From the Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research
Karolinska Institutet, Stockholm, Sweden

FROM GENES TO FUNCTION IN AUTOIMMUNITY – A SALTY STORY

Daniëlle Vaartjes

Karolinska Institutet

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From genes to function in autoimmunity – a salty story

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By

Daniëlle Vaartjes

Principal Supervisor:
Rikard Holmdahl
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research

Co-supervisor(s):
Liselotte Bäckdahl
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Medical Inflammation Research

Opponent:
Bryce Binstadt
University of Minnesota Medical School
Department of Pediatrics
Division of Pediatric Rheumatology
Center for Immunology

Examination Board:
Gunnar Pejler
Uppsala University
Department of Medical Biochemistry and Microbiology

Niklas Björkström
Karolinska Institutet
Department of Medicine Huddinge
Center for Infectious Medicine

Louise Berg
Karolinska Institutet
Department of Medicine
Division of Rheumatology

To my loving family

"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less."

NL: "Niets in het leven is om bang voor te zijn, het moet alleen begrepen worden. Nu is de tijd om meer te begrijpen, zodat we minder bang kunnen zijn."

Marie Curie (1867-1934)

ABSTRACT

Autoimmunity is a condition in which the ability to tolerate self breaks down, resulting in immune responses against the body's own healthy cells and tissues. Autoimmune diseases are complex and multifactorial, and both genetic and environmental factors are known to play a crucial role. Using animal models, it is possible to study different aspects of arthritis disease development in an environmentally and genetically controlled setting. In this thesis, I have investigated the effect of genetic risk loci and the single environmental factor sodium chloride (NaCl) on immune cell function and the development of autoimmune diseases using mouse models.

In Paper I, using four congenic sub-loci within the arthritis susceptible *Cia9* locus on chromosome 1, we found that the NOD.Q polymorphic Fc gamma receptor gene (FcγR) cluster located within sub-loci *Cia9i* and *Cia9k*, regulated arthritis. Polymorphic FcγR2b and FcγR4 were contained in both *Cia9i* and *Cia9k*, whereas *Cia9i* mice also carried polymorphic FcγR3. FcγR2b gene and protein expression were downregulated in *Cia9i* and *Cia9k* mice, whereas FcγR3 was upregulated in *Cia9i* mice and found downregulated in *Cia9k* mice compared to littermate control mice. This difference in FcγR3 expression affected killing by NK cells and phagocytosis by macrophages *in vitro* and PC61 antibody induced regulatory T cell depletion *in vivo*. Interestingly, arthritis development was regulated by interaction between FcγR2b and FcγR3 without affecting anti-collagen type II antibody secretion. These results show that polymorphisms in both FcγR2b and FcγR3 regulate the severity of inflammatory responses.

In paper II, we investigated the importance of the system A family of amino acid transporters (SNAT), based on an identified congenic locus, as mediators of immune cell function and arthritis development. We demonstrated that SNAT proteins affect the initial stages of lymphocyte activation by regulating glutamine uptake in the presence of Na^+, and that the effector phase of arthritis could be suppressed by blocking SNAT proteins.

Paper III describes the effect of salt (e.g. NaCl) on the development of autoimmunity. Here we show that a moderate salt intake affects both T cell and macrophage phenotypes *in vitro* and *ex vivo*. However, these moderate levels of salt intake did not alter the development of T cell-dependent autoimmunity, whereas the dextran sulphate sodium (DSS)-induced colitis was exacerbated in mice pre-exposed to salt.

Taken together, I have shown that the interplay between two genes enhances arthritis disease development, whereas a single environmental factor has no impact on arthritis despite triggering the immune system. These results contribute to the understanding of the mechanism behind complex multifactorial diseases as a small building block towards therapeutic intervention.

SUMMARY FOR MY FAMILY

Dutch: Samenvatting

Ons immuunsysteem beschermt ons dagelijks tegen verschillende ziektekiemen die aanwezig zijn in en rondom ons lichaam. Als we toch ziek worden, werkt ons immuunsysteem hard om van die infectie af te komen. Dit gaat vaak gepaard met koorts en, op celniveau, met infiltrerende immuuncellen die de infectie aanvallen en doden. Om een voorbeeld te geven: als een infectie ons lichaam binnenkomt, vallen cellen (zoals macrofagen) deze infectie aan om ze te doden. Soms hebben deze macrofagen hulp nodig van B-cellen, welke antilichaampjes aanmaken, en T-cellen om van de infectie af te komen. In het geval van een auto-immuunziekte worden lichaamseigen cellen aangevallen door het eigen immuunsysteem. Het is niet precies bekend waarom dit gebeurd. Wel weten we dat auto-immuunziektes complex zijn en dat ze kunnen ontstaan door een combinatie van meerdere factoren. Een persoon met een "verkeerde" genetische aanleg die bovendien blootgesteld wordt aan "verkeerde" omgevingsfactoren heeft een verhoogde kans om een auto-immuunziekte te ontwikkelen.

Een welbekend voorbeeld van een auto-immuunziekte is reumatoïde artritis, in de volksmond vaak reuma genoemd. Reuma is een ziekte waarin het kraakbeen in gewrichten langzaam afgebroken wordt en botvergroeiingen kunnen ontstaan. Symptomen zijn vaak zichtbaar in de vingers/handen van patiënten, deze kunnen rood, gezwollen en erg pijnlijk zijn. Tegenwoordig kunnen botvergroeiingen vaak voorkomen worden door op tijd in te grijpen met het nemen van medicatie en het soepel houden van gewrichten door middel van sporten en fysiotherapie. Helaas kunnen reuma en andere auto-immuunziekten tot op heden nog niet worden voorkomen. Daarvoor is meer onderzoek nodig.

Om controle op de genetische aanleg en omgevingsfactoren te hebben, worden muismodellen voor verschillende auto-immuunziekten gebruikt. Muizen worden ziek nadat ze geïnjecteerd zijn met lichaamseigen eiwitten of moleculen voor de desbetreffende ziekte. In deze scriptie heb ik voornamelijk gewerkt met muismodellen voor reuma. Met behulp van muismodellen, heb ik onderzoek gedaan naar het effect van genetische risico factoren en naar het effect van keukenzout (NaCl) op de functie van immuun cellen en de ontwikkeling van auto-immuunziekten.

In artikel I, heb ik gebruik gemaakt van speciaal gefokte muizen die een genetische aanleg hebben voor reuma. Reuma ontwikkeld nadat de muizen geïnjecteerd zijn met collageen type

II (CII), aanwezig in het kraakbeen, of met antilichaampjes tegen CII. Met behulp van deze muizen heb ik gekeken naar de functie van specifieke receptoren die antilichaampjes kunnen binden (Fc gamma receptoren, FcγR) en aanwezig zijn op immuuncellen zoals macrofagen en B cellen. Een defect in het DNA en eiwit van twee van deze receptoren (FcγR2b en FcγR3) leidde tot meer agressieve reuma. Uit mijn onderzoek blijkt ook dat dit afhankelijk is van de functie van macrofagen.

In artikel II, heb ik onderzocht wat de functie is van bepaalde aminozuur transporters, genaamd SNAT, op de ontwikkeling van reuma. SNAT eiwitten reguleren een gedeelte van immuuncelactivatie (voornamelijk T-cellen) door het opnemen van glutamine in de aanwezigheid van een zout ion (Na^+). Het blokkeren van deze eiwitten onderdrukt reuma in muizen.

In artikel III, heb ik gekeken naar het effect van keukenzout op de ontwikkeling van drie verschillende auto-immuunziekten (reuma, multipele sclerose (MS) en inflammatoire darmziekten). Op celniveau zien we dat immuuncellen zoals T-cellen en macrofagen meer autoreactief worden, wat inhoudt dat ze ziekten kunnen veroorzaken. Muizen krijgen een zoutwater oplossing (1% NaCl) of normaal kraanwater te drinken alvorens ze worden blootgesteld aan het stofje dat ze ziek maakt. Gedurende de ziekte worden de symptomen van de muizen in de twee verschillende groepen (zoutwater en normaal kraanwater) bijgehouden. We zien dat het innemen van keukenzout in deze concentratie geen effect heeft op de ontwikkeling van reuma of MS, maar dat het de symptomen voor de inflammatoire darmziekten verergerd.

Samengevat heb ik in artikel I aangetoond dat de nauwe samenwerking van twee Fc gamma receptoren de ontwikkeling van reuma verergerd. Dit bevestigt de complexiteit van de ziekte. Bovendien is mijn onderzoek in artikel III in overeenstemming met onderzoek in reuma patiënten, waarin geen directe link tussen overtallige zout inname en reuma was aangetoond. Desalniettemin, heb ik bevestigd dat overtallige inname van zout een negatief effect heeft op celniveau en op de ontwikkeling van inflammatoire darmziekten.

Al met al heb ik met mijn onderzoek bijgedragen, al is het een klein puzzelstukje, aan het beter begrijpen van de ontwikkeling van complexe auto-immuunziekten zoals reuma.

LIST OF SCIENTIFIC PAPERS

I. **Vaartjes D**, Klaczkowska D, Nandakumar KS, Holmdahl R.
Immune complex receptors FcγR2b and FcγR3 alleles act in concert to regulate inflammation.
Manuscript

II. Raposo B, **Vaartjes D**, Ahlqvist E, Nandakumar KS, Holmdahl R.
System A amino acid transporters regulate glutamine uptake and attenuate antibody-mediated arthritis.
Immunology. 2015 Sep 8. DOI: 10.1111/imm.12531

III. **Vaartjes D**, Nandakumar KS, Holmdahl R, Raposo B.
Increased salt exposure affects both lymphoid and myeloid effector functions, influencing innate-associated disease but not T cell-associated autoimmunity.
Immunology. Accepted on February 25 2018. DOI: 10.1111/imm.12923

CONTENTS

LIST OF ABBREVIATIONS

ACPA	Anti-Citrullinated Protein Antibody
ADCC	Antibody Dependent Cellular Cytotoxicity
ADCP	Antibody Dependent Cellular Phagocytosis
APC	Antigen Presenting Cell
B6	C57BL/6J
B10	C57BL/10J
BCR	B Cell Receptor
CAIA	Collagen Antibody-Induced Arthritis
CCP	Cyclic Citrullinated Peptide
CIA	Collagen-Induced Arthritis
CII	Collagen type II
CNS	Central Nervous System
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DMARD	Disease-Modifying Anti-Rheumatic Drugs
DN	Double Negative
DSS	Dextran Sulphate Sodium
EAE	Experimental Autoimmune Encephalomyelitis
EPV	Epstein-Barr Virus
FcγR	Fc gamma Receptor
FLS	Fibroblast-Like Synoviocyte
GWAS	Genome-Wide Association Studies
HLA	Human Leukocyte Antigen
HS	Heterogeneous Stock
IBD	Inflammatory Bowel Disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleucin
ILC	Innate Lymphoid Cell
ITAM	Immunoreceptor Tyrosine Based Activating Motif
ITIM	Immunoreceptor Tyrosine Based Inhibitory Motif
KO	Knockout Out
LAT	Linker for the Activatin of T cells
LPS	Lipopolysaccharide

M-CSF	Macrophage Colony-Stimulating Factor
MBL	Mannan-Binding Lectin
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NET	Neutrophil Extracellular Trap
NK	Natural Killer
NO	Nitric Oxide
NOD	Non-Obese Diabetic
NSAID	Non-Steroid Anti-Inflammatory Drug
OR	Odds Ratio
PAI	Partial Advanced Inter-Cross
PAMP	Pathogen-Associated Molecular Patterns
PD-1	Programmed cell Death-1
PI3K	Phosphoinositide 3-Kinase
PRR	Pattern Recognition Receptor
QTL	Quantitative Trait Loci
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
ROS	Reactive Oxygen Species
RTX	Rituximab
SE	Shared Epitope
SLAM	Signaling Lymphocytic Activation Molecule
SLE	Systemic Lupus Erythematous
SNAT	Sodium-Coupled Neutral Amino acid Transporter
SNP	Single Nucleotide Polymorphism
T1D	Type 1 Diabetes
TCM	Central memory T cell
TCR	T Cell Receptor
TEM	Effector memory T cell
Th	T helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	Regulatory t cell
VIP	Västerbotten Intervention Program
WT	Wildtype

1 INTRODUCTION

"Autoimmunity is a condition in which the ability to tolerate self breaks down and the body ends up under attack as the immune system mistakes the body's cells for enemies (1)."

Here I intend to give a brief overview of the immune system and what is known about the development of autoimmunity. I will further discuss the important role of genetics and environmental factors in the pathogenesis of different autoimmune diseases, with the main focus on rheumatoid arthritis and mouse models thereof. Furthermore, I will explain why the use of animal models is so important to study genetics and environmental factors. The genes and their functions studied in this thesis are the genes for the sodium-coupled neutral amino acid transporters and the Fc gamma receptors. "A salty story" reflects on the impact of increased salt exposure on immune cell function and autoimmunity as described in paper III. The overall aim of this thesis is to contribute to the understanding of mechanisms behind complex multifactorial diseases as a small building block on the road to therapeutic intervention.

1.1 THE IMMUNE SYSTEM

From birth and onwards we live in symbiosis with different microorganisms, such as bacteria, fungi, viruses and parasites, without constantly developing diseases. It is the immune system that protects us. The immune system is our body's defence mechanism and consists of numerous cell types (immune cells), tissues and molecules that help us fight infections and to remove dead and damaged cells from the body. A classical division is made between the innate and adaptive immune system. Once pathogens have crossed the physical barriers of the body, such as skin, the gastrointestinal and the respiratory mucosa, the innate immune system gets activated. This mechanism of first line defence is nonspecific and fast, acting within minutes or hours after exposure to an infectious pathogen, either by acute inflammation or through antiviral defence. When activated, innate cells differentiate into short-lived effector cells to clear the pathogen. If needed, innate immune responses stimulate adaptive immunity through antigen-presentation. The adaptive immune system consists of humoral immunity and cell-mediated immunity, and is characterized by specificity, diversity,

specialization and memory. It therefore requires several days to get activated. The innate immune system is evolutionary older than the more specialized adaptive immune system and is found not only in vertebrates, but also in invertebrates and plants. Studies in insects have demonstrated the absence of adaptive immunity and therewith that of autoimmune diseases (2). Although immunological memory is considered to be exclusive for the adaptive immune system, there is an increasing body of evidence that suggests that innate immune responses exhibit memory characteristics after the first encounter with a pathogen. As reviewed by Netea *et al*., this memory of trained immunity is defined as an increased response to a secondary infection that can be exerted towards the same microorganism or cross-protection observed as phenotypic changes in NK cells and macrophages (3).

In the following section, the various cell types and characteristics of the innate and adaptive immune system, as well as the connection between both, will be reviewed.

1.1.1 The innate immune system

Cells of the innate immune system are both from myeloid and lymphoid lineages. Myeloid cells include granulocytes (neutrophils, basophils and eosinophils), monocytes, macrophages, dendritic cells and mast cells and are involved in acute inflammation. These cells cannot distinguish between individual pathogens, since they lack specific receptors, but they express pattern recognition receptors (**PRR**) with which they can recognize pathogen-associated molecular patterns (**PAMP**) present on pathogens, and can thus distinguish self from non-self. Damaged or necrotic cells are also cleared by innate immune cells through their recognition of damage-associated molecular patterns (**DAMP**) on those cells. One of the most described innate immune cell receptors are toll-like receptors (**TLR**) (4). An example is TLR-4, which is specific for bacterial lipopolysaccharide (LPS) and this receptor is often activated in *in vitro* macrophage assays (5). Activated TLRs either enhance expression of cytokines (which are soluble proteins that mediate immune and inflammatory reactions) and other proteins to promote phagocytosis or the production of type 1 interferons (IFN) to mediate antiviral defence. One of the cells involved in the latter, are natural killer (NK) cells. NK cells are, among innate lymphoid cells (ILCs), NK T cells and γδ T cells, cells of the lymphoid compartment of the innate immune system (4). For the scope of this thesis, neutrophils, macrophages and NK cells will be described further. Neutrophils and macrophages are thought to be important regulators in arthritis development (6), studied in

papers I, II and III. Moreover, macrophage activation has been assessed in papers I, II and III, whereas the cytotoxic function of NK cells has been studied in paper I.

Neutrophils are circulating leukocytes that are recruited to the site of infection or tissue damage and are highly abundant in blood. They are short lived cells and are the first cell type to respond to bacterial and fungal infection. After they are recruited to the site of infection or tissue damage, they ingest microbes through a process called **phagocytosis**. Once microbes are phagocytosed, they will fuse with lysosomes and will be killed by enzymes and toxic substances such as reactive oxygen species (**ROS**) and nitric oxide (**NO**), often referred to as oxidative burst. Inflammatory cytokines and immune complexes can enhance this phagocytosis process, after which neutrophils kill themselves either through apoptosis or by forming neutrophil extracellular traps (NETs). The latter is a process called NETosis and is considered to be pro-inflammatory, whereas apoptosis renders anti-inflammatory effects, attracting macrophages to remove the apoptotic cell (7–9).

Macrophages are monocyte-derived cells that reside in different organs and tissues throughout the body. The clearance of apoptotic cells is induced by cytokines such as interleukin (IL)-4 and IL-13. These apoptotic macrophages are important for tissue repair and can produce anti-inflammatory cytokines such as IL-10. Macrophages activated by microbial TLR-ligands and IFN-y are involved in phagocytosis and the production of ROS. Activated macrophages are also important effector cells within the adaptive immune system. A relevant example is their recognition of antibody-coated (opsonized) microbes through their Fc gamma receptors (receptors that recognize the Fc portion of immunoglobulin G (IgG), described in detail below), which induces antibody-dependent cellular phagocytosis (**ADCP**). Another component that can help clear pathogens, either in the absence or presence of circulating antibodies, are **complement** proteins, which are circulating as well as membrane-associated proteins. When activated, they opsonize microbes and stimulate phagocytosis, promote leukocyte (e.g. neutrophils and macrophages) recruitment to the site of inflammation (C3a and C5a) and lyse the microbe (10).

As briefly mentioned, innate immune cells secrete cytokines upon activation. One of the cytokines secreted by macrophages during phagocytosis is IL-12. IL-12 can activate **NK cells** to produce IFN-y that in turn activates macrophages to kill the phagocytosed microbes. A direct function of NK cells is to kill host cells infected by microbes. NK cells contain cytoplasmic granules filled with perforin and granzymes. Upon activation, NK cells release these granules that then enter the infected cells and activate enzymes, inducing apoptosis. NK cells distinguish infected cells from non-infected cells through the expression levels of class I

major histocompatibility complex (MHC-I) molecules. A healthy cell expresses MHC-I, which is recognized by an inhibitory receptor on NK cells, preventing NK cells to attack. A virus-infected or stressed cell has reduced expression of MHC-I, together with high expression of ligands for activating NK cell receptors, which results in NK cell activation and killing of the infected cell (11). One such activating receptor is CD16, or Fc gamma receptor 3, which binds to antibody-coated cells and results in killing by antibody-dependent cellular cytotoxicity (**ADCC**).

A further link between the innate and adaptive immune system is the process of antigen presentation by antigen presenting cells (**APCs**) such as dendritic cells (DCs). DCs are present in the epithelia and subepithelial tissues and use various membrane receptors to bind microbes. After endocytosis of the microbe antigens, the engulfed proteins get processed into peptides for loading onto MHC molecules (12). This process leads to release of inflammatory cytokines such as TNF and IL-1. Subsequently, DCs upregulate co-stimulatory molecules such as CD40, CD80 and CD86 and the chemokine receptor CCR7. This induces migration to lymph nodes where they mature into APCs. By presenting the peptide antigens on their MHC molecules to T cells, T cells will get activated.

1.1.2 The adaptive immune system

As mentioned above, the adaptive immune system is antigen specific and gets triggered several days into the progression of an infection.

Both T cells and B cells originate from bone marrow hematopoietic stem cells, but whereas B cells develop and mature in the bone marrow, **T cells** migrate to the thymus to develop. Here these thymocytes undergo a series of maturation steps, expressing different surface markers. During early development, thymocytes don't express the T cell co-receptors CD4 and CD8 and are called double negative (DN: CD4-CD8-) T cells. These DN cells are further subdivided by CD44 and CD25 into four stages (DN1-4). At DN3 (CD44-CD25+) β-selection and rearrangement of the T cell receptor (TCR) occurs. This leads to expression of both CD4 and CD8 (double positive) after which the α-chain of the TCR is rearranged. This is followed by positive and negative selection. Only thymocytes that don't interact too strong or too weak with MHC class I or MHC class II are positively selected and eventually become $CD8^+$ or $CD4^+$ cells, whereas other cells are killed by apoptosis. These positive selected cells

then undergo negative selection, where thymocytes that interact too strongly with self-antigen will be killed by apoptosis or selected to become Regulatory T cells (Tregs) (4, 13).

After maturation, naïve $CD4^+$ and $CD8^+$ T cells migrate to peripheral lymphoid organs, where they can get activated after their TCR recognizes peptide antigens presented by APCs on their MHC-II or MHC-I molecules, respectively. This leads to T cell expansion and differentiation into effector cells. These effector cells enter the circulation and migrate, together with other leukocytes, to the site of infection where they encounter the antigen. CD4 T cells activate (help) leukocytes to induce phagocytosis and CD8 T cells kill infected cells either directly or through release of cytokines that activate macrophages to kill. However, T cells need a second and third signal to become activated, otherwise they will become anergic instead. A second signal is provided by the APCs through co-stimulatory molecules (CD28 on T cells and CD80/CD86 molecules on APCs), and cytokines are signal three.

CD4 T cells are also called T helper cells (Th). Upon activation these cells differentiate into specific subsets, including Th1, Th2, Th17 and Tregs. They differ in their cytokine production profile, surface marker receptors and effector functions. Th1 cells secrete IFNγ, IL-2, IL-10 and TNF and promote macrophage activation and cytotoxic T cell proliferation to induce phagocytosis and killing of mibrobes. Upon antigen recognition, Th1 cells express CD40L on their surface that binds to the CD40 receptor on macrophages and professional APCs. Differentiation of Th1 cells is induced by cytokines such as IL-27, IL-12 and IFNγ. Th2 cells expand in the presence of IL-4 and IL-2 or IL-7. They mount immune responses against large extracellular pathogens such as parasites and are required for humoral immunity. Th2 cells secret IL-4, IL-5, IL-9, IL-13 and IL-25. Th17 cells secrete IL-17 and IL-22 that stimulate chemokine secretion and recruitment of neutrophils and macrophages as mentioned above. In mice, Th17 cells develop in the presence of IL-6 and TGF-β, whereas IL-1β, IL-6, IL-21 and IL-23 are needed in humans (4, 14).

After an immune response has been carried out and the pathogen has been cleared, T and B cell responses should be terminated to avoid inflammatory tissue damage. Activated T cells start expressing inhibitory receptors such as cytotoxic T lymphocyte antigen (CTLA)-4 that outcompetes CD28 for binding to costimulatory CD80/86 molecules on APCs. It also dampens downstream TCR and CD28 signaling. The CTLA-4 protein thus acts as a negative regulator of T cell activation (15). Another inhibitory receptor is programmed cell death-1 (PD-1), which inhibits T cell proliferation and cytokine production.

Towards the end of the immune response, most antigen-specific T cells die. However, in the presence of IL-7 a small percentage develops into memory T cells that provide long-lasting immunity. Although memory T cells proliferate less, their activation threshold is lower than that of primary T cells, resulting in a greater effector response (16). There are two types of memory T cells: effector memory T cells (TEM) and central memory T cells (TCM). TEMs migrate to the inflamed peripheral tissues and display immediate effector function; they are characterized by high expression of CD44 and the lack of CD45R0, CCR7 or CD62L expression. TCMs home to T cell areas of secondary lymphoid organs and provide reactive memory by proliferating and differentiating into effector cells in response to inflammatory processes. TCMs express CD44, but also CD45R0, CCR7 and CD62L (17).

B cells provide the humoral (antibody) mediated adaptive immune response. They recognize antigens through their B-cell receptor (BCR), which is a membrane-bound form of immunoglobulin (Ig). Fully differentiated B cells become plasma cells, which is when they secrete soluble Ig (antibody) of the same antigen specificity as their BCR. Upon antibody-pathogen binding, other cells and molecules are recruited to destroy the pathogen.

B cells develop in the bone marrow from pro-, pre- and immature to mature B cells through different stages of V (variable), D (diversity) and J (joining) gene recombination of the Ig heavy and light chain, which will form the BCR. As with T cells, B cells undergo positive and negative selection processes to get their required specificity. Positive selection requires the pre-BCR and BCR to bind to their ligand. B cells that survived the positive selection process are immature B cells expressing IgM. IgM is the first antibody class secreted by activated B cells and is produced upon B cell activation by microbial antigens alone, without T cell help. Negative selection is the process where immature B cells binding to self cell-surface antigens are removed from the repertoire, through clonal deletion, anergy, receptor editing or ignorance. The immature B cells that survive will migrate from the bone marrow to the spleen where they become mature B cells, co-expressing IgM and IgD. B cells mature in follicles in the spleen, they transition from T1 B cells to T2 B cells into follicular B cells or marginal zone B cells depending on the activating signals provided by other cells in the follicles. These mature B cells can then be activated to secrete antibodies (4).

Antibodies bind to different antigens by recognizing specific epitopes with their antigen-binding or variable (V) region. The constant (C) region exists in five different forms, called isotypes, and engaging of each one results in different effector mechanisms. Once the BCR recognizes and binds to a specific epitope on an antigen, the B cell gets activated, leading to clonal expansion and antibody production. Five different classes of antibodies are known:

IgM, IgD, IgG, IgA and IgE. IgM is present in the blood stream as a pentamer after initial B cell activation. To increase the affinity and variety of antibodies, B cells undergo somatic hypermutation and class switching with the help of T cells in peripheral lymphoid organs. When activated, the BCR internalizes and degrades the antigen to present it on its MHC class II molecules to T cells. T cells then upregulate CD40L on their surface and bind to CD40 on B cells. This co-stimulation together with T cell cytokine secretion stimulates the B cells and induces B cell proliferation, class switching, and affinity maturation. The latter, as the name implies, is a process to increase antibody affinity to target antigens in order to more effectively eliminate infections. Affinity maturation occurs by somatic hypermutation of proliferating B cell V genes in germinal centers in lymphoid follicles. Eventually, B cells will differentiate into long-lived plasma or memory B cells.

IgA antibodies are dimers, whereas IgE and IgG are monomers and these antibodies are therefore smaller compared to IgM. IgA-secreting plasma cells are predominantly found in the lamina propria, just below the surface epithelia of skin and mucosa. IgA is secreted as a dimer in the lamina propia and transported across the epithelium of the gut, the respiratory epithelium, the lactating breast and various other glands. IgA antibodies protect epithelial surfaces from infectious agents and are also very important in regulating the gut microbiota (18, 19). IgE is mainly bound to receptors on mast cells, found just beneath epithelial surfaces of the skin and mucosa. Upon antigen binding, mast cells release chemical mediators such as histamine that can induce processes such as sneezing, coughing and vomiting, to eliminate the pathogen. IgE levels are elevated during allergic reactions. For the scope of this thesis, IgG and its effector functions will be discussed in more detail.

IgG antibodies are found in the blood and in the extracellular fluid. It is their high affinity for antigen and their ability to diffuse easily throughout the extracellular fluid that make IgGs the primary antibodies to neutralize toxins in tissues. IgG antibodies are subdivided into IgG1, IgG2, IgG3 and IgG4 in humans, named by decreased order of abundance in the serum. Mice don't have IgG4 but have IgG1, IgG2a, IgG2b and IgG3. Each IgG molecule consists of two identical class γ heavy (H) chains and two identical light (L) chains that can be either κ or λ. The two heavy chains are linked to each other and each light chain is linked to one heavy chain by disulfide bonds, resulting in a Y-shaped antibody. The N-terminal parts of the two H and L chains are the variable region (V_H and V_L), whereas the C-terminus is constant. Through simultaneous binding of two identical antigens by both of the N-terminal parts, the total antibody-antigen interaction strength is increased, resulting in a higher avidity. The N-terminal region is also called the F(ab')2 fragment, whereas the constant region (C_H2 and

C_H3) is named the Fc fragment. The Fc part defines the IgG isotype and interacts with effector molecules. Class switching occurs in the presence of cytokines produced by T cells. For example, B cell activation in the presence of IL-4 will induce switching to IgG1 and IgGE, but inhibits switching to IgM, IgG2a and IgG3. IFN-γ induces IgG2a and IgG3, TGF-β induces IgG2b, and IL-21 induces IgG1 and IgG3 (4, 20). These different isotypes are specialized to function in different compartments of the body. All four isotypes of IgG are important for pathogen neutralization, whereas IgG1 and IgG3 are also important for opsonization, NK cell cytotoxicity, activation of the complement system and to a lesser extend sensitization of mast cells. IgG2 can act as an opsonin in the presence of the right Fc receptor.

1.1.3 Fc gamma receptors

Antibodies binding to smaller pathogens neutralize the pathogen and protect the body against infection. However, they need help to destroy the pathogen. One way is by antibody-dependent cellular phagocytosis through Fc gamma receptors (FcγRs) on effector cells expressing all FcγRs. Humans express six different FcγRs, including the high affinity activating FcγR1, the low affinity activating FcγRs FcγR2A, FcγR2B, FcγR3A and FcγR3B, and the low affinity inhibitory FcγR2B. FcγR1 and FcγR2B are also present in mice, whereas

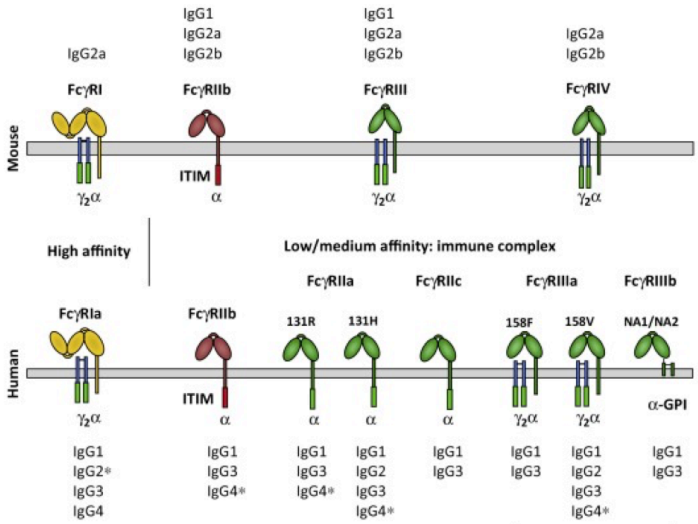

Figure 1 Mouse and human Fc gamma receptors (FcγRs). Yellow: high affinity FcγR. Red: inhibitory FcγR. Green: low/medium affinity activating FcγRs. Indicated which antibody isotype can activate the FcγR. The ITAM and ITIM motifs are indicated in green and re, respectively. The antibody isotypes that can activate the individual FcγRs are listed. For the human FcγRs, the different haplotypes are listed. Mouse FcγRI can also bind IgG2b and IgG3 at low affinity. Adapted from (21).

mouse FcγR4 is the orhologoue of human FcγR3A and mouse FcγR3 is the orthologue of human FcγR2A (Figure 1). In both human and mice, the FcγR genes are clustered in close proximity on chromosome 1 (22, 23). Polymorphisms of the FcγR gene cluster in mice have been studied in paper I.

Antigen:antibody complexes, also called immune complexes, can be cleared by FcγRs alone or with help of the complement system. Complement can bind to the Fc parts of antibodies in immune complexes after which they bind to the complement receptor CR1 on phagocytes and together with the FcγR induce phagocytosis. However, mice deficient in the classical, alternative or mannose-binding lectin (MBL) pathway of the complement cascade showed unchanged antibody activity (24). Moreover, although C5a was found to be important in a model for autoimmune haemolytic anaemia, C5a acted as a second messenger by upregulating activating FcγRs rather than directly in cytotoxic reactions (22, 25). Here I will further describe the function of FcγR specific mechanisms.

When binding to immune complexes, FcγRs get activated and can trigger activation of innate effector cells and regulation of B cells (23, 26). B cells only express the inhibitory FcγR2b that regulates activating signals transduced by the BCR and delivers apoptotic signals to plasma cells (23). Mouse NK cells only express the activating receptor FcγR3 that is involved in ADCC, leading to NK cell degranulation, cytokine production and target cell apoptosis (23). Some effector cells, such as monocytes and macrophages, express all four FcγRs and the net signaling outcome is determined by the ratio of activating vs. inhibitory FcγR expression on the cell surface. Engagment of FcγR in these cells results in the release of immune modulators that are cytotoxic and/or (pro-) inflammatory and in phagocytosis.

Mouse and human FcγRs differ in their binding affinity. Whereas in humans IgG1 and IgG3 antibodies are more pro-inflammatory, in mice this is the case for IgG2a and IgG2b, indicated *in vivo*. This difference seems to be caused by the medium-affinity FcγR4, which selectively interacts with these isotypes. On the other hand, IgG1 selectively binds to FcγR3, which has been demonstrated in mouse models using FcγR3 knockout (KO) mice that showed abrogated antibody activity. Moreover, the activity of IgG2a and IgG2b was not altered in these KO mice. Furthermore, using FcγR2b KO mice, it has been shown that FcγR2b negatively regulates the activity of IgG1 when co-expressed with FcγR3 on the same effector cell (21, 23, 27).

Activating FcγRs consist of a ligand binding α-chain and a signal-transducing adaptor molecule, containing immunoreceptor tyrosine-based activator motifs (ITAM) in its

cytoplasmic domain. In monocytes and macrophages, this adaptor molecule consists of two γ-chains that are involved in downstream signalling and the assembly of cell-surface transport of the respective α-chain. Upon immune complex binding, activating FcγRs crosslink and induce phosphorylation of the ITAM motif by SRC kinases. This generates a docking site for SYK that in turn activates various downstream targets, including the linker for the activation of T cells (LAT) and phosphoinositide 3-kinase (PI3K). PI3K is responsible for the recruitment of membrane molecules, which leads to an increase in intracellular calcium levels and activation of further downstream signalling events (e.g. ERK, JNK and p38), and eventually to cell activation (e.g. ADCP, ADCC, oxidative burst and cytokine release) (23).

The inhibitory FcγR2b exists in two forms: FcγR2b-1, solely expressed on B cells, and FcγR2b-2, expressed on all phagocytes. On B cells, FcγR2b functions as a checkpoint of humoral tolerance. During peripheral B cell development FcγR2b 1) inhibits the activation and expansion of autoreactive B cells into IgG positive plasma cells, 2) excludes autoreactive IgG positive B cells from the follicles, and 3) induces apoptosis of autoreactive plasma cells upon immune complex binding. It has been shown that plasma cells from autoimmune-prone mouse strains have reduced FcγR2b expression and are resistant to FcγR2b dependent apoptosis induction. The expression of FcγR2b goes together with expression of its activating counterpart. Moreover, the FcγR activation threshold of a cell is determined by the activating to inhibitory (A/I) FcγR binding ratio on a given cells. FcγR2b expression can be altered by cytokines such as IL-4 and IFNγ. Whereas IL-4 reduces FcγR2b gene expression on B cells, increased levels are found upon stimulation with IFNγ or LPS. The opposite was true for FcγR2b gene expression on monocytes (28).

Signalling by FcγR2b occurs through a single α-chain, which contains an immunoreceptor tyrosine based inhibitory motif (ITIM) in its cytoplasmic tail. In B cells, ITIM phosphorylation occurs upon crosslinking of FcγR2b and the BCR followed by recruitment of SHIP that prevents membrane recruitment. This in turn inhibits downstream signalling such as proliferation and calcium flux. On innate cells, such as macrophages and neutrophils, crosslinking of FcγR2b decreases FcγR mediated phagocytosis, oxidative burst and cytokine release. On DCs, FcγR2b inhibits DC maturation and suppresses antigen internalization and presentation on MHC molecules to CD4 and CD8 T cells (23, 28).

An immune response is the balance of positive and negative signals from both arms of the immune system. Simultaneous triggering of activating and inhibitory FcγR signalling pathways and therewith setting a threshold for cell activation is an example of a well-balanced immune response. Disturbing this balance can result in the loss of tolerance and the

induction of autoimmune responses. The involvement of FcγR2b in autoimmune diseases has been shown in both mice and humans, most prominently in systemic lupus erythematosus (SLE) (28). For the scope of this thesis, the role of FcγR2b in rheumatoid arthritis will be discussed in more detail later.

1.2 AUTOIMMUNE DISEASES

Autoimmune diseases affect 5-10% of the world population with a high female preponderance. They are chronic multifactorial diseases in which the immune system neglects the distinction between self and non-self (29). This causes inflammation and tissue damage, which can be paired with severe pain, loss of function and disability. More than hundred human diseases are considered autoimmune, targeting nearly every tissue. They can be organ specific like type I diabetes (T1D) and inflammatory bowel disease (IBD) or systemic such as SLE, rheumatoid arthritis (RA) and multiple sclerosis (MS). Although affecting different organs, they all have a common denominator: a break of tolerance by regulatory T and B cells. Albeit the exact trigger for this remains elusive, both genetics and environmental factors are known to play a key role in the development of autoimmune diseases (30, 31) (Figure 2).

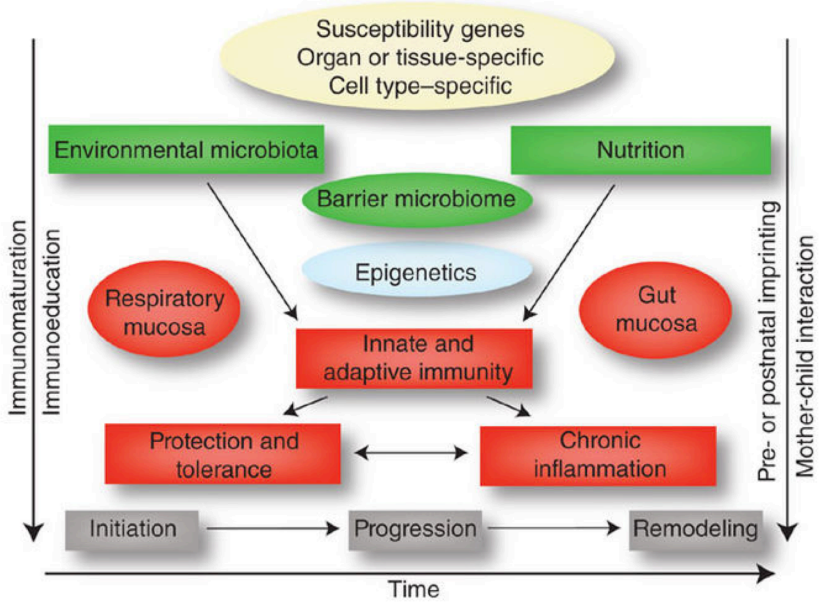

Figure 2 Representation of the development of autoimmune diseases. Showing the involvement of genetics (yellow), environmental factors (green) and epigenetics (blue). Disease development (initiation, progression, remodelling) depends on interaction between intrinsic (red) and extrinsic (green) mechanisms. Adapted from (32).

Despite the numerous treatments that are available for various autoimmune diseases, none of them cures from disease. It is therefore very important to gain a more complete understanding of the mechanisms behind these different autoimmune diseases. Since the genetic and environmental background is easily controlled in mice, candidate genes causing a particular disease phenotype can be identified using mouse models of human diseases (33). These mouse data together with data from genome-wide association studies (GWAS) and statistical linkage analysis in humans can be used to develop new drugs and to stratify patients into groups for better treatment approaches (34).

One of the most successful treatments of the past decade is the use of therapeutic antibodies (IgG monocloncal antibodies) (35). They have so far been used in the treatment of SLE, MS and RA.

In the present thesis I studied the role of genetic factors and a single environmental factor on the development of disease in mouse models of MS, IBD and RA. A description of these diseases will be given here with the main focus on RA.

1.2.1 Multiple sclerosis

Multiple sclerosis is among the most common causes of neurological disorders in young adults, arising between early and middle adulthood. Women are twice as likely to be affected than men. MS is characterized by inflammation, focal demyelination and axonal damage of the central nervous system (CNS) (36, 37). Many clinical symptoms for MS have been described and most MS patients develop one or more of these symptoms, including sensory and motor problems ranging from muscle spasms to partial paralysis, difficulties in coordination and speech, visual disturbances, cognitive impairment, depression, pain and fatigue (38). Even though the exact etiopathogenesis of the disease is complex and still has to be clarified, it is suggested to be T-cell mediated (36). $CD4^+$ Th1 and Th17 T cells specific for myelin have been shown to play a role in the initiation of MS (39, 40). Moreover, genetic and environmental factors are involved. Since there is no cure for MS, the available treatments aim at managing MS symptoms (e.g. physical therapy), speeding up the recovery from attacks (e.g. corticosteroids) and slowing disease progression (e.g. beta interferons or ocrelizumab (anti-CD20)).

1.2.2 Inflammatory bowel disease

Inflammatory bowel disease is an overarching term for disorders such as ulcerative colitis and Crohn's disease that are chronic relapsing disorders of the gastro-intestinal tract. Since the mid-twentieth century, the incidence of IBD has increased dramatically and has this been linked to a westernized lifestyle. Alterations of the microbiota, exposure to antibiotics, diet, smoking and lack of vitamin D have emerged as modifiers of systemic and intestinal immunity. IBD patients are usually diagnosed at young age (15-35 years old) and men and women are equally affected (41). As nicely reviewed by de Souza & Fiocchi, IBD is triggered by 1) a dysfunctional immune response, 2) the environment, 3) the genetic make-up and 4) the gut microbiota (42). One of the earliest signs of intestinal inflammation in IBD is the infiltration of the gut mucosa and epithelium by neutrophils. However, the immunological trigger of IBD is still unclear since many abnormalities exist in both innate and adaptive immunity (42). Current treatment consists of classic anti-inflammatory drugs, immunosuppressive drugs and biological treatments such as anti-TNF (43).

1.2.3 Rheumatoid arthritis

Rheumatoid arthritis is a chronic inflammatory disease characterized by circulating levels of autoantibodies against the Fc part of IgG, called rheumatoid factor (RF) (although not specific for RA) (44), antibodies against joint-specific proteins (45) and citrullinated autoantibodies or ACPAs (specific for RA) (46). Patients have inflammation in the articular joints, which can lead to joint deformation. Disease symptoms include swelling, stiffness and pain in multiple joints, which often starts in wrists, hands and knees, and more general symptoms including fever, weight loss and fatigue. RA patients have a reduced quality of life with a shorter life expectancy compared to the general population (47). Moreover, patients can suffer from systemic immune responses such as infections and cardiovascular diseases, which are common co-morbidities and have been shown to increase premature death in RA patients (48). RA affects approximately 0.5-1% of the world's population with a higher prevalence in native-American populations and in North European and North American countries (49). Disease onset is around 30-60 years of age with a two to three times greater incidence in women compared to men (50). Both genetic and environmental factors predispose individuals to RA and influence the disease outcome (30, 51). However the exact cause has yet to be determined. Although various treatments are available, due to the

complexity of the diseases, no cure has been found to date (34, 52, 53).

As described by Holmdahl *et al.*, epidemiological and genetic analyses, together with clinical observations, suggest that RA pathogenesis can be divided into three distinct stages: autoimmunity, subclinical arthritis and clinical arthritis (54). RA patients are diagnosed using classification criteria, which is a 1-10 scoring system, described by the American College of Rheumatology and European League Against Rheumatism (55). A person is classified as an RA patient when reaching a score of 6 (out of 10) or higher (Table 1).

Table 1 Rheumatoid arthritis classification criteria. The scoring is based on the number and site of joints involved (score 0 to 5), serological parameters (score 0 to 3), elevated acute-phase response (score 0 or 1) and symptom duration (score 0 or score 1). RF: rheumatoid factor, CCP: cyclic citrullinated peptide, CRP: C-reactive protein, ESR: erythrocyte sedimentation rate.

Criteria	Points
A) Joint involvement (0-5)	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
> 10 joints (at least 1 small joint)	5
B) Serology (at least one test result is required) (0-3)	
RF (-) and anti-CCP (-)	0
Low titer RF (+) or low titer anti-CCP (+)	2
High titer RF (+) or high titer anti-CCP (+)	3
C) Acute-phase reactants (at least one test result is required) (0-1)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D) Duration of symptoms (0-1)	
< 6 weeks	0
≥ 6 weeks	1

Patients can be divided into serological positive or negative groups (RF and ACPA). Both ACPA and RF positive patients are at a higher risk of developing a more aggressive form of arthritis, leading to more joint damage (56, 57). For treatment purposes patients are stratified based on their serology or genetics.

1.2.3.1 Pathogenesis

As RA is a complex autoimmune disease, or rather disorder, there are numerous molecular mechanisms underlying RA. Altered post-transcriptional regulation by environmental factors, susceptible genes and epigenetic modifications could lead to self-protein modification, resulting in loss of self tolerance and subsequent autoimmunity presented as autoantibody

production (31). Moreover, dendritic cells could present these altered self-peptides and activate T and B cells. It is however suggested that this process occurs outside of the joint, either at the side of "stress", in secondary lymphoid tissues, in the bone marrow or in the lung (58, 59) unknown signal would then be required to relocate the inflammation to the joint where synovitis is initiated (60). Synovitis is inflammation of the synovial membrane and is the first clinical sign of RA (61). This inflammation is initiated and perpetuated by cells from the innate and adaptive immune system, both resident and infiltrating, and their interaction with synovial fibroblasts, chondrocytes and osteoclasts.

A healthy synovium comprises mainly of macrophages and fibroblast-like synoviocyte (**FLS**). During on-going joint inflammation, the lining layer of the synovium thickens paired with infiltrating lymphocytes ($CD4^+$ and $CD8^+$ T cells, B cells, plasma cells), monocytes, macrophages, mast cells, NK cells and dendritic cells. Neutrophils mainly reside in the synovial fluid where they secrete prostaglandins, proteases and ROS. Macrophages are potent effector cells during synovitis, they are activated via different routes including direct T cell contact, the ligation of TLRs and binding of immune complexes to FcγRs. Upon activation macrophages release a large amount of cytokines such as IL-6, TNF and IL-1, chemokines, reactive oxygen and nitrogen, as well as matrix-degrading enzymes (e.g. MMP). Cytokines such as IL-1 and TNF can activate FLS, which in turn produce high levels of matrix-degrading enzymes, adhesion molecules, chemokines and cytokines, including high levels of M-CSF. M-CSF in turn induces macrophage differentiation from monocytes, creating a more inflammatory environment, and induction of osteoclastogenesis (formation of osteoclasts). Furthermore, MMPs and cytokines such as IL-1 and TNF-a will affect chondrocytes by degrading cartilage. They can also increase osteoclast numbers and breakdown osteoblasts. Moreover, TNF mediates leukocyte activation, endothelial angiogenesis and nociception and is suggested to be involved in processes of the central nervous system, such as depression and fatigue. Secreted by multiple cells, IL-1, TNF-a and IL-6 are abundantly present in RA synovium and have all been targeted for treatment (60).

Another cytokine that is present in RA synovium and activates macrophages, FLS and osteoclasts to promote the release of additional inflammatory mediators, is IL-17, produced by activated **T cells**. Although frequencies of Th17 cells in blood are similar between healthy controls and pre-RA patients, they are elevated in blood and synovial fluid of patients with established disease (14). Despite the great success of blocking IL-17 in a mouse model of RA (collagen-induced arthritis, CIA), to date IL-17 targeting therapeutics have not been effective in patients with established RA (60). Additional cytokines produced by CD4 T cell subsets

are also present in serum and RA synovium. Both Th1 cells and Th1 cytokines, such as IFN-y, IL-2 and TNF-a, are elevated in RA patients. On the other hand, involvement of Tregs in RA is not as clear (62). Given that Tregs are involved in the suppression of autoreactive T cells, one explanation is that the functionality of Tregs has been altered in RA. This has been shown by defective CTLA-4 regulation using KO mouse models (63–65). Another reason could be the responsiveness of T cells to Treg mediated suppression. Furthermore, it has to be noted that T cell subsets can be plastic and their interactions complex. An imbalance between pro- and anti-inflammatory subsets, Th17 and Tregs for example, is often considered responsible for disease development (60, 66).

Clearly, **autoantibodies**, present in serum far before clinical disease onset, are a hallmark of RA, suggesting a major involvement of B cells. **B cells** are indeed present in RA synovial tissue in close contact with T cells, which are believed to provide help to B cells to produce autoantibodies (67). In case of RF and ACPA, natural B cells are activated through germ-line-encoded BCRs (68). The initial low avidity and titers of these antibodies increase with time due to germinal centre selection and the help of T cells (69). However, correlation of joint destruction with low-avidity ACPAs has been reported (70). ACPAs are highly specific for RA (up to 98%), albeit not present in all patients (up to 77%), whereas RF can also be found in other autoimmune diseases. Even though there is no clear proof that RA autoantibodies are pathogenic in humans, studies in mice clearly show that epitope specific anti-collagen type II (CII) antibodies can induce arthritis. Furthermore, it has been shown that CII-specific B cells can give rise to antibodies that attack the cartilage matrix without any clinical signs of arthritis (71–73). Increased pain has been reported as well (74). Moreover, some specific ACPAs can give rise to activated osteoclasts with consequent bone destruction, as demonstrated in both RA and animal models for arthritis (59, 75, 76). Despite the absence of ACPAs in animal models for RA, RFs are found in several animal models including CIA (77). Here, T cells activated by CII, provide help to cross-reactive B cells, with consequent antibody production.

Furthermore, it has been demonstrated that increased numbers of B cells are present in joint tissue and in draining lymph nodes of patients with early RA (78, 79). Furthermore, aside from its role in antibody production, synovial B cells also contribute to disease progression by secretion of pro-inflammatory cytokines such as IL-6 and TNF. The most prominent proof of B cell contribution in RA is the successful therapeutic depletion of $CD20^+$ B cells using Rituximab.

Both the complement system, particularly component C5, and the **FcγRs** get activated by anti-CII antibodies, initiating the inflammatory response in RA (80, 81). In humans, polymorphisms have been described within the coding sequences of FcγR3A and FcγR2B in association to different autoimmune diseases. The different forms are thought to alter ligand binding affinity and receptor-mediated effector functions (82–84). It has been shown in mice that lacking the signalling subunit of the activating receptors FcγR1 and FcγR3 protects from arthritis, whereas mice deficient in the inhibitory receptor FcγR2b develop a more severe disease (80, 85–87). Furthermore DBA/1 FcγR3 KO mice are protected from CIA despite producing similar amounts of anti-CII mAbs as control mice (88). Moreover, when inducing arthritis in mice using single anti-CII mAbs, mice that lack FcγR2b show enhanced disease whereas mice lacking FcγR3 do not develop arthritis (89, 90)ince FcγR bearing cells, including macrophages, neutrophils and NK cells, are a prominent feature of rheumatoid synovium and synovial fluid, the binding of autoantibodies such as RF to these cells is likely to contribute to the inflammatory process in RA (91). Therefore extensive research on the role of the various FcγRs on different cell types in arthritis has been conducted in both mice and humans (92). Polymorphisms in FcγR3a in RA patients can be used to predict treatment response to for example rituximab (anti-CD20) (34, 93).

1.2.3.2 Genetics of RA

It has been suggested from twin studies that the heritability of RA is approaching 65%, which means that in an RA patient up to 65% of the disease could be allocated to genetics (94). Using Genome Wide Association Studies (GWAS) more than 100 genetic risk loci have been associated with RA, also indicating the contribution of environmental factors on disease development and progression (34). Despite large efforts in identifying genetic factors affecting RA, less than 50% of the estimated heritability can be explained by previously identified risk alleles. Moreover, only 16% of the total RA susceptibility can be explained (95–98). This is depicted as “variance explained” in Figure 3 and represents the sum of genetic variance and environmental variance.

The first identified risk genes in RA were the major histocompatibility complex (MHC) genes, with strong association to human leukocyte antigen (HLA)-DRB1*04 (99). To date, the strongest genetic association among autoantibody-positive European RA patients still remains the HLA-DRB1 alleles (100–103).

Another strongly associated risk gene, which was initially found in T1D, is the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) (104, 105) in which a non-synonymous coding SNP (R620W) causes T cell hyporeactivity (106, 107). Moreover, R620W has been linked to the presence of RF and anti-cartilage specific antibodies in RA patients (104, 108). The importance of T cells in RA development is also supported by genetic associations with the CTLA4 gene (109, 110) and the signal transducer and activator of transcription 4 (STAT4, (111)). CTLA-4 deletion in adult mice has been shown to result in more severe arthritis development compared to control mice (63). On the other hand, STAT4 is a transcription factor essential for the development and maturation of Th1 and Th17 cell populations (112).

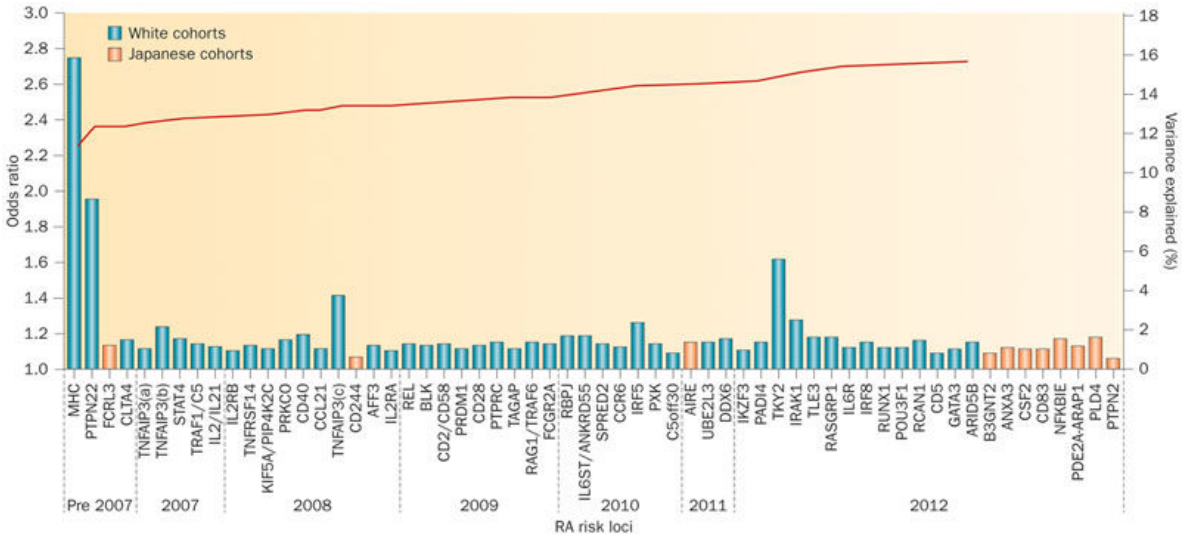

Figure 3 RA genetic risk loci. Odds ratios for the risk loci are presented in a chronological order of discovery. Data of Caucasian (white) and Japanese cohort are shown (98).

A number of genetic studies made it possible to stratify patients as being ACPA positive or ACPA negative with stronger genetic associations in the ACPA$^+$ subgroup (113, 114). A few loci, such as TNFAIP3, STAT4, IL6ST and PTPN22 have been associated to RA susceptibility independent of antibody titers (97, 115). Among others, CD28, CTLA-4, CD40, HLA-DRB1*15, HLA-DRB1*03, HLA-DRB1*1, C5, FCΓR2B and FCΓR3A have been mainly associated with ACPA$^+$ disease (34, 93, 116–119). However, due to genetic heterogeneity, environment variability, gene-gene or gene-environment interactions and various life styles, it has been difficult to find other causative genes within the human population.

1.2.3.3 Environmental factors

Over the years, several environmental factors have been associated with the risk of developing RA (120) with smoking being the most prominent one (121, 122). Smoking has been shown to enhance the risk in serologic positive patients who carry the HLA-DRB1 shared epitope (SE) alleles or PADI4 polymorphisms (123–126). In patients carrying the HLA-DRB1 susceptible allele, increased production of anti-CCP (cyclic citrullinated peptide) antibodies and T cells reacting to citrullinated proteins have been described (127, 128).

The geographical prevalence of RA and other autoimmune diseases has generated the hypothesis that vitamin D levels are an important risk factor. In RA, several epidemiological studies show an association between low levels of circulating vitamin D and an increased risk or severity of arthritis (129–131). However, there has been some conflicting data (132–134). Moreover, based on data from healthy individuals with high exposure to sunlight and vitamin D deficiency, lack of vitamin D might rather be a consequence of chronic inflammation than a cause thereof (135). Another theory is that cellular infections by bacteria or viruses like Epstein-Barr virus (EPV) are the underlying cause for RA (136). EPV is a ubiquitous virus with 95% of the world's population being infected. It has been found in sera and in synovial tissue of RA patients (137, 138).

Another broadly studied environmental factor is eating habits. However, due to the common issue of inaccuracies in assessing dietary intake, evaluating the effect of diet on RA is difficult. Through a nested case-control design in the Västerbotten Intervention Program (VIP) cohort in Sweden, a significant association was found between protein consumption and an anti-CCP-positive disease or smokers (odds ratio (OR) of 1.40 and 1.80 respectively). However, associations were no longer significant when the data was adjusted for sodium intake (139). In this regard, recent studies in animal models and on human cells *ex vivo* have demonstrated the importance of sodium in the induction of pathogenic Th17 cells (140, 141). Since Th17 cells are suggested to be a key player in the early pathogenesis of RA (142), the role of dietary sodium in the disease course of RA in the VIP cohort has been re-evaluated (139). Stratifying patients based on their smoking status at the time of the examination showed that sodium intake more than doubled the risk for RA among smokers. This was not observed among non-smokers. More than 50% of the increased risk of developing RA from exposure to smoking or high dietary salt intake was due to interactions between the two. The risk was further increased for the development of anti-CCP-positive and/or HLA-SE–positive RA (139). On the contrary, we have shown in mouse models for RA, that elevated levels of

sodium, provided in drinking water, did not affect the development of arthritis. Thus even in an environment controlled setting, this single environmental factor does not have an impact on arthritis development (Paper III) (143).

1.2.3.4 Current treatment strategies

The most commonly used treatments for RA are non-steroid anti-inflammatory drugs (NSAIDs) that reduce pain; disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate that reduce structural joint damage progression; and corticosteroids that relieve acute symptoms. The problem with these drugs, and with many drugs in general, is the developed drug tolerance in many patients. During the last years, biologic agents have been introduced as treatment (53). These include monoclonal antibodies and recombinant proteins targeting pro-inflammatory molecules. The first and most successful biological agent used to treat RA is a monoclonal antibody against TNFα, (144). Anti-TFNα agents, such as etanercept are now widely used for RA treatment. Use of anti-IL-6 (e.g. Tocilizumab), CTLA-4 fusion proteins (e.g. Abatacept) or anti-CD20 monoclonal antibodies (e.g. Rituximab) have been very effective. More recently, small molecule inhibitors of the JAK pathways have been very successful in the treatment of early RA (145, 146). However, targeting cytokines such as IL-1, IL-17, IL-21 or IL-22 showed less effective.

To improve the safety and efficacy of drugs used for treating RA patients, pharmacogenetics and pharmacogenomics analyses have been adopted from GWAS data trying to develop personalized treatment strategies (147, 148). However, there is still a gap to be filled in the full understanding of RA pathogenesis for which basic research is fundamental.

1.3 ANIMAL MODELS FOR AUTOIMMUNE DISEASES

Using mouse models and different mouse strains, the observed phenotypes and linked genes can be further investigated in an environmentally and genetically controlled manner (149–156). However, due to the complexity of autoimmune diseases, no single animal model can cover the entire spectrum of the heterogeneous human disease. Nevertheless, the individual models are very useful to study one or more aspects of the corresponding human disease and give the possibility to knock down or enhance the expression of genes. Below I will briefly describe the animal models used in papers I, II and III.

1.3.1 Arthritis models

There are many different mouse models to study RA that are either spontaneous (e.g. TCR transgenic K/BxN mice and SKG mice) or induced. Well-studied induced mouse models for RA are collagen-induced arthritis (CIA), collagen antibody-induced arthritis (CAIA) and glucose-6-phosphate isomerase (GPI)-induced arthritis. These models touch upon individual stages of disease development, mostly covering the clinical phase of arthritis.

CIA, in which mice are immunized with the major articular cartilage protein CII, is dependent on both T cells and APCs such as B cells (157). B cells are important for antibody production against CII (158). CIA resembles several features of RA like pannus formation in the synovium, bone erosion, immune cell infiltration, MHC dependence and engagement of both cellular and humoral responses.

Autoantibodies have long been considered to be involved in the pathogenesis of RA. Using **CAIA**, in which epitope defined anti-CII monoclonal antibodies are injected into mice, the disease dependency on joint specific antibodies (e.g. CII) can be studied. It further allows studying the effector phase of arthritis without involving the priming phase. CAIA is characterized by acute infiltration of neutrophils and macrophages (6). It moreover requires the involvement of complement components and FcγRs (159, 160).

1.3.2 Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is the extensively used animal model for MS having similar clinical, immunological and genetic features as seen in MS (161). Disease is induced in susceptible mice with CNS proteins or peptides such as myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP). The disease development can either be acute progressive, chronic or remitting, depending on the mouse strain used, and is Th1 and Th17 dependent. The model is used to gain more insight into the pathogenesis of the disease, as well as for pharmacological and genetic studies.

1.3.3 Dextran sulphate sodium-induced colitis

Dextran sulphate sodium (DSS)-induced colitis is the most widely used animal model to study IBD and is fast, simple, reproducible and easily controlled. DSS is administered

through drinking water and it affects disease severity depending on its molecular weight (ranging from 5 to 1400 kDa) and administration dose (1.5 – 5%). The disease can be acute, chronic, and relapsing based on the administration dose and frequency. Administering 40-50 kDa DSS in drinking water, most closely resembles human ulcerative colitis (162). Characteristics of the disease are weight loss, faecal and rectal bleedings, splenomegaly, decreased colon length, epithelial erosion and immune cell infiltration in the colon. Furthermore, gut microbiota is a key determinant of the development and severity of colitis, with exacerbated colitis in mice treated with antibiotics (163, 164). Unlike in human disease, T and B cells are not required for DSS-induced colitis disease development. Nevertheless, T cells are amongst the pro-inflammatory infiltrating cells in the colon, together with macrophages (chronic disease) and neutrophils (acute disease) in mice.

1.4 STUDYING GENETICS AND GENE FUNCTION IN ANIMAL MODELS

Identifying quantitative trait loci (QTLs) in family linkage studies was an initial step in identifying and sequencing genes in the susceptibility to a particular disease (165). However, this approach did not generate many linked loci. The only conclusively linked locus identified in RA was the MHC. From the past decade, genetic mapping of complex multifactorial diseases, such as RA, has been done by GWAS in which large cohorts, including patients and healthy controls, are genotyped with whole genome sequencing using coding information from single nucleotide polymorphisms (SNPs). This makes it possible to identify genes and/or proteins likely to regulate the observed phenotype (166). With GWAS, new disease loci are identified after which the risk effect in a particular locus can be fine mapped in order to quantify the heritability and to narrow down which variants/genes in that locus are relevant (167). However, in complex autoimmune diseases it is the involvement of multiple causal variants in many disease-associated loci (168), often working in concert. Moreover, it is not only the common and low-frequency variants (allele frequency of 0,5 – 5%) that account for the genetic heritability of a disease. Albeit poorly studied, rare variants (with an allele frequency of <0,5%) and copy number variations also contribute to complex autoimmune diseases. In RA, copy number variants have been reported for the MHC region, FCΓR3A, FCΓR3B and others (169–171).

Human genome studies require large cohorts due to a large number of variables. Therefore, utilization of animal models is essential. In experimental models, environmental factors and genetic backgrounds can easily be controlled, reducing the variability and thus the number of

animals used. Moreover, animal models allow studying the molecular pathways and the role of candidate genes leading to disease development (33). In order to identify disease-associated loci, several approaches can be used after which the contribution of the candidate gene(s) can be verified using KO, knockin (KI) or transgenic mouse models and gene silencing methods (172, 173). Partial advanced inter-cross (PAI) lines, F2 crosses, heterogeneous stock (HS) mice or congenic mice are models used to identify disease-associated loci and to map susceptible elements and gene(s) (151, 156, 172, 174). A PAI breeding strategy is used to investigate genetic interactions between QTLs on two congenic strains, created on the same genetic background, to identify the disease phenotype associate genes. F2 crosses are used for linkage analysis, in which a particular trait is linked to the identified locus. In this thesis, congenic mice have been used to study the genetic effect on arthritis development in mice and the functionality of these genes. –

1.4.1 Congenic mice

Congenic mice are inbred mice that carry a defined part of a genome (the congenic locus) of one mouse strain (donor) introgressed into another mouse strain (receiver) (175). Thus the background genome of the congenic mice is identical to that of the receiver. Initially, the donor strain, with a different genetic signature, is crossed with the receiver strain resulting in F1 mice (Figure 4). These F1 mice, 50% identical to the respective donor, are further backcrossed to the receiver with the help of marker-assisted selective breeding until 99,9% of the donor genome material outside of congenic locus is eliminated (172). This is usually achieved with 10 backcrosses. The obtained congenic mice can then be kept in homozygous and heterozygous intercross breeding to prevent spontaneous recombinations by meiosis and to create littermate control mice to study the disease phenotype, respectively. The littermate control mice have genetic material identical to the receiver. So when studying the disease phenotype, the observed phenotypic differences

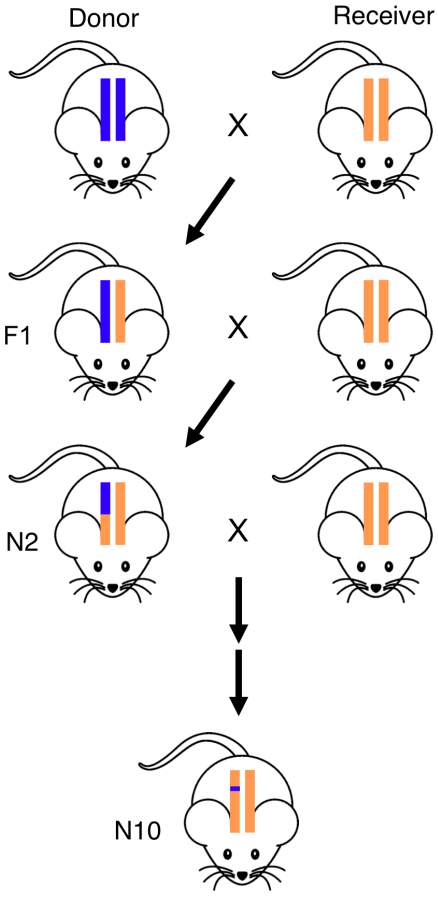

Figure 4 The generation of congenic mice. F1: 50% donor, N2: 25% donor, N10: <0.5% donor.

can be allocated to the genetic fragment obtained from the donor. However, the congenic fragments often consist of multiple genes and fine mapping is not as straightforward, since at a certain fragment size the chances of finding new recombinations will decrease. Nevertheless, a candidate region can be investigated in detail using several approaches: 1) search literature for relevant gene information and 2) assess sequencing databases for SNPs and transcriptional activity between congenic mice and receiver. Once one or several candidate genes have been found, cell specific gene and protein expression can be assessed, followed by functional studies.

1.4.2 Knockout mice

One commonly used method to verify the role of a candidate gene is the use of knockout mice. KO mice have mostly been generated using embryonic stem (ES) cells from the 129 inbred mouse strain. A null-mutation is made in the 129 ES cells, which are then injected into a blastocyst and implanted into the uterus of a pseudo-pregnant female. If the mutant gene is transferred to the offspring, the mutant gene can be bred to homozygosity to get a KO mouse. As with congenic mice, the obtained KO mouse should be backcrossed to the desired background to limit the genetic effect of the 129 background. Even so, tightly linked genes in the flanking region surrounding the null mutation do not separate easily and are often contained within the KO mice. So besides studying the effect of the knocked out gene, the interaction of the flanking genes will have a significant impact on the studied phenotype (173, 176). Therefore, several KOs have been made in the B6 mice directly. A relevant example is the FcγR2b KO mouse. Whereas $Fc\gamma R2b_{129}$ KO mice showed high susceptibility to lupus, $Fc\gamma R2b_{B6}$ KO mice failed to develop disease (177). This clearly indicates the impact of the linked genes. Nevertheless, both KO mouse models are widely used models to study genetic functions.

2 PRESENT INVESTIGATIONS

This thesis comprises of three studies, all of which focus on various functions in the regulation of autoimmunity in mouse models.

2.1 AIMS

The overall aim of the present thesis was to investigate the effect of genetic risk loci and that of environmental factors on the development of autoimmune diseases in a further understanding of the mechanisms behind these complex diseases.

The specific aims of the constituent papers were:

Paper I: To identify the arthritis promoting genes in the arthritis susceptible *Cia9* locus, and to investigate the functional effect of polymorphic Fc gamma receptor genes on immune cells and arthritis disease development in mice.

Paper II: To assess the importance of the system A family of amino acid transporters as mediators of immune cell function and arthritis development in mice.

Paper III: To study the effect of salt on immune cell function and on the development of autoimmune diseases in mouse models.

2.2 RESULTS AND DISCUSSION

2.2.1 Paper I

Immune complex receptors FcγR2b and FcγR3 alleles act in concert to regulate inflammation

When the balance between FcγR2b and the activating FcγRs on the same cells is disturbed, this can lead to the development of chronic inflammatory or immunological diseases (28, 92). Polymorphisms in human FcγRs have been linked to systemic lupus erythematosis (SLE) and rheumatoid arthritis (RA). Moreover, with the use of various FcγR knockout mice, it has been shown that FcγRs are important in the downstream effector pathways driving pathogenesis in autoimmunity (178). However, it has to be taken into account that the expression and function of other FcγRs might be influenced when knocking out an individual FcγR (179), which is due to genetically linked genes. This effect can be diminished with the use of congenic mice. Previous studies have indicated the importance of the FcγR gene cluster (including FcγR2b, FcγR3 and FcγR4), located on the NOD.Q-derived *Cia9* locus on chromosome 1, in arthritis development (174, 180, 181). In **Paper I**, we addressed this by using sub-congenic *Cia9* mice to study arthritis susceptibility and to investigate the functional effect of polymorphic Fc gamma receptor genes.

To identify the arthritis promoting genes in the susceptible *Cia9* locus, CIA and CAIA were run in the four overlapping sub-congenic lines *Cia9b, Cia9c, Cia9i* and *Cia9k*. *Cia9b* spans the region above the FcγR cluster on chromosome 1, *Cia9c* covers the region below the FcγR cluster, containing several genes from the SLAM family, and *Cia9i* and *Cia9k* contain the FcγR gene cluster, excluding the SLAM region. *Cia9b* and *Cia9c* both showed no regulation of arthritis, which restricted the disease-regulating interval to less than 1 Mb of *Cia9*. The sub-congenic *Cia9i* and *Cia9k* loci on the other hand, ~2 Mb fragments, showed significant arthritis regulation, confirming arthritis regulation to a < 1 Mb interval in between the *Cia9b* and *Cia9c* locus. It was indeed the polymorphic FcγR gene cluster that regulated arthritis (*Figure 5*). Polymorphic FcγR2b and FcγR4

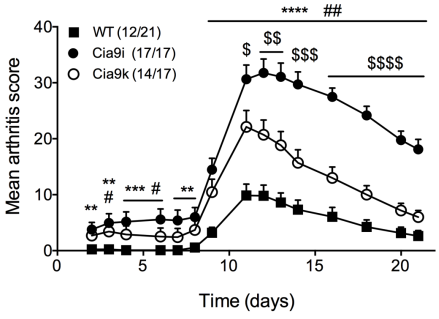

Figure 5 Polymorphisms in the Cia9i and Cia9k fragment exacerbate arthritis onset and development of CAIA. *Mice were injected i.v. with 4 mg of anti-CII mAbs cocktail on day 0 and boosted with LPS i.p. on day 7. Arthritis score was assessed macroscopically. (arthritis/number of mice). Significant differences between WT and Cia9i (*), Cia9i and Cia9k ($) and WT and Cia9k mice (#).*

were contained in both *Cia9i* and *Cia9k*, whereas *Cia9i* mice also carried polymorphic FcγR3. FcγR2b gene and protein expression were downregulated in *Cia9i* and *Cia9k* mice, whereas FcγR3 was upregulated in *Cia9i* mice and downregulated in *Cia9k* mice compared to littermate control mice. No expression differences for FcγR4 were observed.

To further investigate the role of polymorphic FcγR2b and FcγR3, FcγR-dependent functions on B cells, NK cells and macrophages were studied. Since elevated levels of anti-collagen type II (CII) antibodies were found in *Cia9* mice during CIA (181) and the role of FcγR2b (182) has been suggested, we assessed the function of B cells in *in vivo* and *in vitro* antibody secretion. Despite lower expression of FcyR2b on *Cia9i* and *Cia9k in vitro* activated B cells, no differences in anti-CII antibody levels or *in vitro* antibody production by CII primed B cells were observed.

For macrophages, an FcγR3 mediated mechanism was observed. With the use of antibody dependent cellular phagocytosis (ADCP) we studied the effect of activating FcγRs. We show that *Cia9i* macrophages, which have increased FcγR3 expression, induce significantly more phagocytosis compared to macrophages of WT, *Cia9k* and FcγR3 KO mice. The same was true for FcγR3 mediated Treg depletion *in vivo* using PC61 antibody, which has previously been shown to be dependent on macrophages (183). We saw increased depletion of Treg cells in *Cia9i* mice and decreased depletion in *Cia9k* mice compared to WT mice. We furthermore show that *Cia9i* mice have increased oxidative burst both through FcγR dependent and independent pathways. Previous studies in neutrophils show that both FcγR2b and FcγR3 affect ROS production. Using FcγR3 KO mice, ROS production by neutrophils was solely regulated through FcγR mediated pathways (184, 185), whereas in FcγR2b KO mice the oxidative burst was affected after stimulation with LPS as well (186). Our results clearly show a major effect from FcγR3 in both settings. Although not significantly different, *Cia9k* mice show a trend towards higher oxidative burst compared to WT mice. This indicates, and is in line with our arthritis data, a combined role of FcγR2b and FcγR3 and points towards an altered activating to inhibitory (A/I, e.g. FcγR3:FcγR2b) binding ratio, which determines the FcγR activation threshold (28).

We further showed the importance of NOD.Q polymorphic FcγR3 by studying antibody dependent cellular cytotoxicity (ADCC) by NK cells. The specific lysis by *Cia9i* and WT NK cells was linked to FcγR3 expression. Interestingly though, specific lysis by *Cia9k* NK cells was also increased compared to that of WT NK cells. Since FcγR3 expression on *Cia9k* NK cells was slightly reduced, we expected lower or equal NK cell mediated lysis. This points towards the involvement of other linked genes within the congenic fragment. The only gene

within the *Cia9k* fragment that has been associated with NK cell-mediated cytotoxicity is the activating transcription factor 6 (Atf6) (187). It is possible that without NOD.Q FcyR3, NOD.Q Atf6 still controls cytotoxicity.

With our congenic mice, we were able to study the independent and additive effect of FcyR2b and FcyR3 on inflammation without major impact of the NOD.Q flanking region. Our congenic mice could provide a more physiological setting to study FcγR function. Recent data suggest that the activating FcγRs, FcγR4 in particular, on neutrophils in the joint mediate bone erosion during antigen-induced arthritis (188). Since we also showed increased arthritis susceptibility in a model mediated by macrophages and neutrophils, it would be interesting to further investigate this in our congenic mice. Furthermore, a recent study shed light on the involvement of FcγRs in the adaptive immune system. Through APC-mediated presentation of immune complexes, they show that the role of activating FcγRs is redundant (189). In line with this, we did not find an impact from B cells. Nevertheless, the above-mentioned studies have been conducted in complete FcγR knockout mice. It therefore would be interesting to further investigate the role of polymorphic FcγRs on adaptive immune regulation.

In summary, we show that it is the additive effect of genetic polymorphisms in FcyR2b and FcyR3 that regulate arthritis severity and inflammation in our congenic mice, likely through macrophage-mediated mechanisms.

2.2.2 Paper II

System A amino acid transporters regulate glutamine uptake and attenuate antibody-mediated arthritis.

Glutamine is the most abundant amino acid in the circulation and can be used as an alternative energy source in actively proliferating cells. Its metabolic role in immune cell activation, such as macrophages and T cells, has been well-studied, demonstrating its importance in immune function (190–193). During an inflammatory response, immune cells are in high demand for intracellular amino acids that can either be generated endogenously or that have to be transported from the extracellular environment (194). One such transporter for glutamine is the system A family of amino acid transporters (members of the sodium-coupled neutral amino acid transporters – SNAT) (195), which is a unidirectional transporter for sodium ions and neutral amino acids. These transporters, existing of SNAT1, SNAT2 and SNAT4, were shown of importance during several T-cell activation processes (191, 196, 197). Moreover, the genetically clustered SNAT1, SNAT2 and SNAT4, were previously identified in a 800 kb quantitative trait locus (Cia36) that regulated arthritis development in the T-cell dependent mouse model collagen-induced arthritis (152). However, the exact role of these SNAT proteins in arthritis development remains elusive. In **Paper II**, we address this problem by blocking the SNAT proteins during T-cell activation and proliferation *in vitro* and during T-cell dependent and T-cell independent arthritis development *in vivo*.

To evaluate the role of system A proteins during T cell activation, we performed kinetic studies by stimulating spleen cells with anti-CD3/28 *in vitro* in the absence or presence of the amino acid analogue 2-(methylamino) isobutyric acid (MeAIB) to block the system A proteins. We found that *Slc38a1* and *Slc38a2* (corresponding to SNAT1 and SNAT2 proteins, respectively), as well as *Mtor* and *Erk1* gene expression were significantly increased after 2-hour culture and were back to basal levels after 5 and 24 hours, indicating rapid *de novo* synthesis of these proteins to supply the cell's energy demands. MeAIB significantly reduced expression of the genes after 2-hour culture, but expression levels after 5 and 24 hours culture did not reduce to basal level. We furthermore showed that glutamine uptake by T cells is dependent on system A transporters, only in the presence of Na^+ in culture medium. Moreover, under normal culture conditions, T-cell proliferation and activation (shown as IL-2 and IFNγ secretion) were significantly reduced when blocking SNAT proteins.

To test if the same were true in models for autoimmunity, we administered mice daily with MeAIB or PBS, starting 7 days before induction of arthritis. Given the protective role of the

Cia36 locus in arthritis development (152) and the role of SNAT proteins in T cell activation, we expected an effect on T-cell dependent arthritis development when blocking SNAT proteins using MeAIB. Although we did not observe any significant differences in arthritis severity, SNAT blockage did cause a delayed disease onset in CIA. After receiving a second inflammatory trigger, this difference disappeared. When using the antibody-mediated arthritis model, we saw a significant reduction in both arthritis severity and incidence that was paired with decreased numbers of neutrophils and elevated levels of $Ly6C^{lo}$ monocytes in the blood when using MeAIB. The former indicates lower levels of inflammation (6), whereas the latter might indicate increased clearance of formed immune complexes by FcγRIV on these cells (198). No differences in T cells were detected. Nevertheless, in the absence of T cells in TCRβ KO mice, MeAIB had a less pronounced effect on CAIA disease severity, albeit causing a lower disease incidence. This indicates the contribution of SNAT proteins in T cells and innate cells such as neutrophils in CAIA disease development.

Whereas the immune system is overly activated during an autoimmune disease, it is weakened in the presence of tumours. Tumours are also major consumers of glutamine, but they are insensitive to repression of glutamine uptake via SNAT proteins (193, 199, 200). Here, we show that de novo synthesis of SNAT1 proteins occurs upon effective blocking of system A proteins in HEK293T cells, which correlates with the proliferation status of these cells (Figure 6).

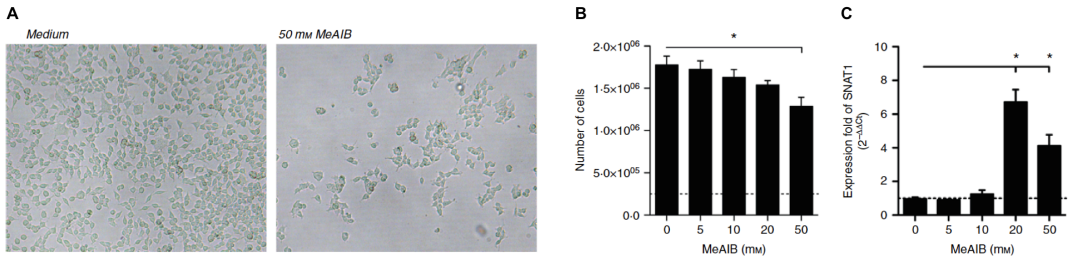

Figure 6 System A substrates affect proliferation of immortalized cell lines. $2.5x10^5$ HEK293T cells were seeded per 25-cm^2 culture flask in medium supplemented with different concentrations of MeAIB. After 72 hr, the total number of cells was assessed (a, b) and total RNA samples were collected for quantitative expression of Slc28a1 gene (SNAT1) (c). Adapted from (201).

Based on our and recently published data (202), our *in vitro* results clearly show the importance of system A transporters in naïve T cell activation and proliferation. Whereas another amino acid transporter, system ASC, is crucial for the development of Th1 and Th17 cells, they did not alter the levels of IL-2 and Treg cells (202). Given the IL-2 and proliferation signature we observed, we suggest that SNAT proteins are involved in the initial activation of lymphocytes. This also seems to be the case during the onset of CIA. After boost however, mice in the different treatment groups get equally sick. One explanation for

this could be the involvement of other amino acids or transporters that could have been activated upon elongated blockage of SNAT proteins. Moreover, T cell activation does not get completely hampered when treated with MeAIB. So upon a second signal, activated immune cells, including T cells, might have found another way to reach their energy demand and increase activation and disease. Nevertheless, we show a significant reduction in CAIA disease severity and incidence upon SNAT blockage.

Collectively, we demonstrate that naive lymphocytes preferentially use SNAT proteins for the uptake of extracellular glutamine. Trans-inhibition of SNAT proteins in immortalized cell lines, although to a lesser extent than lymphocytes, results in a reduction of cell proliferation. And, *in vivo* administration of the SNAT inhibitor MeAIB significantly diminishes the severity of antibody- mediated arthritis, most likely through its effect on metabolically active inflammatory cells.

2.2.3 Paper III

Increased salt exposure affects both lymphoid and myeloid effector functions, influencing innate-associated disease but not T cell-associated autoimmunity.

Short title: Salt affects colitis but not autoimmunity

Dietary salt, in the form of sodium chloride, has over the last couple of years been studied extensively as an environmental factor in several diseases (203–206). Its excessive intake through increased consumption of processed food, has been associated with elevated blood pressure, coronary heart disease and stroke (206, 207). Moreover, there is rising evidence that this Westernized diet can contribute to the pathogenesis of autoimmunity (203, 206, 208, 209). Food is digested in the gut, with help of a person's microbiome. Although the microbiome is relatively stable over time, its composition can be altered by various environmental factors, including diet, which in turn has been linked to various inflammatory diseases (210, 211). Moreover, studies in germ free mice show altered T-cell signatures in the lamina propia of the small intestine. These mice were protected from experimental autoimmune encephalomyelitis (EAE) and colitis (212–214). Furthermore, feeding mice a high salt diet (HSD) lead to induction of Th17 cells and exacerbation of EAE symptoms (140, 141, 215, 216). However, these mouse studies have been conducted with a dramatic increase in dietary salt consumption. A further understanding of a more physiological increased salt concentration on immune cell function and the development of autoimmune disease is needed. In **Paper III** we addressed this issue by studying the effect of increased salt exposure on immune cell functions *in vitro* and *in vivo* and on the development of several autoimmune diseases using mouse models.

To assess the impact of salt on homeostasis and immune cell function, we provided mice with normal drinking water (NDW) or salt drinking water containing 1% NaCl (SDW) for a period of three weeks during which fresh urine and stool samples were collected. Albeit increased water intake, neither urine-specific gravity nor feacal IgA levels were affected by SDW. IgA is a measure of impairment in the intestinal mucosa; it is excreted from the lamina propria after translocation of microbial products when the epithelial barrier gets impaired. Nevertheless, pre-exposure to SDW lead to elevated numbers of $F4/80^{+}CD11b^{+}$ peritoneal cells and to increased production of IL-2, TNFα and IL-17A by $CD4^{+}$ T cells *ex vivo*. In line with previous studies (140, 141, 217–219), we show that *in vitro* exposure to salt also effects T-cell and macrophage effector functions. However, reduced viability for both was observed

as well. Moreover, we indicate that the effects were partially mediated through osmotic mechanisms since similar results were obtained by D-mannitol.

To determine if SDW can affect the development of T-cell mediated autoimmunity, we studied EAE and CIA in mice provided with NDW or SDW. However, and contrary to that observed in EAE under HSD, we did not observe differences in disease development in either EAE or CIA. It has to be noted that the salt concentration used in our study is drastically lower compared to that used as HSD in previous EAE studies. Moreover, our mice express a MHC-II H-2q molecule, whereas B6 mice used in the previous studies express H-2b, which might cause a difference in the strength and duration of the generated T-cell responses (220). Furthermore, all mice developed a very severe form of EAE, leaving a very small window to worsen the disease.

Macrophages are known to play a crucial role in the development of antibody-mediated autoimmunity (6, 221). Since we found increased numbers of macrophages and expansion of both pro- and anti-inflammatory cytokines, we assessed whether antibody-mediated arthritis could be influenced by SDW. During the initial phase of CAIA, mice exposed to SDW developed less arthritis. However, this was completely abrogated after LPS stimulation. A possible explanation for this is increased infiltration of inflammatory macrophages and neutrophils after LPS stimulation (6, 201).

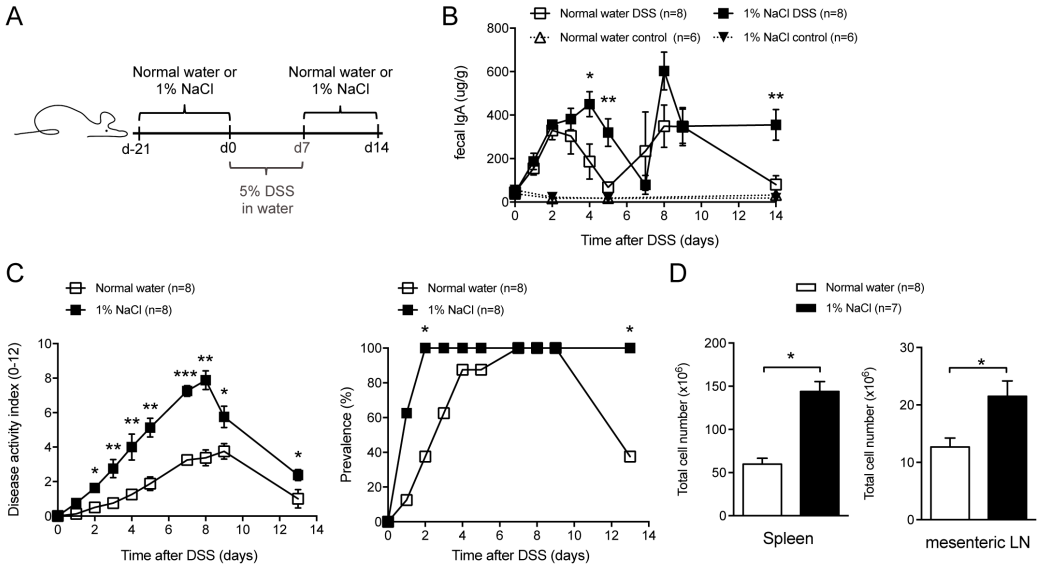

Figure 7 Exposure to high salt intake exacerbates the severity of dextran sulphate sodium (DSS)-induced colitis. (a) Mice were pre-exposed to 1% NaCl in water or standard drinking water for 3 weeks, followed by 5% DSS for 1 week, and again 1% NaCl in water or standard drinking water for another 1 week. (b) Faecal IgA titres from colitis mice described in (a) compared with naïve mice under identical salty water regiments. (c) Colitis disease symptoms were monitored daily and presented as disease activity index (DAI) score and prevalence. (d) Absolute cell numbers in spleen and mesenteric lymph nodes of mice 14 days after DSS exposure. Adapted from (143).

Since dietary factors have shown to influence the microbiota and immune cell signatures in the gut, we hypothesized that salt would have an adverse effect on DSS-induced colitis in mice, an inflammatory disease of the gut. This was indeed the case and in line with recently published studies (222, 223). Mice with SDW have exacerbated colitis symptoms (Figure 7), increased levels of IgA, decreased colon length caused by increased microscopic inflammation, increased number of lymphocytes, elevated levels of macrophages in the colon and higher number of TNFa on both $CD4^+$ T cells and macrophages in the colon. Interestingly, the observed disease phenotype was independent of its osmotic properties, since D-mannitol resulted in a similar disease phenotype as mice on NDW.

Taken together, we show that exposing mice to moderate salt concentrations influenced the effector functions of naïve T lymphocytes and myeloid cells, in particular macrophages, with pathological consequences during the development of inflammatory diseases, particularly leading to exacerbated disease symptoms in colitis, but not in T-cell mediated autoimmunity.

2.3 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

With the work presented in this thesis, I have contributed to a further understanding of the complexity that is autoimmunity. I have addressed both the effects of genetic and environmental factors on the development of autoimmune diseases using mouse models. **Paper I** identifies that polymorphisms in both FcγR2b and FcγR3 regulate the severity of inflammatory responses. This observation highlights the importance of genetic interplay in the regulation of autoimmunity and the relevance of using congenic mice. **Paper II** determines that SNAT proteins regulate T cell activation *in vitro* and that blocking them diminishes the severity of antibody-mediated arthritis *in vivo*. This reduction most likely occurs through MeAIBs effect on metabolically active inflammatory cells. Targeting immune cell metabolism might thus be beneficial for treatment purposes. **Paper III** concludes that moderate salt intake exacerbates DSS induced colitis phenotypes caused by changes in T cell and macrophage signatures *in vivo*. Phenotypic immune cell changes were not translated into T-cell dependent autoimmunity. These findings highlight the regulation of macrophage-dependent pathologies by salt.

In **papers I** and **II**, I have shown the importance of using congenic mice to identify arthritis causative genes. Although congenic mice were not used as such in paper II, it was the information obtained from congenic mouse studies that led us in this direction. The use of MeAIB to target SNAT proteins for treatment of arthritis in human disease is not likely unfortunately due to its high uptake by the liver. However, it is likely that current arthritis treatments, affecting cell proliferation, already target SNAT proteins. Nevertheless, this has yet to be investigated, as well as a therapeutic approach that could solely affect the metabolism of immune cells.

Clinical data has demonstrated the role of FcγR polymorphisms and their binding affinity in treatment responses in RA patients. Moreover, various strategies for FcγR targeting exist for clinical intervention in autoimmunity, such as blocking FcγRs, neutralizing circulating immune complexes, use of bispecific ligands (e.g. crosslinking FcγR2b with the BCR on B cells) or antibody modification to manipulate binding affinity and reduce inflammatory responses. However, there is still need for a better understanding of the individual FcγR function on individual cell types.

Taken together, I have shown that the interaction between two genes enhances arthritis disease development, whereas a single environmental factor has no impact on arthritis albeit triggering the immune system. These results contribute to the understanding of the

mechanism behind complex multifactorial diseases as a small building block towards therapeutic intervention.

3 ACKNOWLEDGEMENTS (DANKWOORD)

"Doe maar gewoon, dan doe je al gek genoeg." "Just act normal, then you're acting crazy enough as it is.", is the motto of my fellow Dutch people… But what is normal? The journey of a PhD definitely is not.

Going through a PhD is like being on a very very long rollercoaster ride. The thrill, the excitement, lots of ups, but also lots of downs. For me this started in December 2012, or officially in August of 2013. It has been tough, very tough, at times, but I have also enjoyed this period of my life to the fullest. I have had the pleasure to meet and work alongside some very talented scientists and I have learned to appreciate the small victories in life. Finishing this PhD would not have been possible without the help and support of so many people and I would therefore like to take this opportunity to thank everyone who has contributed to this.

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