids. However, TLR11 is only a pseudogene in humans while TLR12 and -13 are absent. All thirteen are present in mice. (Murphy et al. 2008, Shi, Cai et al. 2011, Pifer, Benson et al. 2011, Yarovinsky 2014) The general signal pathway of TLRs is recognition of Pathogen-associated molecular patterns (PAMPs) by their respective TLRs, initiating recruitment of adaptor proteins for example MyD88, which leads to recruitment and activation of protein kinases, which activate transcription factors leading to gene transcription. The gene transcription leads to expression of inflammatory cytokines (for example TNF, IL1

and IL2), chemokines, endothelial adhesion molecules, costimulatory molecules and antiviral cytokines (IFNα/β). (Murphy et al. 2008)

For this thesis, the most important TLRs are TLR2 and -4. TLR2 was concluded to be the main driver of CAIA (Kelkka, Hultqvist et al. 2012), while TLR4 was concluded not to be as important. However, TLR4 has been proven to bind to mannan (Abbas et al. 2007). Furthermore, a study using *Streptococcus pyogenes* induced arthritis have indicated a role for TLR2 in the initiation of disease but a later role for TLR4, possibly indicating a role for this receptor in development of a more long-term disease (Abdollahi-Roodsaz, Joosten et al. 2008).

2.1.6 FcγR

The cell surface receptors responsible for recognizing structures specific for the carboxy-terminal constant region of the immunoglobulin (Ig) molecule are called Fc receptors (FcRs); normally containing both signaling components and the Ig binding region. There are several different types of FcRs, recognizing a wide range of IgG isotypes, IgE and IgA. The FcRs mediate an extensive range of the cells effector functions after antibody interaction such as phagocytosis, activation of mast cells and both targeting and activating the NK cells. The receptor designated for the IgG antibodies are the Fcγ receptors (FcγR). FcγRs come in different types, ranging from FcγRI-IV in mice to FcγRI-III in humans, differing in their affinity for different IgG isotypes. All, but FcγRIIb that is connected to a de-activating ITIM, are connecting to an immune activating ITAM motif. They are all transmembrane proteins in innate cells such as neutrophils, macrophages, B cells, NK cells, eosinophils and basophils etc. FcγRI will cause activation and phagocytosis of phagocytes as will FcγRIIa and FcγRIIIb but in a less efficient manner. FcγRIIb is a negative feedback receptor in B cells. FcγRIIIa is involved in cell-mediated cytotoxicity. (Abbas et al. 2007, Lunemann, Nimmerjahn et al. 2015, Nimmerjahn, Ravetch 2008) The signaling pathways initiated by the binding of IgG to FcγRs (excellently reviewed in Nimmerjahn, Ravetch 2008) lead to either (a) cell inhibition of calcium flux, (b) activation after initiation of FcγRIII, (c) proliferation after simultaneous FcγRIIb: B cell receptor (BCR) activation or (d) apoptosis if no BCR is present.

2.2 Adaptive immunity

The adaptive immunity is initiated by the innate immunity through antigen presenting cells (APC), such as for example macrophages and dendritic cells. Perhaps the most striking characteristic of the adaptive immune system is its ability to

remember previously encountered antigens and thus mount a quick response towards the pathogen. All receptors in the adaptive immunity have in common that they: (a) are encoded in multiple gene segments, (b) require gene rearrangement, (c) have a large clonal distribution and (d) are able to discriminate between even closely related molecular structures. The adaptive immune system consists of αβ- and γδ-T cells, B cells, natural killer (NK) cells and NKT cells. (Murphy et al. 2008, Abbas et al. 2007)

2.2.1 T and B cells

αβ-T cells can be further divided into CD4+ helper T cells, CD8+ cytotoxic T cells and regulatory T cells (Treg). The CD4+ T cells are important for B cell differentiation and can also make macrophages more active, CD8+ T cells are involved in elimination of cells infected with pathogens and in the killing of tumour cells, while the Tregs are important as suppressors of the immune system by supressing other T cells and thus in extension maintaining self-tolerance. γδT cells are important as helpers of the innate system in clearing pathogens and also have cytotoxic functions. The role of B cells is primarily antibody production while the NK cells are in charge of killing the virus infected cells. NKT cells can, depending on the stimuli they get, either activate or supress both the innate and the adaptive immune system. The B and T cells are activated by the APCs via co-stimulating molecules and the BCRs or T cell receptors (TCR) respectively. (Abbas et al. 2007)

Depending on the cytokine milieu the CD4+ T cells can differentiate into different subsets; T helper (Th) 1, Th2, T follicular (Tfh), Th17 and the above mentioned Tregs. An IL12 and IL18 milieu, produced by activated DCs, activates a proliferation of Th1 cells. Th1 cells produce IFNγ, important for activating macrophages and to induce them to kill intracellular bacteria. IL-4 both induces and is produced by Th2 cells. IL4 can also induce type II macrophages. Tfh cells can make the IL4, IL12, IL18, and IL21 cytokines. They also express cell-surface molecules required for effective B cell responses and production of high-affinity, class-switched antibodies. In the presence of IL6 and tumour growth factor (TGF)-β, CD4+ T cells will develop into Th17 cells. Th17 cells produce IL17 and IL22 and are promoting infiltration of neutrophils to inflamed sites via IL17. IL22 will induce the production of antimicrobial peptides. (McKee, MacLeod et al. 2010)

The role of T cells is explored in paper III, where T cells specific to specific collagen locus are investigated and in paper I, where the dependence of the arthritis model is investigated in the RAG1 mouse; a mouse lacking T and B-cells. (Mombaerts, Iacomini et al. 1992)

2.2.2 Immunoglobulins

There are five different classes of Ig; IgM, IgG, IgD, IgA and IgE. They are all constituted of two heavy (H) chains and two light (L) chains (Figure 1), but can be distinguished by their C and V region; the latter accounting for the specificity of the antigen binding. The H chain is defining the class, which in turn correlates to the effector function of the antibody. IgG is the most abundantly common antibody and it has several subclasses (IgG1-4 in humans). The Fc part of the immunoglobulin is formed by the bottom of the two H chains, while the combination of the L chain and the other part of the H chain were L chain binds is called Fab (figure 1). The binding of an antibody to a pathogen will induce phagocytosis by the innate immunity and activation of the complement system via the classical pathway as previously described. (Murphy et al. 2008)

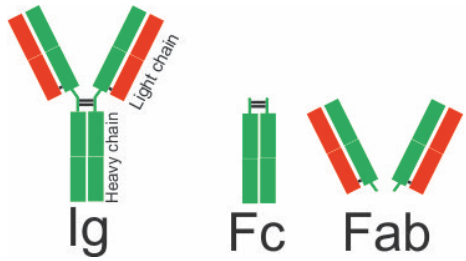

Figure 1. Immunoglobulins (Ig) have two heavy chains (green) and two light chains (red). The different parts of the immunoglobulins are kept together with disulfide bonds (illustrated in black). The bottom part of an Ig is called the Fc fragment and the top part consists of two Fab fragments. An Ig can be divided into these parts by exposure to the protease papain.

2.2.3 ACPA and citrullination

Citrullination is when peptidyl arginine deiminases (PADs) change arginine to citrulline by post-translational modifications. There are five PADs 1-4 and 6. PADs are found in numerous different cell types and tissues, such as epidermis and uterus (PAD1); brain, skeletal muscle, inflammatory cells and cancer cells (PAD2); hair follicles and keratinocytes (PAD3); several different cancer types and granulocytes (PAD4); while PAD6 is present in embryos and oocytes. All PADs are found intracellularly in the cytoplasm of the cell. (Trouw, Toes 2016, Bicker, Thompson 2013)

Autoantibodies against citrullinated peptides (ACPAs) are antibodies of IgG or IgA isotype that binds to proteins that have been citrullinated. Once bound, ACPA

have been indicated to activate the immune system in various ways such as activating the complement system, inducing bone loss and osteoclastogenesis, and is important for the formation of neutrophil extracellular traps (NETs). NETs, produced by neutrophils in response to triggers, contains decondensed chromatin and granule enzymes. NETs works as an antimicrobial attack "capturing" and breaking down the pathogen. (Trouw, Toes 2016)

2.3 Autoimmunity

Autoimmunity is when the immune system starts mounting a reaction towards self-antigens instead of pathogenic antigens. In healthy individuals, the cells in the body are protected thanks to the education of the adaptive immune system in thymus and the immune regulatory cells *e.g.* Treg and type II macrophages.

There are many different autoimmune diseases such as RA, Ps/PsA, Type 1 Diabetes (autoreactive T cells against pancreatic islet cell antigens rendering the loss of insulin production), Multiple Sclerosis (MS, autoreactive T cells against oligodendrocytes rendering the patient unable to control its nervous system), Systemic lupus erythematosus (SLE, autoantibodies and autoreactive T cells against DNA, chromatin, protein and ubiquitous ribonucleoproteins affecting most organs in the body) and Sjögren's syndrome (autoantibodies and autoreactive T cells against ribonucleoprotein affecting the moisture-producing glands of the body and causing dryness of mucus lining organs such as month and vagina but also joint pain). (Murphy et al. 2008) In this thesis two of these have been further investigated; RA and Ps/PsA.

2.3.1 Rheumatoid arthritis (RA)

RA has an incidence of 0,5-1 % in the human population, with lower incidence in the north hemisphere compared to south and also lower incidence in urban compared to rural areas. It is a chronic inflammatory joint disease, which causes cartilage and bone damage, rheumatoid nodules, pulmonary involvement or vasculitis, and systemic comorbidities, as well as disability. RA is a substantial burden for both the individual (declining physical function due to musculoskeletal deficits, pain and a cumulative comorbid risk lowering the quality of life) and the society (economic burden due to heavy medications and loss of work force). It is partly a genetic disease; the human leukocyte antigen (HLA), MHC in mouse, is considered the major genetic factor but other genetic loci have also been identified such as protein tyrosine phosphatase, non-receptor type 22 (PTPN22) and Ncf1. ACPAs

and autoantibodies against IgG (*e.g.* rheumatoid factor [RF]) are linked to increased risk for RA and are present in 50–70 % of patients at diagnosis and often remain at stable levels during the course of the disease. ACPA positive patients are often more severely sick compared to ACPA negative patients. Both ACPAs and RF are regularly used to diagnose RA., along with joint pain and destruction and morning stiffness. Environmental factors *e.g.* smoking and exposure to certain pathogens have been reported as risk factors for development of RA. Macrophages, dendritic cells, disease specific T cells against antigens of the joints, *e.g.* collagen, and autoantibodies produced by B cells (examples are RF and ACPAs) have been suggested as drivers of the disease. It is a disease which varies in severity and can thus in periods be better or worse both because of natural variations in the disease or successful medications. (Smolen, Aletaha et al. 2016, Olofsson et al. 2003, Olsson, Nerstedt et al. 2012, Kinne et al. 1995, Barrera et al. 2000, Turunen, Huhtakangas et al. 2016, Trouw, Toes 2016)

2.3.2 Psoriasis (Ps) and Psoriatic arthritis (PsA)

Ps affects 2-3 % of the world-wide population and PsA affects 25 % of those getting Ps. Ps and PsA are thus both common diseases in human, but are unfortunately insufficiently understood. Induced by genetic contributors *e.g.* psoriasis susceptibility locus (PSORS) and unknown environmental factors, they become chronic inflammatory diseases causing mainly skin lesions while PsA also causes joint inflammation and destruction. Both Ps and PsA also cause systemic manifestations. Although Ps lesions have been shown to be initiated or exacerbated by inflammation-induced stimuli, physical injuries to the skin (the "Koebner response") and/or various infections (such as *Candida* infections), there are still questions regarding the reasons of disease development. Both type I and II macrophages have been shown to play an important role in models for Ps and PsA and macrophage depletion have been shown to lead to protection. Neutrophils, dendritic cells and mast cells are also of importance and can be found in abundance in the Ps plaques. Mast cells are most likely the main source of IL17 rather than T cells. However, T cells have been seen as the major mediators of Ps were CD8+ T cells and CD4+ T cells are producing cytokines such as IL2, IFNγ and TNFα. NKT cells will drive inflammation and produce IFNγ and in Ps the Treg cells have a deficient functional suppressor activity both in the skin and blood of the patients. Furthermore, oxidative stress has been believed to be a key factor in the pathogenesis of Ps. Discharge of ROS from cells in the plaques might have chemotactic effects on neutrophils and phagocytic processes, that might cause a high superoxide (O_2^-) production. However, recent animal studies have shown a protective role of ROS in Ps and PsA by supressing the T cell response. Also, a trial treatment of Ps using hyperbaric oxygen therapy and thereby increasing

intracellular ROS has previously been demonstrated to be successful. (Michael, Boehncke 2005, Khmaladze et al. 2014, Kopp, Riedl et al. 2015, Khmaladze et al. 2015, Bowcock, Krueger 2005, Gaspari 2006)

2.4 Animal models

Since many autoimmune diseases start long before symptom onset, studying them in humans is hard. Also, they are often systemic diseases while human research is restricted to blood samples and occasional biopsies. This, together with the need for models to perform initial drug screenings, has made animal studies important in RA research.

For RA, there are many different models to utilize, however most of them are acute and therefore heal after a short time. Thus, they fail to model the whole disease. Generally, arthritis models are either inducible or genetic spontaneous models. Examples of genetic models are; the K/BxN, NZB/NZW, HuTNF Tg, IL-1RA$^{-/-}$, Tristetraprolin$^{-/-}$ and TNF gene mutation in AUUUA motif. Examples of induced models are; Collagen-induced arthritis (CIA), Pristane-induced Arthritis, Proteoglycan-induced arthritis, Zymosan-induced arthritis, Immune complex arthritis and Serum transfer models. (Kannan, Ortmann et al. 2005)

Spontaneous chronic, progressive inflammation is developed in the K/BxN mouse (KRN T cell receptor transgenic mouse on the C57BL/6 x NOD background) and as early as three weeks of age, the first symptoms of clinically visible joint inflammation can be observed which progressively evolves into a severe chronic inflammatory arthritis. The joint destruction is promoted by B cell produced autoantibodies and T cell involvement, making the model similar to the CIA model. As in CIA, arthritis can be transferred by serum to induce disease and can in this way be induced in most strains regardless of MHC. (Kannan et al. 2005, Kouskoff, Korganow et al. 1996) Another strain developing spontaneous arthritis is the NZB/NZW mice, the F1 generation of a cross between NZB and NZW mice. Similar to human disease the NZB/NZW mice produce IgM and IgG rheumatoid factors and have therefore also been used to model SLE. (Kannan et al. 2005)

One example of an induced arthritis model is Zymosan-induced arthritis where Zymosan, a polysaccharide from the cell wall of *Saccharomyces cerevisiae* is injected intra-articularly into the knee joints of mice where it binds to TLR2 in macrophages. This leads to the induction of pro-inflammatory cytokines, arachidonate mobilization, protein phosphorylation and activation of the complement system via the alternative pathway. This disease is a proliferative inflammatory arthritis.

It has mononuclear cell infiltration, synovial hypertrophy and pannus formation. The peak of disease is around day three and inflammation subsiding by day seven. (Asquith, Miller et al. 2009) Another example is pristane-induced arthritis (PIA), induced by the mineral oil 2,6,10,14-tetramethylpentadecane (known as pristane), causes a chronic inflammatory arthritis in mice similar to RA. Main symptoms of PIA are synovial hyperplasia, cartilage erosions, bone erosions, inflammatory cell infiltrates, and pannus-like formation. The disease establishes as a prolonged, delayed clinical time course of joint inflammation, normally starting between 60 to 180 days after pristane administration. (Potter, Wax 1981, Patten, Bush et al. 2004)

Inflammatory arthritis can be studied in various strains of mice, when primed with antigens like methylated bovine serum albumin in complete Freund's adjuvant and subsequent intra-articular challenged by an injection of the same antigen. In the investigation of hierarchical roles for given factors in the adaptive, immune-mediated articular damage of the joints these models can be of use. The pathology encompasses immune complex-mediated inflammation with a successive response of articular T-cell-mediated responses. However, the antigen-induced model does not recapitulate the endogenous breach of tolerance that is typical of RA pathogenesis. Antigen induced arthritis has thus limitations in applicability in the studies of arthritis. (Asquith et al. 2009)

In 1991 Keffer *et al.* (Keffer, Probert et al. 1991) created a transgenic mouse overexpressing human TNF-α, which develops chronic inflammatory and erosive polyarthritis. The dependency of the model on TNF-α is cemented by the treatment with a monoclonal antibody against human TNF-α, which completely prevents the development of arthritis. The model has a chronic progressive nature and bears close resemblance with RA, in contrast to CIA and adjuvant-induced arthritis, which are acute and self-limiting. (Asquith et al. 2009)

Existing inducible chronic models of arthritis have in common a dependency on infiltrating T- and B-cells to draining lymph nodes and a long priming period before arthritis is developed. Examples of these models are CIA, COMP induced arthritis (COMPIA), Proteoglycan-induced arthritis (PGIA) and antigen- and collagen induced arthritis (ACIA). Lasting for about a month, PGIA is a clinically manifesting disease model that sometimes manifests as a relapsing disease. PGIA is initiated by proteoglycan aggrecan emulsified in dimethyl-dioctadecylammonium bromide (DDA) adjuvant and injected intraperitoneally. The model is limited to BALB/c mice. Another limiting factor is that the proteoglycan used is isolated from human cartilage during joint replacement surgery. Another, recently developed model for chronic arthritis is a combination of CIA and antigen-induced arthritis called antigen- and collagen induced arthritis (ACIA). ACIA is inducing a chronic, subclinical mouse model limiting it to *ex vivo* analyses. Both CIA and

COMPIA are adjuvant dependent. (Carlsen, Hansson et al. 1998, Carlsen, Nandakumar et al. 2008, Corthay, Johansson et al. 1999, Baddack, Hartmann et al. 2013, Glant, Mikecz et al. 1987, Finnegan, Mikecz et al. 1999, Kannan et al. 2005) In this thesis, two arthritis models were used; CIA and a novel chronic version of collagen antibody induced arthritis (CAIA).

Models for Ps can either be spontaneous, genetically engineered, induced, or human skin transplant models. There are two spontaneous models, flaky skin model based upon the Ttc^{fsn}/ Ttc^{fsn} mice (however this model does not express all the characteristics of Ps) or chronic proliferative dermatitis (CPD) based on the $Sharpin^{cpdm}$/ $Sharpin^{cpdm}$ mice. At the age of 5-6 weeks, CPD mice develop skin lesions characterized by epidermal hyperplasia, hyperkeratosis, parakeratosis and necrotic keratinocytes mainly caused by an infiltration of the dermis and epidermis by granulocytes and macrophages. (Kauffman, Aria et al. 2004, Khmaladze et al. 2015, HogenEsch, Gijbels et al. 1993)

One genetically engineered model is the K14-IL-$17A^{ind/+}$ mouse which develops skin inflammation showing many characteristic hallmarks of Ps. Another genetically engineered model is the involucrin enhancer/promoter-dependent expression of human amphiregulin (INV-AR) model. It is a model located in the supra-basal epidermis of transgenic mice. These mice have epidermal hyperkeratosis, acanthosis, parakeratosis, an exaggerated dermal vasculature, exaggerated infiltration of neutrophils and CD3+ T lymphocytes in the lesions. (Cook, Brown et al. 2004, A. L. Croxford, Karbach et al. 2014, Khmaladze et al. 2015)

There is only one existing induced model for Ps called the imiquimod (IMQ) model. IMQ treatment, topical administration, targets TLR7/8 which induces and exacerbates IL-23/IL-17 axis in the mice causing Ps. IMQ-induced Ps is characterized by inflamed and scaly skin lesions, resembling plaque-type Ps. The model shows an increased epidermal proliferation, neoangiogenesis, and an abnormal differentiation of CD4+ T cells, dendritic cells and neutrophil infiltration. (Asquith et al. 2009, Kannan et al. 2005, Potter, Wax 1981, Patten et al. 2004)

Human skin can be transplanted on severe combined immunodeficient (SCID) and AGR129 mice. Both mouse strains lack T and B cells, but only SCID lacks NK cells. The xenografts are tolerated for several months in the SCID mice and even slightly better in the AGR129 mice. (Cook et al. 2004, A. L. Croxford et al. 2014, Khmaladze et al. 2015) Interestingly, it has been demonstrated (Boyman, Hefti et al. 2004) that grafts from Ps patients not currently showing lesions and transplanted on AGR129 mice spontaneously develop Ps plaques.

Models for PsA are spontaneous, genetically engineered or induced. Currently, there is only one spontaneous model for PsA occurring in the DBA/1 strain. The DBA/1 male mice, which develop the disease if grouped after puberty causing stress due to intermale aggressiveness. This leads to severe arthritis; swollen joints, inflammation and enthesitis. The disease is believed to be the result of an exaggerated healing response. (Holmdahl, Jansson et al. 1992, Corthay, Hansson et al. 2000, Khmaladze et al. 2015)

An example of a genetically engineered PsA model is the JunB/c-Jun epidermal inducible double-knockout mouse. It is a model targeting epidermal keratinocytes. JunB is a component of the AP-1 transcription factor and localized in the Ps susceptibility region PSORS6, while c-Jun is considered to be an antagonist to JunB. An epidermal deletion of both JunB and c-Jun causes the development of a Ps-like disease phenotype and arthritic lesions in mice. Interestingly, the T and B cells seem to play a minor role in the etiology of this disease. A PsA-like disease in 'humanized' (HLA-transgenic, mice lacking their own MHC), was described (Bardos, Zhang et al. 2002), in which animals of four transgenic lines (HLA-DR2.Ab^o, DR4.Ab^o, DQ6.Ab^o and DQ8.Ab^o) developed hyperkeratosis and parakeratosis, nail deformities and bone resorption. This was associated with significantly fewer CD4+ cells and reduced NK cell activity compared to the disease-resistant HLA-DR3.Ab^o transgenic mice. (Khmaladze et al. 2015, Zenz, Eferl et al. 2005) In this thesis, the novel and currently only available, induced PsA model have been utilized; MIP.

2.4.1 CIA

Traditionally, CIA is induced by an initial injection of collagen type II (a variety of sources have been used including bovine, porcine, chick, and human; response varies with strain and injection conditions), intradermally in an emulsion consisting also of complete Freud's adjuvant (CFA). The initial injection is often followed by an immune stimulation of collagen and incomplete Freud's adjuvant (IFA). The model has frequently been used, because it shares both immunological and pathological features of human RA. CIA is primarily an autoimmune disease of joints. Immunity to autologous type II collagen (CII) from both T and B cell is essential for disease manifestation. Genetically susceptible strains of mice for CIA all have MHC haplotypes H-2q or H-2r, these strains are DBA/1, B10.Q, and B10.RIII, a model which is highly reproducible. The role of anti-collagen antibodies in CIA is clearly proven, and it is also postulated that the binding of these to the joints initiates the complement cascade. The auto-antibody response in CIA is predominated by the IgG2 subclass with high levels of both IgG2a and IgG2b present at the peak of arthritis. Furthermore, the disease generates collagen-specific T cells. (Asquith et al. 2009,

Kannan et al. 2005) ROS produced by macrophages partly protects against CIA (Gelderman et al. 2007), completely ROS deficient mice and rats develop even more severe disease especially in comparison to ROS sufficient controls (Hultqvist et al. 2004, Hultqvist et al. 2011). The model can become chronic (Holmdahl, Rubin et al. 1986, Corthay et al. 1999, Bajtner, Nandakumar et al. 2005).

2.4.2 CAIA

Antibodies directed to CII can induce an arthritis model called CAIA. It is a B- and T- cell independent model, causing an acute arthritis lasting about two-three weeks. The model demonstrates some low severity symptoms of arthritis directly after the anti-CII (aCII) antibodies have been *i. v.* injected, but requires an immune stimulation using a substance stimulating TLR2 and/or TLR4 for proper disease development. Substances proven to stimulate CAIA are for example LPS, monophosphoryl lipid A and lipomannan. (Nandakumar, Holmdahl 2005, Nandakumar, Backlund et al. 2004, Kelkka et al. 2012). The disease is driven by the complement system, shown in C3a Receptor knock-out mice, C5a Receptor knock-out mice, complement protein C6 knock-out mice, and by neutralizing the C5a in vivo, all lowering the severity of disease. (Nandakumar, Jansson et al. 2010, Banda, Hyatt et al. 2012). Infiltration of macrophage and polymorphonuclear inflammatory cells further characterizes CAIA. (Asquith et al. 2009) Interestingly, ROS deficiency lowers the disease severity but if ROS is produced in macrophages the disease can develop (Gelderman et al. 2007).

2.4.3 MIP

MIP is a novel induced PsA mouse model based upon a single intraperitoneal injection of *Saccharomyces cerevisiae* derived mannan. The disease manifests with both Ps-like and PsA-like symptoms in mice, such as swelling and redness of the joints and lesions forming in both paws and ears. The erythema and edema of the joints starts day one after disease initiation, reaching maximum severity around days four-five. The Ps lesions appear from approximately day three. MIP represents PsA's inflammatory phase, in which the major drivers of the disease are the macrophages, γδ T cells and IL-17A. This was shown by the treatment with clodronate liposomes, anti-Ly6G or anti-IL-17A, respectively, either completely blocked or significantly decreased both joint and skin inflammation. The disease becomes more severe by ROS deficiency. (Khmaladze et al. 2014, Khmaladze et al. 2015)

2.4.4 Mouse models and human disease

In general mouse models have the limitation that they are modelling specific parts of the disease phenotype. Spontaneous models of RA for example are mimicking the genetic predisposition of the disease a lot of them becoming chronic. However, they are often the cause of modifications and limited to that specific genetic setting, while patients have a larger genetic variety. Thus, these genetic models are possibly limited towards certain subgroups of patients or are missing parts of the disease picture; some showing the adaptive dependency of the disease very well (for example K/BxN), other showing the clear role of TNF-α which have led to successful treatment in patients (human TNF-α transgenic mouse) or the NZB/NZW mouse that gives a resemblance towards the autoantibodies seen in RA. Interestingly, the humanized HLA mouse model of PsA show many characteristic of the PsA but for T cell dependency; possibly illustrating a subgroup of patients or that adaptive immunity play a minor role in PsA. Transplant models are mostly used in Ps studies of the plaques, they are limited to severely immunologically deficient mice for the transplant to stay and are thus not showing the full immunological response that the patient would have. Induced models are more targeting the environmental aspects of autoimmune disease induction. Antigen induced models in general are good at illustrating the inflammatory aspects of many autoimmune diseases, such as RA, PsA and Ps, but fail to model the breach of self-tolerance connected to autoimmunity. PIA COMPIA, PGIA, and to some extent CIA (dependent upon the right genetic background), are exception of this; causing chronic models with the characteristic cell infiltration and damage of the joints. No induced model has thus shown proper systemic chronicity of severe Ps and PsA. Generally, models of disease should, when the results are interpreted into known data of corresponding human disease, be regarded from the limitation of each model and every finding in a mouse model are strengthen if similar result patterns can be found in other models of the same disease. (Asquith et al. 2009, Kannan et al. 2005, Potter, Wax 1981, Patten et al. 2004, Khmaladze et al. 2015, Holmdahl et al. 1986, Carlsen et al. 1998, Glant et al. 1987, Finnegan et al. 1999) All models will have their pros and cons in regards to the disease they are trying to model, but by adding the results collectively from different models' important hints can be found on how the immune system works and what can be the cause of diseases seen in humans. Clues that cannot be found in patients for ethical reasons nor in cell cultures because of the systemic nature of autoimmune diseases.

3 AIMS OF THE STUDY

The aim of these studies was to investigate the role of the innate immune system and ROS in autoimmune diseases such as chronic arthritis and psoriatic arthritis;

1. Generate and characterize a chronic arthritis model independent of the adaptive immune system.

2. Investigate the role of MR as a receptor of interest for the development of RA, Ps and PsA.

3. Generate and utilize a ROS inducible mice model for the investigation of ROS regulatory role in different phases of disease.

4. Address the differences in data seen between the mice deficient in ROS due to a natural occurring point mutation in the *Ncf1* gene and KO mice for the same protein.

4 MATERIALS AND METHODS

4.1 Mice

All mice have been backcrossed for at least three generations to the C57BL/10.Q/rhd mice with the Ncf1^{m1J} mutation (Hultqvist et al. 2004). The generation of the Ncf1^{m1J}, MN, Ncf1^{m1J}.TCRβ$^{-/-}$, Ncf1^{m1J}.C5$^{-/-}$ and C5$^{-/-}$ mice has previously been described (Guerard, Holmdahl et al. 2016, Gelderman et al. 2007, A. M. Croxford, Whittingham et al. 2013, Hultqvist et al. 2004). Mice with the Ncf1 knockout allele Ncf1^{Tm2Utu} (B6N.TN2) were analyzed for the possession of only the causative mutation using a 10-k SNP typing chip (Sareila, Hagert et al. 2015). MR deficient mice, first described by (Lee, Evers et al. 2002), were characterised using the primers 5'-GAC CTT GGA CTG AGC AAA GGG G-3', 5'-AGC TCG ATG CGG TTC ACC AG-3', 5'- CTG AGA ATC CCC GCG TCC TC-3', utilizing an adapted protocol from (Lee et al. 2002). The B6.129P2-Fcgr3$^{tm1Sjv/J}$ mice were acquired from the Jackson Laboratory (Bar Harbor, ME) and characterized using the 5'-GTG GCT GAA AAG TTG CTG CTG-3', 5'-CTA CAT CCT CCA TCT CTC TAG-3' and the 5'-GCA CGA GAC TAG TGA GAC GTG-3' primers. The B6.129S4-Mbl1^{tm1Kata}.Mbl2$^{tm1Kata/J}$ mice were from the Jackson Laboratory (Bar Harbor, ME) and was genotyped for the MBL1 gene using the primers 5'-CTG AAG GGC CAG TTC ATG TAC GTG ACA GGG GGG-3', 5'-CCA GAG AGG AGG CAA GGA GAA TAT GGA GG-3' and 5'-GAT CTC CTG TCA TCT CAC CTT GCT-3'. The MBL2 gene was genotyped using the 5'-AGT GAA GGC CCT GTG CTC CGA ATT C-3', 5'-GCG CAT CGC CTT CTA TCG CCT TC-3' and 5'-CCC ACA GAG CAC AAG AGT CAT AAA TG-3' primers. The B6.129S7Rag1$^{<tm1Mom>/J}$ (stock 002216) mice were acquired from Jackson Laboratory (Bar Harbor, ME) and genotyped in accordance with Jackson laboratory's directions. The Conditional Ncf1^{Tm1Rhd} mice, produced upon request from Ozgene Pty Ltd. using C57BL/6J BAC clones RP23-42C7 and RP23-41A15 (Roswell park cancer institute) for the vectors in accordance with (Sareila, Hagert et al. 2017) and subsequently crossed with the inducible CreERT2 gene originated from BQ.B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj/J}$ mice from Jackson laboratories.

All mice in the experiments were sex- and age-matched, 6–20 week-old. If not otherwise noted littermates were used in the experiments. Mice were housed under specific pathogen-free conditions as described earlier in open cages (Sareila et al. 2015) or in closed cages (Khmaladze et al. 2014) under specific pathogen free conditions. The study was approved by the national Animal Experiment Board in

Finland (Eläinkoelautakunta, ELLA), ethical permit numbers ESAVI-0000497/041003/2011 and ESAVI/439/04.10.07/2017, or in case the experiments were performed at Karolinska Institute by the local ethics committee (Stockholms djurförsöksetiska nämnd) permit number N490/12.

4.2 Animal models

The arthritis development was followed by blindly scoring of each red and swollen joint. Each mouse could get one point for each swollen and red toe or knuckle and five points for each inflamed wrist or ankle, rendering a scoring scale of 0-60 points per mouse.

The psoriatic lesions were scored based on severity on a scale of 0-3 for each paw and ears, rendering a maximum score of 15. (Khmaladze et al. 2014)

4.2.1 mCAIA

mCAIA was induced by an injection of a four monoclonal cocktail of aCII antibodies (Table 1) produced from the clones CIIC1, M2139, CIIC2 and UL1 as described in Nandakumar *et al.* (Nandakumar, Holmdahl 2005, Holmdahl et al. 1986, Mo, Holmdahl 1996) i. v. and subsequently injected with 2 mg of mannan (#M7504 Sigma-Aldrich CAS: 9036-88-8) intra peritoneal injection at day 5 and 60 as an immune stimulating substance.

Table 1. The four monoclonal cocktail of anti-collagen type II antibodies. Ig; immunoglobulin.

Clone	Ig subclass	Epitope	Reference
CIIC1	IgG2a	C1	Holmdahl et al., 1986
M2139	IgG2b	J1	Mo and Holmdahl, 1996
CIIC2	IgG2b	D3	Holmdahl et al., 1986
UL1	IgG2b	U1	Nandakumar et al 2005

4.2.2 MIP

A singular intra peritoneal injection of 10mg mannan (when used in SPF open cages at Turku University) and 20mg (when used in IVC cages at Karolinska Institute) from Saccharomyces cerevisiae (Sigma-Aldrich #M7504) induces MIP; dose difference due to an observed higher sensitivity to MIP in open cages compared to mice in closed IVC cages (unpublished observation Hagert et al). (Khmaladze et al. 2014)

4.2.3 CIA

The mice were immunized using *i.d.* injection of 100 µg bovine CII (#804001; MD Biosciences, Zürich, Switzerland) emulsified in Complete Freund Adjuvant (CFA; Difco #263810; BD Biosciences, New Jersey, US). Re-immunization was performed on day 21 post immunization (if not otherwise stated) by 50 µg of bovine CII emulsified in IFA.

4.2.4 Tamoxifen recombination

In mice expressing the inducible CreERT2 was induced the Ncf1-iKI allele was recombined by three intraperitoneal injections of tamoxifen (Sigma; T5648 CAS: 10540-29-1) on day 0, day 1, and day 5 as described in (Sareila et al. 2017).

4.2.5 EndoS

To render the CAIA antibodies unable to bind to the Fc receptors (Collin, Olsén 2001, Baruah, Bowden et al. 2012), EndoS (which specifically hydrolyzes the β-1,4-di-N-acetylchitobiose core of the asparagine-linked glycan of human IgG) was used in accordance with the protocol published in (Nandakumar, Collin et al. 2007).

4.3 ELISA

To assess the aCII antibody levels in mice sera, Nunc Maxisorp 96-well plates (Thermo Fischer Scientific Inc.) were coated with rat, pepsin-digested CII (#8041006-B, MD Biosciences) and thereafter blocked with bovine serum albumin (fraction V, Immuno Diagnostic Oy). Sera were diluted in PBS and incubated for 2h at RT. The levels of bound aCII antibodies were detected using biotinylated Ig, κ light chain (clone 187.1, BD Biosciences) and streptavidin-conjugated Europium (PerkinElmer, Turku, Finland). Victor 1420 multi-label counter (PerkinElmer) was used to measure time resolved fluorescence. (Hultqvist et al. 2004, Sareila et al. 2015)

4.4 Flow cytometry

Flow cytometric analyses were performed as described earlier (Sareila et al. 2015, Kelkka et al. 2014). In short, organs were sampled and when needed and the red blood cells were lysed by hypotonic buffer. Fc-receptors were thereafter blocked and surface antigens were subsequently stained with fluorescently labelled antibodies. For intracellular staining, the cells were first fixed and permeabilized using Cytofix/Cytoperm™ solution (#554722, BD Biosciences) according to manufacturer's protocol. The samples were measured in LSR II or LSR Fortessa (BD Biosciences) and analyzed using the FlowJO software V10 (Tree Star, Inc.).

4.5 Measurement of ROS

Measurement of the ROS production was performed as described (Sareila et al. 2015, Kelkka et al. 2012, Kielland, Blom et al. 2009) by an extracellular, an intracellular and an *in vivo* method.

4.5.1 Intracellular measurement of ROS production

In short, cells were stimulated with phorbol myristate acetate (PMA; 200 ng/ml, 20 min) ex vivo according to the protocols previously described by (Vowells, Sekhsaria et al. 1995, Hultqvist et al. 2011) for intracellular detection of oxidative burst by dihydrorhodamine-123 fluorescence. The samples were measured using either LSR II or LSR Fortessa (BD Biosciences) and analysed in FlowJO software V10 (Tree Star, Inc.).

4.5.2 Extracellular measurement of ROS production

As previously described in (Sareila et al. 2015, Sareila, Jaakkola et al. 2013), the cells were washed with PBS, and thereafter treated with 100 µl isoluminol reagent buffer (isoluminol 10 µg/ml, HRP-type II 4 U/ml, and PMA 200 ng/ml). Immediate data collection of the produced luminescence signal as a result of the extracellular ROS production at 37°C was performed by Infinite M200 plate reader and analyzed using Magellan data analysis software (Tecan Group, Männedorf, Switzerland). Data was presented as the detected signal after 30 min of incubation or as a time course.

4.5.3 In vivo measurement of ROS production using L-012

As described by Kelkka *et al.* (Kelkka et al. 2012, Kielland et al. 2009), sedated mice were injected i.p. with 20 mg/kg L-012 probe (Wako Chemicals, Neuss, Germany) dissolved in physiological saline and the subsequent luminescent signal was detected using the IVIS 50 bioluminescent system (Xenogen, Alameda, CA. Living Image software version 2.50 (Xenogen) was used for image acquisition and analysis.

4.6 ELISPOT assays

To count the IL-17A-producing cells Mouse/rat IL-17A ELISPOT Ready-SET-Go!® (eBioscience) was used according to manufacturer's protocol and Sareila *et al.* (Sareila et al. 2017).

4.7 RNA extraction, genome-wide gene expression analysis, and quantitative real-time PCR

Two independent CIA experiments, with a similar experimental setup, were used for genome-wide expression analysis and the results were analyzed together. RNA preservation, isolation, and purification as well as RNA amplifications, in vitro transcription, cRNA quality check, and the microarray were performed as recently described (Kelkka et al. 2014). The data was anlysed as described in Sareila *et al.* (Sareila et al. 2017).

4.8 Histology

Paws were collected in 4 % paraformaldehyde upon termination of experiment. The paws were then decalcified and paraffin embedded. The resulting blocks were cut in 7µm slices, stained with H&E or Safranin staining to assess the general inflammatory levels. Pannoramic 250 Slide Scanner was used to digitalize the slides and Pannoramic viewer version 1.15.4 was used for analyzations (both from 3D Histech).

4.9 Statistics

Cellular analyses were evaluated using either one-way ANOVA with Bonferroni posttest or Mann-Whitney T-test. Disease scores were analyzed with Mann-Whitney's test. GraphPad Prism Software version 5 was used to make graphs and for all statistical analyses. *$p<0.05$ was regarded as significant, **$p>0.01$ and ***$p<0$.

5 RESULTS

5.1 Creating and characterizing a novel chronic arthritis model (I)

A novel mouse model was needed to investigate the role of the innate immune system in chronic arthritis. Utilizing the knowledge gained from establishing the CAIA model (Nandakumar, Svensson et al. 2003, Nandakumar, Holmdahl 2005) and systematically testing different immune stimulants (Kelkka et al. 2012) and knock-out mouse strains (Nandakumar et al. 2004, Nandakumar et al. 2010) a novel model for chronic arthritis could be established. mCAIA creates a chronic active disease lasting for over 150 days after disease initiation. It is initiated by a single injection of aCII antibodies followed by two immune boosting injections of mannan at day five and sixty after experimental start. Histological studies showed cell infiltration even in the late stages of disease. Illustrating the chronic arthritic scorings of the disease in a heat-map and also in a short movie of the individual joints, the disease was shown to be very active. In short, it shows that the fluctuation in severity is within the same joint and not primarily because of spreading to new joints, although spreading to new joints do exist.

To test the role of the aCII antibodies, the disease was followed when no antibodies were injected, but mannan was administered at day five and sixty as in normal mCAIA (run in parallel). These mice do develop mild symptoms that get enhanced by the second injection. The disease is however significantly milder in both severity and prevalence compared to a setup, in which aCII antibodies are used. Injection with only aCII gives no or mild symptoms. Thus, both are needed for proper disease induction.

5.2 Chronic arthritis can be driven independently of the adaptive immunity (I)

Since the LPS induced CAIA is independent of T and B cells (Nandakumar et al. 2004), this novel mCAIA model was tested for dependency on these cells as well. Joint inflammation induced by mCAIA was comparable in RAG knock-out compared to wildtype littermate control. Since RAG knock-out lack αβ- and γδT cells and B cells, this excludes involvement of the adaptive immunity in this model (Mombaerts et al. 1992). This was further confirmed by the ability to develop disease in the $\beta TCR^{-/-}$ mice.

5.3 Innate immunity in chronic arthritis (I)

5.3.1 Macrophages and neutrophils

Using ROS deficient Ncf1^{m1j} mice (Sareila et al. 2013, Hultqvist et al. 2004) and the MN mice otherwise deficient in ROS except for the macrophages which are ROS sufficient (Gelderman et al. 2007) the mCAIA model was determined to be down-regulated by ROS from macrophages. In fact, severe arthritis is primarily developed in the ROS deficient mice. Macrophages and neutrophils were also present in the synovium and blood of chronically sick mice.

5.3.2 Fc receptors

Since antibodies can affect the immune system via the FcRs, present on T cells, macrophages and neutrophils among others, it is of importance to test the model for the effect of these receptors. The antibodies used to induce mCAIA (IgG2a and IgG2b) are binding with the highest affinity to FcγRIII. (Guilliams, Bruhns et al. 2014, Nimmerjahn, Ravetch 2008, Nandakumar, Holmdahl 2005) Surprisingly, the mCAIA model was successfully run in the FcγRIII$^{-/-}$ mouse strain, this receptor was excluded as important for driving mCAIA.

The aCII antibodies were thus treated with EndoS, specifically hydrolyzing the N-linked glycan in the Fc-region of native IgG rendering them incapable of binding to any of the FcRs. The EndoS treated antibodies did render the aCII antibodies to cause a less severe disease; in fact the disease became as mild as the mice receiving only two mannan injection, but no aCII antibodies. This could suggest involvement of other FcRs. However, EndoS also affects the antibodies ability to activate the complement pathway via the classical pathway. (Collin, Olsén 2001) This indicates a role for the complement in this disease. The other FCRs bind IgG2a and b with much less affinity than the FcRγIII receptor and are thus unlikely to create the difference in arthritis severity and prevalence seen.

5.3.3 The complement pathway

The presence of neutrophils in the synovium and blood of chronically arthritic mice and the effect on severity and prevalence of disease by EndoS treatment but not by knocking out the FcRIII indicates a role of the complement cascade in this model. Indeed, when C5 knock-out mice were subjected to mCAIA they were

protected. To test mannan's role in activating the complement pathway via the mannan binding lectin (MBL), the model was also run in mice knocked-out for both MBL1 and MBL2. The $MBL^{-/-}$ mice developed mCAIA to the same extent as their littermate controls and the lectin pathway was hence concluded non-important. This means that the complement cascade is either initiated via the alternate and/or the classical pathway in this model. The EndoS data points towards the classical pathway but further research is needed to conclude this.

5.4 Mannose receptor (II)

5.4.1 mCAIA

Since mannan binds to MR, both in humans and in mice (National Center for Biotechnology Information, U.S. National Library of Medicine 2016a, National Center for Biotechnology Information, U.S. National Library of Medicine 2016b) the mCAIA model was tested in MR knock out mice. Interestingly, the MR knock-out mice are significantly sicker compared to the wild type control mice that are protected. Also on an $Ncf1^{m1j}$ background the mice gets more severe disease. Hence, lack of MR is enough to break tolerance or enhance an already existing disease in the acute part of mCAIA

5.4.2 MIP

Also in MIP, the loss of MR causes more severe disease in mice, both in regards to arthritis symptoms and the psoriatic lesions. Unlike mCAIA, the role of MR in MIP disappears in a ROS deficient environment arguing for different roles and/or regulatory pathways in the two different models. Also, in vitro studies indicate an ability of ROS to downregulate the expression of MR.

5.5 Creating a transgenic mouse for inducible ROS (III)

To further investigate the role of ROS in autoimmunity, an Ncf1 knock-in mouse was created which lacks ROS until induced. The mouse was created by targeted modification into the Ncf1 locus. Introducing a single amino acid change (T153M) into the mouse NCF1 protein by recombinant *in vitro* mutagenesis which causes lowered capacity of the NCF1-M153 to activate the NOX2 complex and thus a significantly reduced ROS production, which is similar to the one seen in the naturally occurring rat mutation (Hultqvist et al. 2011, Hultqvist et al. 2004, Sareila

et al. 2017). However, a knock-in of the NCF1-M153 together with the corresponding wild-type exon in reverse orientation into the Ncf1 locus causes complete abolishment of the NOX2-dependent ROS. Further studies concluded that the Ncf1-iKI mouse totally lacks the NCF1 protein possibly caused by the palindromic exons in the locus in Ncf1-iKI mice. By also inserting a CreERT2 recombinase, this Ncf1 deficient mouse strain can be inducible with tamoxifen (TAM) thus rendered Ncf1 sufficient. The function of the model was confirmed using both intracellular and extracellular measurements of ROS, showing no ROS before induction and levels similar to littermate wild types after recombination (Sareila et al. 2017).

5.6 ROS is important both in the priming and the effector phase of arthritis (III)

Effectively creating an equivalent of a Ncf1 wild type mouse, by recombining the Ncf1 gene in adulthood prior to initiation of CIA, the Ncf1-iKI mice were effectively protected against CIA to the similar extent as their littermate controls. Furthermore, it also lowered the levels of inflammatory cells such as T cells and myeloid cells. The aCII specific response from IL17 expressing T cells is also affected by the recombination. Furthermore, if the Ncf1 gene is recombined after disease priming it is still protective in the effector phase. However, here the specific aCII response is not affected, whereas the myeloid cells still are.

5.7 CIA induces altered gene expression in ROS deficient mice (III)

Ncf1 deficient and wild type mice were exposed to CIA and Ncf1-dependent changes in genome-wide gene expression and the up- and down-regulation of genes were followed over the course of the disease. These changes in expression were observed both between the priming and disease onset and between the onset and late, clinically severe disease. The largest changes were observed between the clinically sick and the healthy ROS deficient mouse. Interestingly, naïve Ncf1 deficient mice were genetically different from wild type, a difference that grew over the course of the disease. During the course of disease primarily genes related to inflammation (upregulated) and lymphocytes (downregulated) were affected. Furthermore, a strong interferon signature could be observed in naïve Ncf1 deficient mice, reversible upon recombination to the wild type gene. In fact, studying some of the more prominent genes affected by the Ncf1 deficiency expression levels could be adjusted back towards wild type levels during CIA by utilizing the inducible mouse. The effect was more prominent if the recombination was done before

the priming of the disease, but the trend was still there if the recombination was performed 10-15 days after the disease initiation.

5.8 Direct comparison of the point mutated Ncf1 mice with mice with a targeted deletion in the Ncf1 (IV)

Recently, there have been some controversy between the reported immunological phenotypes for the Ncf1 knock-out mice and point mutated Ncf1 mice. Perhaps most clearly exemplified by the difference in the multiple sclerosis model EAE, were the knock-out mouse is completely protected (van der Veen et al. 2000) whereas the Ncf1 mutated mice have shown higher severity in both EAE and CIA (Hultqvist et al. 2004). By comparing knock-out mice to littermate point-mutated, mice subjected to CIA, a difference in severity and prevalence in the females was noted; where the Ncf1 mutated becomes significantly sicker compared to the knock-out mice. This effect was reversible with ovariectomy, in this case indicating a hormonal disturbance in the knock-out mice.

6 DISCUSSION

Existing chronic models such as COMPIA, CIA, PGIA and PIA have in common a long phase prior to arthritis development; during which T- and B-cells are primed and differentiated into effector cells. (Carlsen et al. 1998, Carlsen et al. 2008, Corthay et al. 1999, Bajtner et al. 2005, Olofsson et al. 2003, Hultqvist et al. 2011, Goldschmidt, Holmdahl 1991, Holmdahl et al. 1986, Finnegan et al. 1999, Glant et al. 1987) Thus there was a need for a new chronic model (mCAIA) that not only lacks the time period were no arthritis symptoms can be seen but also is independent upon adaptive immune system to be able to visualize the role of the innate immune system in arthritis. In fact, innate cells have previously been thought to have a role in arthritis but more studies are needed to further establish their role in disease (Kinne et al. 2000, Kinne et al. 1995, Barrera et al. 2000). Paper I-II validates the importance of further investigation of the innate immune systems role in immunity, in which the mCAIA model can be of great value. Furthermore, it has been unclear were during arthritis the innate cell produced ROS (by the NOX2 receptors) and we thus utilized a newly produced ROS inducible mouse strain showing the importance of ROS in both induction and effector phase of disease.

6.1 aCII antibodies' role in mCAIA and RA

In paper I, an active chronic arthritis model independent of the adaptive immune system was successfully created (induced by initial injection of aCII day zero and subsequent injections of mannan at day five and sixty). Instead there seems to be innate factors such as the complement pathway, macrophages and neutrophils that are of importance for mCAIA. Furthermore, the aCII antibodies injected initially (day zero) had left blood circulation before chronicity starts, excluding them from being the main drivers of the disease. However, the aCII antibodies can still be present in the mice, bound for example to cartilage as previously shown possible (A. M. Croxford et al. 2013). Although it is likely that these have broken down before the chronic phase begins. An interesting observation is that the mice can receive antibodies without developing the disease depending mostly on the background but also about 0-20 % of the mice in each experiment will never develop disease although susceptible. This is similar to the situation in humans were subjects with detectable levels of autoantibodies only have a 40–70 % risk of developing RA within a four-year period. Actually, recent data are indicating a rather heterogeneous group, where the levels and the specificity of the autoantibodies can vary. (Trouw, Toes 2016, Figueiredo, Bang et al. 2017). mCAIA is similar to RA in that it seems sensitive to the environment; different housing conditions will

affect the severity of arthritis. Most likely it varies depending on the amount of pathogens and/or microflora present. The style of cage (open or closed) could also have an impact since it alters the stress levels in form of more or less noise etc. (Gerlag, Norris et al. 2016)

6.2 Mannan most likely activates macrophages

Mannan's role as an immune stimulating agent in these models are most likely in activation of the macrophages, probably via pattern recognition receptors such as TLR4 and/or Dectin-2 (Drummond, Saijo et al. 2011, Tada, Nemoto et al. 2002). TLR2 and TLR4 have previously been linked to arthritis, both in humans and in mice. (Kelkka et al. 2012, Arend, Firestein 2012, Abdollahi-Roodsaz et al. 2008). However, this needs to be studied further and are thus outside the scope of this thesis.

6.3 ROS role in rheumatism

ROS has previously been shown to have a role in arthritis and other autoimmune diseases, both in mice (Hultqvist et al. 2011, Olofsson et al. 2003, Zhao, Ma et al. 2017, Khmaladze et al. 2014) and humans (Olsson et al. 2007). Paper I indicate an important role for ROS protecting against arthritis when only the ROS deficient mice truly develop severe disease. The ROS produced from macrophages was determined as enough to protect against disease. However, paper II shows that lack of MR also breaks tolerance, thus ROS is only one piece of the puzzle. In paper III the effect of ROS is more clarified. It is shown to have an effect both in the initiation and effector phase of disease. In initiation, ROS role seem to be to regulate both T cells and macrophages, while the effect in the primary phase might be primarily macrophages dependent since the T cells are not affected. (Sareila et al. 2017) This need to be further studied to be clearly decided, however, the data fits with paper I, which shows chronic disease is macrophage dependent but not T cell dependent.

6.4 Initiation of the complement cascade results in activation of macrophages which drives the disease

A hypothesis is that initial binding of aCII antibodies to cartilage most likely leads to the initial joint damages during the first acute phase. This primes the joints to

be more susceptible for further damages during the chronic phase due to an antibody mediated induction of the complement cascade, via either classical or alternate pathway. This is most likely due to activation of the complement cascade by the antibodies binding to C1q, however the alternate pathway has not been excluded. We hypothesize that the C3a and C5a will thus attract hyper activated innate cells (due to ROS deficiency) via their specific receptors expressed on for example neutrophils and macrophages (Bohlson et al. 2014, Banda et al. 2012, Murphy et al. 2008, Abbas et al. 2007) to the joints, as illustrated in figure 2. Thus, the second injection of mannan causes the initiation of the chronic activation of the ROS deficient, primed and already hyper activated macrophages and neutrophils, which are believed to drive the chronic disease. Interestingly, the role of the complement pathway has also been shown utilizing the traditionally adaptive immune dependent K/BxN arthritis model. (Ji, Ohmura et al. 2002)

6.5 MR down-regulates arthritis and PsA; in mice and humans?

Since the aCII antibodies binds to collagen in the joints a large infiltration of the neutrophils and macrophages will occur there. This will in part be down-regulated by the MR on the type II macrophages, in an attempt to protect against the disease. Deficiency in ROS in these macrophages will drive the disease due to the loss of the down-regulation that ROS can have on signaling pathways.

The important role of ROS in MR regulation of disease becomes evident in the PsA model, MIP, where the effect of MR is lost if the mice are ROS deficient. The involvement of ROS in macrophages is furthermore shown *in vitro* where macrophages are indeed affected by ROS in regulation of MR expression. Perhaps most clearly shown by; (a) the lower amounts of ROS expressed in MR knock out mice compared to MR sufficient mice *in vivo* (b) the increased proliferation of cells in peritoneum upon *in vivo* stimulation by recombinant mouse IL4. The exact manner in which ROS regulated the MR signal pathway is not determined. It is probable that activation of MR after stimulation by mannan induces IL10 and thus initiates IL10 production, which in turn will activate even more M2 and down-regulate M1. (Gordon 2003, Murray, Allen et al. 2014) This hypothesis is shown in figure 3. mCAIA is most likely regulated by MR in a similar manner, illustrated in figure 2. Interestingly, patients with early RA was recently reported to have elevated levels of soluble MR. These patients were also observed to have a decrease in the levels of soluble MR when patients were successfully treated with anti-TNFα and DMARDs. (Heftdal et al. 2017)

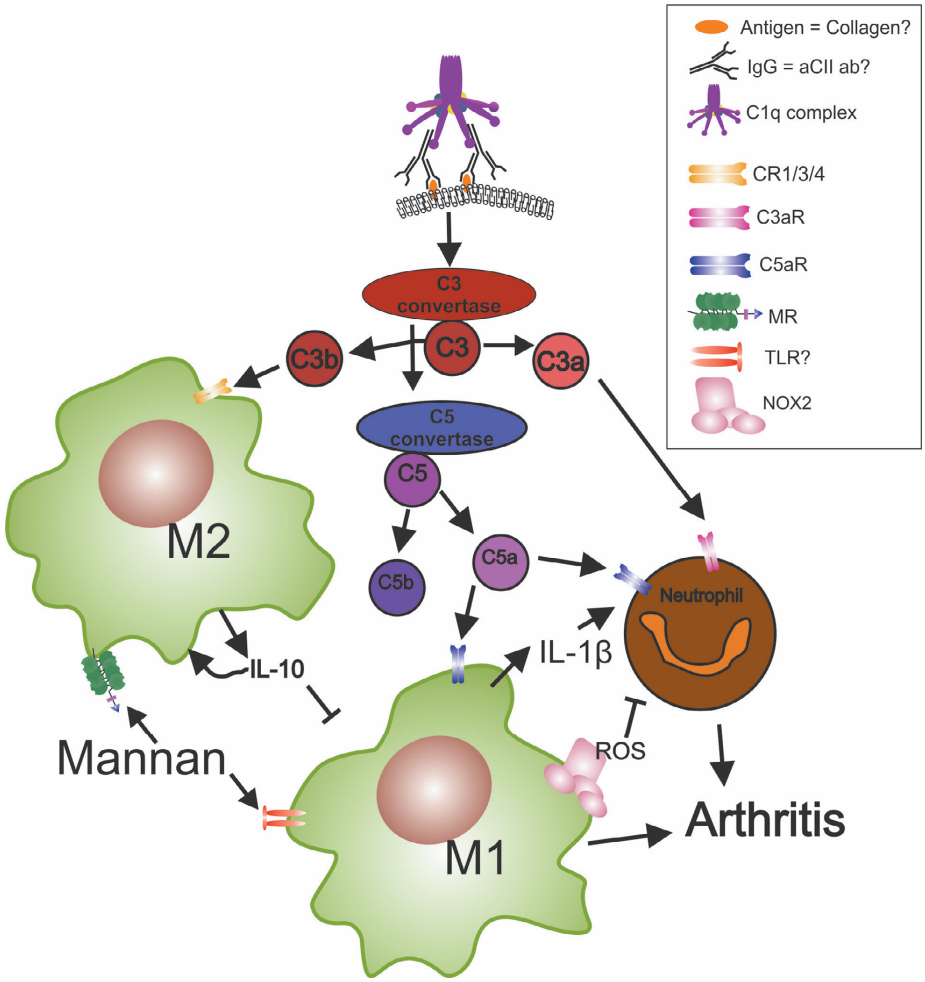

Figure 2. A possible pathway for induction of mCAIA. It is probable that the injected aCII antibodies bind to the C1q complex, thus initiating the complex cascade via the classical pathway; although the alternate has not been excluded. This initiates the formation of C3b that could initiate the M2 cells and the C3a and C5a that will recruit M1 and neutrophils. The disease is further boosted by mannan binding to an unknown receptor, probably a TLR, initiating the M1. In an ROS sufficient milieu this will not develop into a chronic disease due to the NOX2 and ROS dependent down-regulation of the subsequent activation of other macrophages and neutrophils. However, in a ROS deficient milieu the macrophages will become hyperactive and induce arthritis and most likely induce an activation of neutrophils via IL1β. Mannan will also bind to the mannose receptor activating the M2, which can down-regulate the inflammation via secretion of IL10. IL10 also activates new M2 cells. IgG; immunoglobulin G, M1/M2; macrophage type 1/2 MR; macrophage mannose receptor, NOX2; NADPH oxidase 2, R; receptor, TLR; toll-like receptor

The MIP pathway is not dependent on antibodies and no aCII antibodies are produced in this model (unpublished data Hagert *et al*). However, (Khmaladze et al. 2014) postulate a role for γδT cells in this model, as presented in figure 3. Shortly, the M1 will be activated by the mannan, leading to secretion of TNFα, which activates γδT cells. γδT cells will secrete IL17 and thus active neutrophils. This signaling cascade will drive MIP.

Indeed, an enhanced expression of MR in patients with atopic dermatitis and psoriasis compared to normal human skin biopsies has been found (Wollenberg et al. 2002, de Koning et al. 2010). Taken together, the upregulation of MR in human disease and our results utilizing the MR deficient mice indicates that an activation of MR initiates a protective pathway in autoimmune diseases.

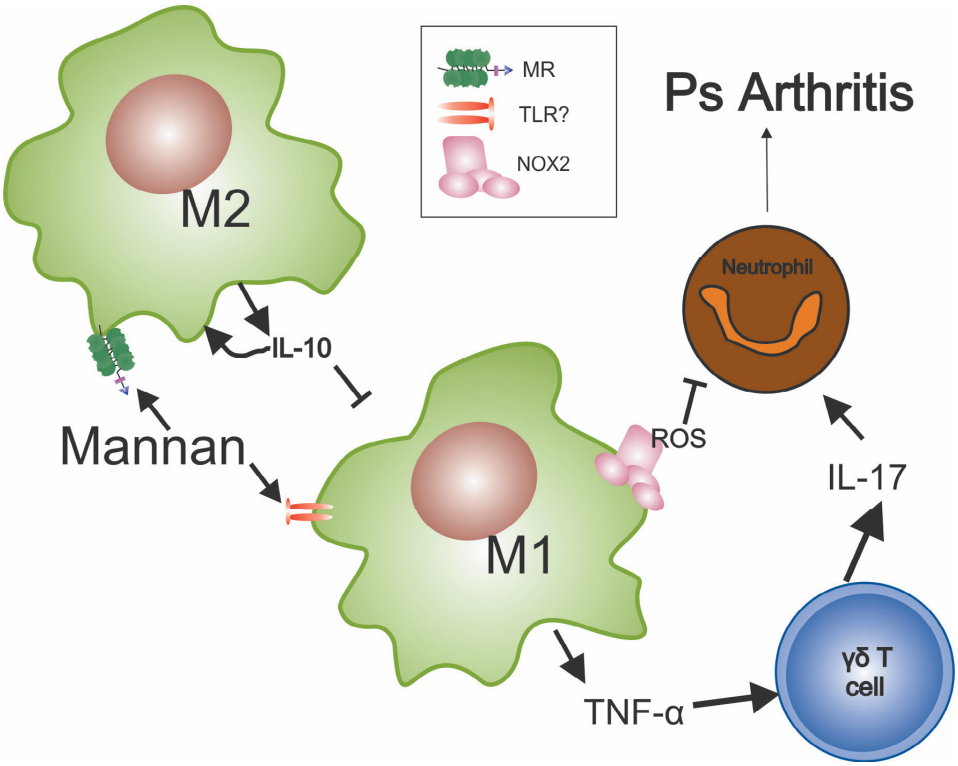

Figure 3. A schematic presentation of a possible pathway for induction of MIP. Mannan can bind both MR and other PAMPs, the former leading to a downregulative pathway via M2, most likely carried out by the secretion of IL10. A probable PAMP activating the M1 cells is the TLRs, which will lead to secretion of TNFα, activation of γδT cells and neutrophils. This can be downregulated via ROS from the NOX2 complex. MR; macrophage mannose receptor, NOX2; NADPH oxidase 2, TLR; toll-like receptor

6.6 mCAIA vs RA

This description, autoantibodies' activating the complement cascade leading to activation of innate immune cells and subsequent arthritis development, of how chronic arthritis can be driven is in part in agreement with data already published about the complement systems in RA. (Arend, Firestein 2012, Stumer, Biermann et al. 2017, Ji et al. 2002) In fact, (Stumer et al. 2017) have shown that RA patients express an increase of immune complexes IgG-C1q and IgG-C3c compared to healthy controls. Furthermore, deficiency in the C1q complex has been associated with the autoimmune disease SLE. In fact, disproportionate or inappropriate complement activation is connected with nearly all inflammatory or inflammation-related diseases (Bohlson et al. 2014); further validating the model's value for further studies of chronic arthritis.

ACPA has been implicated in activating the complement system via classical and alternate pathway, but not lectin pathway, (Trouw, Haisma et al. 2009) further validating our model as a valid model for investigating RA. Indeed, citrullination has been seen in patients' synovium in correlation to both PAD2 and PAD4, expressed on macrophages and leucocytes respectively. It is therefore a possibility that macrophages drive RA through citrullination of proteins in the joints, causing binding of ACPA and initiation of osteoclastogenesis and bone loss. (Trouw, Toes 2016, Turunen et al. 2016) Further studies are needed to confirm this.

Mice subjected to mCAIA suffer from relapses of the arthritis during the chronic phase. The relapses are localized in individual joints. The arthritis moves between already affected joints and to some extent new joints will develop arthritis, making mCAIA an active, relapsing chronic arthritis model. This is similar to RA (Holmdahl, Malmstrom et al. 2014, Boissier, Semerano et al. 2012) and to some degree to other models of arthritis such as CIA (Bajtner et al. 2005) and PGIA (Finnegan et al. 1999).

Innate immunity has previously been implicated having a role in RA, both in the primary and chronic phase, most recently by (Firestein, McInnes 2017). However, this is the first animal model in which this can be appropriately studied since it is the first chronic arthritis model that is B and T cell independent.

6.7 Adaptive immunity seems important as initiator of disease while innate immunity seems to drive chronicity

RA has been characterized to have distinctly different phases; (1) initiation of the disease by unknown environmental factors and (un)favorable genes, (2) production of autoantibodies, (3) that, possibly several years later, causes the clinical symptoms with the characteristic relapses. (Holmdahl et al. 2014, Boissier et al. 2012) Our data indicate that the presence of ROS from the NOX2 complex is of no importance for the developing of a functional immune system in the mice. However, paper I-III clearly indicates a regulatory role for NOX2 produced ROS in the defense against development of an autoimmune disease. In fact, these papers indicate that the primary function of NOX2 regulation, is performed by the macrophages, and does not necessarily involve the T cells in later stages of disease. Whereas the primary phase is most likely regulated by both macrophages and T cells, the chronic phase seems independent of adaptive immunity. Both phases are regulated in a NOX2 dependent manner.

In fact, in paper III the induction of CIA was linked with an increase in both the M-MDSCs and G-MDSCs monocyte populations in Ncf1-deficient mice. A reduction of these populations was seen in spleen after activation of Ncf1. Both disease severity and increasing number of IL-17A+ cells were associated with expansion of MDSCs. However, when the NOX2 complex was activated in the effector phase the effects on the monocyte population was not evident initially, nor was the effect on the autoreactive T cells or IL17A+ cells. Interestingly, over time, the pre-expanded population of splenic monocytes decreased towards the levels of the healthy mice. A difference in the T cell population between sick and non-sick mice was not observed. MDSCs have been proposed pathogenic in CIA (H. Zhang, Wang et al. 2015, Guo, Hu et al. 2016), and the results in paper III support this role. However, the possibility that these cells expanded as a response to inflammation and that the role of MDSCs is to fight the emerging inflammatory attack cannot be excluded. (Wang, Jiao et al. 2015, L. Zhang, Zhang et al. 2014, Crook, Jin et al. 2015, Fujii, Ashihara et al. 2013) All that in mind, the results of paper I-III together point towards an important role for T cells and autoantibodies in the initial phase of the disease development: but a decrease in importance in the later phases of disease. Instead macrophages, most likely in combination with the complement cascade and neutrophils, seem to play an important role in chronic disease regulation.

Interestingly, by genome-wide gene expression a remarkable downregulation of T lymphocyte related genes was identified in the arthritic Ncf1 deficient mouse compared to the wild type. It is previously known that activated T cells downregulate

CD3 and T cell-specific genes (such as Thy1, Cd6, Lck, and the CD3 antigen polypeptides) by interferon regulated genes. Therefore, the down-regulatory effect seen in the lymphocyte population might be a consequence of the ROS-dependent upregulation of the IFN signature. In short, a hypothetic pathway could be that:

ROS deficiency → up-regulation of the IFN pathway → down-regulation of T lymphocytes

It might also be an effect by ROS to activate regulatory T cells, as has earlier been suggested by (Gelderman, Hultqvist et al. 2006). In fact, the interferon pathway was another highly affected pathway identified to be upregulated in the ROS deficient mice compared to the wild type controls (paper III). Other causes may be differences in the cell populations due to the inflammation or it may partly be a more direct effect caused by the lack of ROS in Ncf1 mutated mice. It has previously been shown that the Ncf1 mutated mouse up-regulates STAT1 already in naïve state, an important part of the Interferon signaling pathway (Kelkka et al. 2014).

6.8 The impact of genetic backgrounds on animal experiments

Lastly, in paper IV, a difference in the phenotype of CIA was observed between an Ncf1 point mutated mouse and an Ncf1 knock-out mouse. Importantly, this highlights that genetic manipulation may impact complex genetic phenotypes in a manner that is not equivalent to the one observed in a natural mutant of the same gene. Other common reasons for different outcomes in genetic experiments can be, for example (a) related to differences in genetic backgrounds, (b) not using littermates when needed, (c) differences in environmental settings, or (d) unbalanced sex or age. Thus, great care should be taken when designing animal experiments so that the investigated phenotype is a natural one and not an artificial effect. (Holmdahl, Malissen 2012)

6.9 Prospects and potential weaknesses of these studies

Some may say that the greatest weakness of this study is that it is performed in mice models and that its conclusions are not drawn from human data. However, I regard the mice models, if properly executed, as a strength and not a weakness. Mice models enable us to look deeper into the immune system and to make discoveries we for ethical reasons cannot do in humans. There are examples both of when a finding has led to new treatments such as the successful TNFα story

(Peppel, Crawford et al. 1991) and other less successful stories; were an effect was seen in mice but the effect was absent in humans. But in general, the immune system in mice and humans is similar. It is vital to continue exploring the immune system *in vivo* to see all aspects of it, both in health and disease, something that obviously is not ethically possible in humans due to its invasive nature. Due to the complexity of autoimmune diseases it is important to utilize *in vivo* tools and multiple models, since no animal model has ever managed to illustrate the whole spectrum of autoimmune diseases. By combining the data from both fields, human and animal models, we get a chance to understand the disease and in the future to understand them well enough to treat them.

Too continue the studies presented in this thesis one could block macrophages in chronic disease and investigate if this would be enough to inhibit this disease model, as seen previously by others in different mice models and to some extent in humans (Barrera et al. 2000, Kinne et al. 1995, Kinne et al. 2000).

The role of complement in RA has partly been confirmed (Ji et al. 2002), but never before in a model independent of adaptive immunity. In fact, the method used in this thesis does not conclusively state if complement is an essential part in the driving the chronicity or if it effects rather is in initiating the disease. This could possibly be addressed by histology and /or inhibitory treatments e.g. antibodies against complement factors in chronic mice.

Furthermore, it remains to be investigate wheatear the role of MR is dependent upon mannan being the initiator of the disease or if it is a more general down-regulative pathway. This is of course easiest addressed by exposing the MR KO mice for different, non-mannan dependent arthritis, Ps and PsA models.

ROS role in these models are not fully characterized. Here the inducible ROS model will be vital too further characterize the role of ROS in autoimmune diseases both in regards timing and in regards to which cells are of importance for carrying out the effect.

Also, fibroblast is a likely cell involved in the mCAIA model and should be investigated for its role here. Fibroblast have a known role in the synovium of RA where they are; (a) key integrators of inflammatory signals in the inflamed rheumatoid synovium, (b) mediating direct tissue damage and persistence of cellular infiltrates in already established disease. (Turner, Filer 2015) Fibroblast are as such of interest as treatment target and as part of verifying the mCAIA model's correlation to RA.

6.10 RA and innate immunity

The data presented in this thesis indicates a role of pathogenic macrophages and complements as drivers of arthritis (paper I). It also indicates a regulative role of type II macrophages (paper II). ROS was identified as immunoregulative effect both in primary and effector phase (paper III). Furthermore, we found an indication that ROS can have a regulative effect on MR and that MR loses its downregulative abilities in PsA if ROS from NOX2 complexes are lost (paper II). ROS have previously been implicated to play a role in autoimmune diseases, for example SLE (Olsson et al. 2017) and enhanced copies of the Ncf1 gene have been associated with less likelihood to develop arthritis in humans and rodents (Olofsson et al. 2003, Kelkka et al. 2014, Olsson et al. 2012) PsA have been connected to ROS in mice models (Khmaladze et al. 2014). Both arthritis and PsA have previously been linked to regulation of disease via macrophage produced ROS (Khmaladze et al. 2014, Gelderman et al. 2007) and treatment with the macrophage inhibiting clodronate showed indications to milder disease in humans and rodents (Barrera et al. 2000, Kinne et al. 1995, Valleala, Laitinen et al. 2001). It is however likely that the RA patients consists of a heterogenous group and thus the treatment will very likely be personal and not all is necessarily helped by down-regulating the innate immunity. For those patients that are in need of innate regulating medicine, my hypothesis is that it will need a multifactorial drug attacking both the macrophages and the complement cascade; especially in ACPA positive patients since the lingering ACPAs might upregulate the complement system and thus drive disease. This however would need further studies to firmly conclude. (Trouw et al. 2009, Stumer et al. 2017, Bohlson et al. 2014)

Another interesting aspect is anti-TNFα such as for example etanercept and infliximab drugs. TNFα can enhance macrophage differentiation towards M1, enhance activation of B cell and fibroblast proliferation and is a power full inducer of inflammation, among others. Anti-TNFα drugs will work on many different immunological pathways simultaneously; (a) blocking IL6 production thus lowering inflammation, (b) some activates FcR receptors and thus provide them the ability to be involved in antibody-dependent cell-mediated cytotoxicity (ADCC), (c) some of them will cause production of IL10 which will cause immunosuppression partly via M2 cells, (d) some can also induce T cell activation-induced cell death and (e) inhibit T cell proliferation and cytokines, (f) they will also induce monocyte and lymphocyte apoptosis. Hence, it is not clear if the positive effect of anti-TNFα is because of it downregulating the innate or adaptive immune system or if it indeed is because of its activation of M2 cells that downregulates the immune system. It is therefore, interestingly, plausible that one of the most common drugs exert its downregulative effects due to down-regulation of inflammatory innate cells and/or

upregulation of MR positive M2 cells. The exact mechanism of anti-TNFα drugs on RA and PsA is not known. (Martinez, Gordon 2014, Sedger, McDermott 2014) More investigation is hence needed to find out the true driver of RA and PsA, and it is likely that it is not the same cell for all patients.

7 CONCLUSIONS

In conclusion, this thesis presents a hypothesis that the adaptive immune system (e.g. T cells and autoantibodies) is of importance primarily in the initiation of arthritis, but that the chronic arthritis is can be primarily driven by the innate immune system (e.g. macrophages and complement cascade). This conclusion is drawn primarily from the results from paper I and III. In paper I, a novel chronic arthritis model was established and proven to be independent of adaptive immunity, but it was observed that macrophages and the complement cascade play an important role. Paper III illustrates the role of ROS in different phases of arthritis, the primary phases and the effector phase. It also concludes that ROS regulates disease in both phases, but the mechanism with which it regulates the disease differs. In the priming phase a switch from ROS deficient to wild type levels affects the T cells and the monocytes/macrophages. In the effector phase the T cells are unaffected and the effect is dependent on CD11b+ monocytes and neutrophils. Taken together, this research postulates the adaptive immune system as the initiator and the innate immunity as the driver of the disease. In paper II, we could find a down-regulatory effect on the disease severity by MR in models for both RA and PsA, further indicating an importance of macrophages in autoimmunity. Further studies are however necessary to confirm this hypothesis. If true, this hypothesis could affect the manner we treat RA patients. It is possible that a more innate immune regulating treatment might be needed for treating already chronic disease.

We can also conclude that the difference between KO and point mutated Ncf1 mice might very well be down to poor back-crossing. Thus pin-pointing how important it is to do carefully create and characterize new animal models.

To summarize;

1. A new mice model for chronic arthritis was generated and characterized to be independent of the adaptive immune system. Chronic arthritis was driven by macrophages and complement system.

2. MR was concluded as important for regulating arthritis and PsA.

3. By the utilizing the ROS inducible mice model, we showed the importance of ROS in both primary and effector phase of arthritis

4. By careful crossing between a mouse with a point mutation in *Ncf1* and Ncf1 KO mice an important lesson on back-crossing and proper planning of how to design new mice was learned.

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