From the DEPARTMENT OF MEDICAL BIOCHEMISTRY AND BIOPHYSICS
Karolinska Institutet, Stockholm, Sweden

THE GENETICS OF EXPERIMENTAL ARTHRITIS IN RODENTS

Michael Förster

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To my family and friends,

Failure is the key to success, each mistake teaches us something.
Morihei Ueshiba (1883 – 1969)

Do the difficult things, while they are easy and do the great things, while they are small. A journey of a thousand miles must begin with a single step.
Lao Tzu (6th century B.C.)

Perception is strong and sight weak. In strategy it is important to see distant things as if they were close and to take a distanced view of close things.
Miyamoto Musashi (1584 – 1645)

ABSTRACT

Unravelling the genetic susceptibility to complex autoimmune diseases and understanding these pathologies on a mechanistic level are major obstacles to improve our possibilities for therapeutic intervention and an increase in the quality of life of affected patients. Studies in experimental rodent models, that can be run under stable environmental conditions, which itself can be subjected to experimental manipulation, and in cohorts of potentially unlimited size, hold significant promise for the understanding of genes and pathways involved in complex autoimmune diseases. In this thesis, which is based on five scientific manuscripts, we initially investigated the influence of the genetic background on the ability to detect three major genetic loci (*Pia4/Cia12*, *Pia5/Cia3*, *Pia7/Cia13*) for pristane induced arthritis (PIA) in the rat. We also investigated the effect of *Pia1*, which includes the RT1 region (major histocompatibility complex (MHC) in the rat). We could show that the major arthritis regulator NCF1 as well as the MHC are silent in certain genetic backgrounds, whereas their genetic effect on PIA susceptibility can be detected in other, distinct genetic setups, arguing for the importance of genetic interactions between MHC and non-MHC genes for PIA development. In the second and third paper, we used a unique approach with a heterogeneous stock (HS) derived inbred-outbred mouse cohort that had been backcrossed to the arthritis susceptible C57BL10/Q (BQ) mouse strain, in order to map clinical phenotypes and the autoantibody response during collagen induced arthritis (CIA) development. We defined numerous novel loci and fine mapped already described quantitative trait loci (QTL) associated with clinical disease and/or autoantibody production providing the to date most comprehensive mapping study in CIA. The papers 4 and 5 concern the positional identification of candidate genes for the CIA loci *Cia21* and *Cia22* in the mouse. We propose the costimulatory molecule CD2 as a female specific genetic risk factor for autommunity in the joint and the central nervous system (CNS). We also pinpoint the chitinase like gene *Chi3l3*, also denoted as *Ym1*, as an important immunomodulator in experimental murine arthritis models based on both active immunization with collagen (CII) and passive transfer of arthritogenic antibodies. Hopefully, the findings presented in this thesis will have clinical implications based on the novel genetic targets, we identified. In addition, our data demonstrate the difficulties and pitfalls that are associated with gene identification using a hypothesis free positional cloning approach in experimental rodent populations.

LIST OF PUBLICATIONS

I. **Detection of arthritis-susceptibility loci, including Ncf1, and variable effects of the major histocompatibility complex region depending on genetic background in rats.**
Carola Rintisch, Michael Förster, Rikard Holmdahl.
Arthritis Rheum. 2009 Feb;60(2):419-27.

II. **High-resolution mapping of a complex disease, a model for rheumatoid arthritis, using heterogeneous stock mice.**
Emma Ahlqvist, Diana Ekman, Therese Lindvall, Marjan Popovic, Michael Förster, Malin Hultqvist, Dorota Klaczkowska, Ivanka Teneva, Martina Johannesson, Jonathan Flint, William Valdar, Kutty Selva Nandakumar, Rikard Holmdahl.
Hum Mol Genet. 2011 Aug 1;20(15):3031-41.

III. **Genetic control of antibody production during collagen induced arthritis development in heterogeneous stock mice.**
Michael Förster[*], Bruno Raposo[*], Diana Ekman, Dorota Klaczkowska, Marjan Popovic, Kutty Selva Nandakumar, Therese Lindvall, Malin Hultqvist, Ivanka Teneva, Martina Johannesson, Emma Ahlqvist, Rikard Holmdahl.
Arthritis Rheum. 2012 (in press)

IV. **Positional identification of CD2 as a risk factor involved in sexual dimorphism of murine autoimmune susceptibility.**
Michael Förster, Katarina Vlachou, Sara Lind, Erik Lönnblom, Martina Johannesson, Rikard Holmdahl
Manuscript

V. **Positional identification of the chitinase like gene locus as a risk factor for experimental arthritis in mice.**
Michael Förster, Bruno Raposo, Manuel Kulagin, Ivanka Teneva, Martina Johannesson, Nicola L. Harris, Rikard Holmdahl.
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[*] These authors contributed equally to this work.

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
APC	Antigen presenting cell
CAIA	Collagen antibody induced arthritis
CD	Cluster of differentiation
CIA	Collagen induced arthritis
CFA	Complete Freud’s adjuvant
CNV	Copy number variant
CTLA-4	Cytotoxic T lymphocyte antigen - 4
CII	Collagen type II
EAE	Experimental autoimmune encephalomyelitis
GPI	Glucose 6 phosphate isomerase
HLA	Human leukocyte antigen
HS	Heterogeneous stock
IFA	Incomplete Freud’s adjuvant
IFNγ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MS	Multiple sclerosis
EAE	Experimental autoimmune encephalomyelitis
Ncf	Neutrophil cytosolic factor
QTL	Quantitative trait locus
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
TCR	T cell receptor
TH	T helper cell
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
T1D	Type 1 diabetes

THE IMMUNE SYSTEM

Our body is constantly under exposure of invading pathogens such as bacteria, viruses, fungi and large multi-cellular parasites. It is our immune system's task to defend us against these foreign invaders. Generally, our immune defences are divided into two distinct, but not mutual exclusive categories, namely the innate and the adaptive immune system. The innate immune system contains immediate, first line defence mechanisms that prevent pathogens from gaining entry into the host at the contact site with the environment (skin barrier and mucous secretion); that lead to the activation and production of molecules for disposal or destruction of pathogens [complement system, reactive oxygen species (ROS), reactive nitrogen species (RNS)]; and expression of receptors that recognize pathogen- (PAMPs) or danger associated molecular patterns (DAMPs) subsequently leading to cell activation. Pattern recognition receptors were postulated by Charles Janeway in 1989, who inferred that prior to clonal expansion of the adaptive immune system, activation by PAMPs recognized by receptors with broad substrate specificity are an absolute necessity for the efficient mounting of an immune response (pattern recognition model) [1]. Of particular importance in this process are Toll like receptors (TLRs) that recognize bacterial or viral products and have been initially described in fruit flies and later found in all higher organisms including mice and humans [2,3,4]. Additionally, specialized phagocytic immune cells that function as antigen presenting cells (APCs), ingest and digest pathogens and present fragments as antigens via the MHCII to the adaptive immune system [5]. The discoveries of TLRs and the unravelling of their importance for the innate immune system as well as the identification of dendritic cells (DCs) that act as APCs, were jointly awarded the Nobel Prize in Medicine and Physiology in 2011. An alternative model, proposed by Polly Matzinger, suggested that innate immune cells primarily discriminate disturbances of tissue homeostasis by pathogens, through the recognition of DAMPs (danger model) [6]. Very recently, two papers indicated CLEC9A (DNGR1) as a receptor for filamentous actin of necrotic cells as the first DAMP receptor [7,8]. Activation of the adaptive immune system comprised of B and T lymphocytes results in a specific response against the invading pathogen, whereby B lymphocytes produce antibodies and constitute the humoral part-, whereas T lymphocytes provide either help to B lymphocytes aiding antibody production or directly mediate a cytotoxic response constituting the cellular arm of the adaptive immune system. Characteristically, both the humoral- and the cellular branch of adaptive immunity rely on the ability to recombine variable (V), diversity (D), joining (J) gene segments in order to generate their B cell and T cell receptors of potentially unlimited specificity using the recombination activating gene (RAG) proteins [9,10]. A central aspect for the understanding of the adaptive immune system is the concept of clonal selection proposed by Burnet [11]. It states that during its activation in response to a pathogen only clones with correct specificity are expanded, leading to the production of antibodies that mediate destruction of the respective target. Upon re-infection with the same pathogen the host mounts a more efficient immune response, as the adaptive immune system unlike the innate immune defence, is able to generate long lived, persisting cells that mediate immunological memory.

AUTOIMMUNITY

Autoimmunity is generally referred to as the failure of an organism to maintain immunological tolerance to self, which leads to an attack of the host's own immune system against potentially any of its tissues, resulting in their destruction and

subsequently loss of function. Autoimmune diseases are generally classified as either organ specific [multiple sclerosis (MS), type 1 diabetes (T1D), autoimmune thyroiditis (AT), myasthenia gravis (MG)] or systemic, as they lead to manifestations in multiple tissues and organs [rheumatoid arthritis (RA), systemic lupus erythematosus (SLE)]. The term autoimmunity was derived from the paraphrase "horror autotoxicus" coined by German immunologist and Nobel Prize laureate Paul Ehrlich, who falsely assumed that autoimmunity as a consequence of self-immunization with an antigen is not possible [12]. Reasonable estimates state that approximately up to 5% of the human population are affected by an autoimmune disease [13].

The introduction of the clonal selection theory and the concept of immunological tolerance by Burnet and Medawar provided the intellectual framework for our current understanding of autoimmunity, as a consequence of the breach of immunological tolerance to self. Despite of decades of extensive research, the aetiology of this phenomenon is far from being understood. Several genetic as well as environmental factors may play a role, but their exact contribution and their complex interplay with each other remain to date elusive.

Interestingly, some autoimmune pathologies were found in the case of a few, rare, monogenic diseases with Mendelian inheritance pattern, arising from highly penetrant mutations in a single gene that proved to be a key regulator of immunological tolerance. As an example serve patients with loss of function mutations in the autoimmune regulator gene *AIRE* that develop autoimmune polyendocrine syndrome type 1 (APS) also known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), displaying hypothyroidism, adrenal insufficiency, hypogonadism and recurrent infections with *Candida* species [14]. Recent work indicated that these patients develop autoantibodies against a cytokine named IL-17, implicated to be crucial for host defence against fungal pathogens in mice and humans [15,16,17]. Mechanistic studies, using AIRE deficient mice, identified this transcription factor as a major regulator of central tolerance in the thymus, where it provides a molecular switch for the expression of tissue associated autoantigens in medullary thymic epithelial cells (mTECs) [18].

Accordingly, a major regulator of peripheral tolerance was defined, when loss of function mutations in the *FOXP3* gene (forkhead box P3) were found to cause immunodysregulation polyendocrinopathy enteropathy X linked syndrome (IPEX) in humans, which manifests in lymphadenopathy, T1D, psoriatic or eczemic dermatitis, food allergies and autoimmune skin conditions such as alopecia universalis or bullous pemphigoid [19,20]. Similar phenotypes were described in mice with a point mutation leading to FOXP3 deficiency underlying the IPEX comparable *scurfy* phenotype [21]. In both species loss of FOXP3 leads to a dramatic reduction in numbers and function of $CD4^+$ $CD25^+$ T regulatory cells, found to be crucial for the maintenance of peripheral tolerance and tissue homeostasis [22,23,24,25].

Apart from these rare, monogenic, primary immunodeficiency diseases exhibiting a strong autoimmune component, autoimmune pathologies are generally considered to be complex diseases, with a multitude of genetic and environmental factors interacting to govern incidence, severity and clinical outcome of a particular disease.

AN INTRODUCTION TO MOUSE GENETICS

The completion of the mouse and the human genome sequence projects, it became apparent that there is a large degree of homology between both species [26,27,28]. The mouse genome is contained in 20 chromosomal pairs (19 autosomes; X and Y sex chromosomes) and current estimations range from 2.7 to 3.4 billion base pairs for its size, which is comparable to the human genome. In the initial draft of the mouse genome, it has been estimated that approximately 99% of all mouse genes have a homologue in humans, 96% of these genes map to the same syntenic region and 80% of the murine genes are 1 to 1 orthologues descending from a common ancestor [28]. The Ensembl genome browser release 68 for the *Mus musculus* genome lists 23140 genes (including putative pseudogenes, immunoglobulin and T cell receptor (TCR) genes) and an additional 37 genes for the mitochondrial genome. Interestingly, more than 98% of the human and the mouse genome do not encode for proteins, and in turn are made up by seemingly functionless DNA sequences, for which in the term "junk" DNA has been coined by Susumu Ohno in 1972 [29]. These sequences comprise intronic and intergenic DNA, often consisting of repetitive sequences, mobile genetic elements (transposons, retrotransposons), pseudogenes (pseudo-, retropseudogenes) as well as non-coding RNA molecules of different size. Very recent data obtained by the ENCODE consortium strongly argued against the notion of the existence of "junk" DNA, instead indicating that up to 95% of the genomic sequence are used to initiate transcription and gene expression [30].

Novel gene variants can arise during the process of faulty copying of the DNA molecule during cell division, whereby many changes affecting the DNA sequence are minor point mutations of single nucleotides that lead to the formation of single nucleotide polymorphisms (SNPs). SNPs may alter codons, coding for critical amino acids of a protein resulting in gain- or loss of function mutations that account for differences in genetic susceptibility to complex autoimmune- and/or chronic inflammatory conditions. This is illustrated by a naturally occurring SNP in codon 153 of the neutrophil cytosolic factor (*Ncf1*) gene in rats that affects ROS production by the NADPH oxidase complex (NOX2) and was proven not only to alter susceptibility to autoimmune disease, but also to be causal for differences in psychosocial stress behaviour in the laboratory rat providing an example for pleiotropy [31,32,33]. Concordantly, an A to C transversion mutation at the -2 position of the 5' end of exon 8 in the *Ncf1* gene that spontaneously occurred in a colony of C57BL6/J-m (lpr(db/db)) mice, altered a splice site and led to functional deficiency of NOX2 and increased autoimmunity [34,35]. A non-synonymous coding SNP encoding for structural polymorphism Asp105Gly in the *Nramp1* gene leads to increased susceptibility to insulin dependent diabetes in NOD mice and prove to be the causative gene for the *Idd5.2* locus, elegantly shown by in vivo RNA interference by Kissler and colleagues [36,37]. This loss of function polymorphism in the phagosomal ion transporter NRAMP1 also associated with decreased susceptibility to infection with intracellular pathogens like *Salmonella typhimurium*, *Leishmania donovani* and certain *Mycobacterium* species [38,39]. An additional example of differential expression of alternatively spliced transcripts that mediate the loss of humoral tolerance to nuclear antigens, is the identification of the *Ly108* gene as part of the murine lupus susceptibility locus *Sle1b* [40,41,42]. This gene is encoded in the SLAM/CD2 gene cluster on murine chromosome 1 and implicated to alter the threshold of T cell activation and NKT cell numbers [40,43].

As only few coding or splice site mutations were unambiguously positionally cloned, speculation arose that these variations may not be sufficient to primarily account for

genetic differences in quantitative phenotypes. In turn polymorphisms affecting promoter, silencer, enhancer elements or transcription factor binding sites that influence gene expression were suggested to underlie differences in heritability of complex traits [44]. This notion poses a significant obstacle for definitive identification of polymorphisms underlying QTLs defined by linkage, as it is much more difficult to assess functional consequences of a certain associated SNP on the development of an expression QTLs (eQTL), which may not be as apparent as for coding- or splice site mutations. This problem received increasing attention as large association studies in humans often produce strongest hits in gene deserts and non-coding regions of the genome [45].

Other polymorphisms that may underlie complex traits are minor structural alterations of the genome leading to insertion or deletion (INDEL) of few nucleotides. These alterations have historically been used in mapping studies as they produce micro- or macrosatellite markers that can be resolved by gel electrophoresis after PCR amplification. Examples, for which INDELs were implicated in genetic susceptibility to complex immunological traits, include a 2 bp deletion at positions 62 and 63 of an 83 base pair exon near the 5' end of the *Hc (C5)* gene leading to a premature stop codon and a truncated protein [46]. This mutation is found in the following mouse strains: A/HeJ, AKR/J, DBA/2J, NZB/B1NJ, SWR/J, B10.D2/oSnJ and renders these mice susceptible to ovalbumin (OVA) induced allergic asthma [47]. Other minor structural variants in a candidate gene for murine SLE like autoimmunity present in multiple mouse strains (NZB/BlNJ, BXSB/MpJ, MRL/MpJ, NOD/ShiLtJ, 129S1/SvlmJ, NZW/LacJ, SJL/J and SWR/J) are deletions in the promoter and the third intron of the *Fcgr2b* gene, the sole murine inhibitory Fc receptor, expressed on B cells and macrophages [48,49,50,51]. In addition, coding non-synonymous polymorphisms, found in this gene, form two distinct haplotypes in the laboratory mouse (Ly17.1/2) [52]. Though these SNPs lead to amino acid alterations in the extracellular D2 domain that interacts with the CH2 domain of the IgG Fc region, there is no evidence of a functional impact of these coding variants [53,54].

Large genomic alterations involving insertions, deletions, inversions or translocations of big stretches of DNA sequence have been described and are increasingly appreciated in complex genetics. In mice, a translocation and duplication of the *Tlr7* gene locus from the X to the Y sex chromosome was found to increase B cell reactivity and autoantibody formation manifesting in a systemic lupus erythematosus (SLE) like syndrome in Yaa males (Y autoimmune accelerator) [55,56]. Additional evidence for the causal role of this mutation was obtained, when the autoimmune phenotype was recapitulated by transgenic overexpression of the *Tlr7* gene [57,58]. However, other genes, included in the translocated DNA segment, may also contribute to increased lupus susceptibility [59]. In addition, a copy number variant (CNV) of *Fcgr3b* gene was described to underlie increased susceptibility to glomerulonephritis and SLE across species in both rats and humans [60,61]. These and other examples illustrate the importance of CNVs as a driving force for genome evolution and genetic susceptibility to complex traits [62]. During the last couple of years several research groups investigated the global distribution of copy number polymorphisms across the mouse genome by massive parallel sequencing of multiple mouse strains [63,64,65]. Thereby, the chitinase like gene locus on murine chromosome 3 was implicated to harbour a CNV, resulting in a duplication in the 129P2/OlaHsd, 129S1/SvlmJ, NOD/ShiLtJ, LP/J, FVB/NJ, CAST/EiJ, C57BL/6NJ mice [66]. This relates to work presented in paper 5, where we positionally identified the murine chitinase like gene *Chi3l3* as a candidate for the control of experimental arthritis in mice.

A SHORT GENEALOGY OF THE LABORATORY MOUSE

Of the > 500 inbred mouse strains and substrains currently used in biomedical research are hybrids between the *musculus* and *domesticus* subspecies of the house mouse *Mus musculus*. Per definition, a mouse strain is considered to be inbred, when it has been repeatedly brother sister mated for more than twenty consecutive generations. Under the assumption that no genetic drift occurs, all mice are at this stage genetically identical (isogenic) and can be traced back to a single ancestral breeding pair. The currently used inbred mouse strains were derived from "fancy" mice maintained as pets and initially caught at the beginning of the 19th century. Clarence Cook Little bred the first laboratory mouse strain by successive brother sister mating of an initial breeding pair carrying the recessive genes for diluted, brown and non-agouti (DBA) [67,68]. The most commonly used mouse strain in biomedical research are C57 black derived strains, initially derived by Little at the Cold Spring Harbor Laboratories from a breeding pair he received from Miss Abbie Lathrop. With the completion of the mouse genome project significant hopes were attained to unravel novel gene functions in a complex model system and thereby gain a better understanding of the genetic basis of pathological situations in humans [28].

FORWARD VERSUS REVERSE GENETICS

Identification of genes and pathways controlling quantitative phenotypes are of major interest and can potentially be achieved in two distinct not mutual exclusive ways in the laboratory mouse. Firstly, a reverse genetic approach is gene centered, hypothesis driven, often involving the introduction of an artificial mutation in the gene of interest, which in turn is then evaluated for its capacity to influence a particular phenotype [69]. Alternatively, for a forward genetic approach novel gene variants can be introduced by de novo mutagenesis with N-ethyl-N-nitrosourea (ENU) [70]. Genetic susceptibility to autoimmune disease was studied using ENU mutagenesis in mice and led to the identification of a loss of function mutation of *Rc3h1* (roquin), a posttranscriptional repressor of the inducible T cell co-stimulator (ICOS) that mediates the accumulation of follicular helper T cells and subsequently anti-DNA antibody formation culminating in a lupus like disease [71,72]. Moreover, this phenotype is accompanied by decreased susceptibility to *Salmonella typhimurium* infection [73].

On the other hand mapping of disease genes in animal models can be achieved in hypothesis free manner by the identification of naturally occurring gene variants with varying effect sizes, as large cohorts of experimental animals of phenotypic distinct inbred mouse strains are available. Initially, identification of quantitative trait loci for a particular phenotype is carried out by a correlation of genetic markers with the phenotype in a segregating population, defined as genetic linkage analysis. Following the identification of the locus, one then isolates the respective region in a congenic strain, and tries to narrow down the critical interval that is associated with a particular phenotype by recombination assisted breeding. Once the critical interval is sufficiently small, one aims to positionally identify candidate genes and polymorphisms that may explain the observed phenotype, which in the last step need to be functionally proven to firmly establish a quantitative trait gene for a particular QTL.

Genome wide mapping strategies

Following a hallmark paper by Lander and Botstein in 1989 genome wide studies on complex phenotypes in experimental rodent populations were performed using F2 intercrosses or N2 backcrosses [74]. For these, two phenotypically distinct inbred strains are intercrossed to produce an initial F1 cohort, of which all individuals are genetically identical. Depending on the type of cross these individuals are either further backcrossed to one of the background strains or they are subsequently intercrossed to accumulate recombinations of parental genetic information. A typical F2 cohort of mice consists of several hundreds of individuals that are genotyped on genome wide level with molecular markers spanning evenly over the complete genome followed by genetic linkage analysis. Bateson and Punnett initially introduced the concept of genetic linkage that is defined as the tendency of genes that are located in close proximity on a chromosome to be inherited together during meiosis, as the chance of a crossing over between chromatids that separate two distinct loci decreases with physical proximity [75]. In following, Morgan developed the idea that the amount of recombinations observed in a genetic cross could be used for the construction of linkage maps, which laid the foundation of modern genetic analysis [76]. Consequently, in any experimental cross, genetic markers and genes regulating the phenotype of interest that are in close proximity, are very likely to be co-inherited in a filial generation.
A genome scan using an F2 population usually yields a mapping resolution of 10 to 30 cM with chromosomal intervals that typically contain hundreds of genes. To date approximately 50 QTLs for CIA have been described using these methods, many of which overlap with QTLs for other arthritis models as well as other autoimmune models like experimental induced autoimmune encephalomyelitis (EAE), clearly indicating a common genetic component governing susceptibility to these pathologies in the mouse [77,78]. However, the low amount of recombinations that occurs in a F2 or N2 population strongly impedes with the mapping resolution that can be obtained with these conventional methods. Therefore, only for a fraction of QTLs a candidate gene or polymorphism has been proposed outlining the difficulties of positional cloning. This led to several attempts to increase mapping resolution and subsequently facilitate gene identification accounting for differences in heritability of quantitative traits in experimental rodent populations.

Advanced intercross lines (AIL)
To overcome the problem that only few recombination events occur during the production of an F2 or N2 filial generation, AILs were initially created. They are produced from a F2 population that is randomly intercrossed to acquire recombinations between two given loci during the additional filial generations [79]. AILs were used for genome wide mapping as well as fine mapping of already existing loci and proof of concept has been obtained in numerous studies. Yu and colleagues used a (DBA/1 x FVB/N)F11/12 AIL to fine map several CIA loci previously identified in a conventional F2 cross [80,81,82]. Another example for successful fine mapping of already existing QTLs that laid the foundation for the work presented in this thesis are studies done on the *Eae2/Eae3/Cia5* susceptibility loci identified in a B10.RIII x RIIIS/J F2 population [83,84]. Using a partial advanced intercross (PAI) between *Eae3/Cia5* and *Eae2* bicongenic mice, taking advantage of genetic interactions between these loci, led to the identification of several subloci on murine chromosome 3 (*Cia5, Cia21, Cia22*) and chromosome 15 (*Cia30, Cia31, Cia32*) that interact in complex fashion and affect clinical CIA as well as inflammation related cellular subphenotypes like alterations in CD4/CD8 T cell populations [85,86].

Heterogeneous stocks (HS)

An additional resource for fine mapping of QTLs that received increasing attention during the last years are HS that are available for both *Mus musculus* and *Rattus norvegicus*. These stocks are advanced intercrosses with contributions of eight inbred strains that have been semi-randomly bred for a multitude of generations. Currently, two eight allele heterogeneous mouse stocks are available; the Boulder stock bred for more than 60 generations (HSIBG) comprised of (A/J, AKR, BALB/C, C3H, C57BL/6, DBA/2, Is/Bi and RIII) and the Northport stock bred for more than 55 generations (HSNPT) comprised of (A/J, AKR/J, BALB/CJ, LP/J CBA/2J, C3H/HeJ, C57BL/6J and DBA/2J) both originally intended to be used for studies of aging related and behavioural phenotypes [87,88]. Of particular importance for this thesis is the HSNPT that has been used to create an arthritis susceptible HS derived mouse inbred outbred cohort by backcrossing it to C57BL/10.Q (BQ) in a F3 cross (HSxBQ), as HSNPT mice itself are resistant to CIA, but not CAIA or EAE induction [89]. The usefulness of HSNPT mice for fine mapping of known QTLs was successfully demonstrated by studies undertaken by Mott and colleagues [90,91]. These have later been expanded to genome wide mapping of a variety of biochemical, immunological, haematological and metabolic quantitative phenotypes [92]. In parallel to our own study involving the genome wide mapping of CIA in HSxBQ mice presented in paper 2, Johnsen et al. utilized the available HSNPT mice to map arthritis severity in an arthritis model involving passive transfer of arthritogenic antibodies [93]. While HS mouse cohorts are readily available to researchers making tedious work of intercrossing to obtain an outbred cohort obsolete, caveats for its use are apparent. Firstly, the number of markers that has to be genotyped in these cohorts is very high, as linkage disequilibrium (LD) blocks are considerably small due to high amount of recombinations acquired during HS construction. Secondly, the cohort size in a single experiment to detect linkage for small and intermediate effect QTLs is big as both the number of segregating alleles and their interactions with the background genome are usually underestimated by bioinformatic methods used for analysis [94]. Lastly, family effects need to be corrected in the statistical analysis in order to minimize the amount of false positive identified QTLs in a particular cross [94,95].

Collaborative cross (CC) and recombinant inbred lines (RILs)

The most recent tool for quantitative trait mapping in rodents, are RILs derived from the CC experiment, aiming to generate hundreds of RILs from an octo parental cross [96]. A RIL is created by repeated intercrossing of two (or more) inbred mouse strains until complete fixation of the genome has been achieved. The idea has initially been proposed almost ten years ago by the Complex Trait Consortium, yet the CC is still not fully available due to the extensive amount of time that is required for the generation of the RILs. The major advantage of the CC approach compared to a conventional HS is the fact that its RILs have only to be genotyped once and with the complete genotyping information available, can be used to map genetic control of multiple phenotypes. Furthermore, F1 progeny of RILs from the CC can potentially be created in limitless fashion through intercrossing and subsequently be used for mapping projects, attempting to model the genetic complexity in humans using genetically normal individuals without artificially introduced mutations [97]. The CC was constructed to maximally increase the diversity by the inclusion of wild derived inbred mouse strains derived from both *musculus* and *castanaeus* subspecies, with allele contributions of the (129Sv/Im, A, C57BL/6, CAST/Ei, NOD/Lt, NZO/HILt, PWK/Ph and WSB/Ei) strains [88]. Very recently, the CC came into focus, as a series of proof of concept studies for genome mapping of different quantitative traits using the first CC derived RILs were

reported, including a study investigating host resistance to *Aspergillus fumigatus* infection as an immunological phenotype [98].

Locus based strategies

Congenic and consomic strains

In order to achieve positional cloning of genes in using the outlined hypothesis free forward genetic approach, it is often necessary to isolate the respective chromosomal region implicated in a segregating cross to make a congenic strain. In theory, congenic animals are isogenic clones that differ only for the congenic locus from the respective background strain. In experimental rodent populations congenics can be produced by classical serial backcrossing to the background strain for more than ten generations. Alternatively, they can also be obtained by so called "speed congenic approach", for which not only the presence of a desired locus, but also the absence of disturbing donor alleles is assessed [99]. Furthermore, consomic mouse strains, in which whole chromosomes of inbred mouse strains were substituted, may provide a shortcut for the construction of congenic strains circumventing tedious backcrossing [100]. A disadvantage for the widespread use of consomic strains is the limited amount of chromosomal substitution strains and strain combinations that are available to the research community.

It is of utterly importance to keep in mind that most of the currently used knock out strains, which have been made in mixed chimeric backgrounds, are in fact congenic strains. During the generation of a targeted mutation in 129 embryonic stem cell lines, a 129 derived fragment bearing "flanking genes" is carried along, if experiments are not performed in this particular background [101]. That these genetic "impurities" can have a great functional impact is illustrated by an increasing amount of literature, outlining the importance of the genetic background and "flanking genes" for the correct interpretation of experimental results, obtained with gene targeting technologies. Several examples in which conflicting results using chimeric knock out mouse strains at different stages of backcrossing include the *Spp1/Opn* (osteopontin) gene and its role in joint and CNS autoimmunity as well as the role of IFNγ signalling in the non obese diabetic (NOD) mouse model of T1D [102,103,104,105]. With genotyping costs dropping, SNP marker panels that are already commercially available may be used to accurately assess the genetic purity of congenic and conventional gene targeted mice alike. Alternatively, experiments using littermate controls, introduction of targeted mutations in the mouse strain of interest using the respective ES cells circumventing backcrossing issues or in silico mapping strategies discussed in following may be used to overcome the problems associated with "flanking genes" in chimeric knock out experiments [101,106]. Noteworthy, all congenic mice that are the basis for the experiments presented in this thesis have been rigorously evaluated for genetic impurities using a custom made 8k Illumina SNP Chip [107].

In silico mapping and expression QTL analysis

Advances in genomic technologies displayed by large genotyping and sequencing projects of multiple laboratory mouse strains, have created hopes that resolution of QTLs can be simplified by utilizing the mosaic structure of the mouse and haplotype reconstruction without the de novo construction of segregating crosses. These methods are based on the fact that all laboratory strains descended from a few founder mice that were initially bred and selected at the beginning of the last century. Some of the currently used mouse strains are thereby closely related, exhibited by the amount of allelic variants that are shared at certain loci between otherwise distinct strains [108]. Under the assumption that a series of markers at distinct loci are always co-inherited, it

is theoretically possible to predict a variant segregating in a cross, from the respective marker information by reconstruction of founder haplotypes that are identical by descent. The usefulness of this approach was illustrated by the identification of transcription factor binding sites for Yin Yang 1 (YY1) and serum response factor (SRF) governing H2-Eα serum levels [109]. Clearly, these assumptions are directly correlated with the amount and the distribution of genotyping information available for a particular panel of mouse strains as rare, relatively recently occurred genetic variants for example mutations acquired during domestication of different mouse strains, that may have strong impact on a particular phenotype, but tend to be underestimated with this type of analysis. Simulations, using sequencing data of the HSNPT panel in a region harbouring an anxiety QTL on murine chromosome 1, illustrated the limitations of this approach indicating a highly complex haplotype structure in the laboratory mouse [110]. This led to the suggestion to use complete genome sequencing technology for different mouse strains to efficiently map quantitative trait associated genetic variation. Large full coverage genome sequencing projects of several commonly used strains have recently been reported and it can be expected that these will speed up the identification of quantitative trait genes [65].

Another technology that has been proposed to aid the hypothesis free identification of candidate genes in experimental crosses is the analysis of gene expression patterns that are associated with the development of a particular phenotype governed by a QTL. The rationale behind this notion is fuelled by the assumption that the majority of polymorphisms underlying heritable differences of quantitative traits, affect mRNA and subsequently protein expression of a particular gene rather than their primary amino acid structure or splice sites [44,111]. Successful combination of gene expression analysis to improve candidate gene identification in QTL mapping studies, has been carried out, none the least for the projects concerning the *Cia5/Cia21/Cia22* loci, including a genome wide gene expression analysis and a QTL restricted gene expression analysis [112]. Likewise, for other arthritis loci, namely *Cia2* and *Cia3* that were identified using a F2 intercross and fine mapped with a DBA/1 x FVB/N AIL, global gene expression analysis of central and peripheral lymphoid organs during different stages of the disease was used to pinpoint candidate genes for these small and intermediate effect QTLs [81,113,114].

Gene identification criteria – "the burden of proof"

To resolve a QTL and successfully define a quantitative trait gene additional evidence beyond positional identification of a candidate gene is required. Flint and Mott illustratively described this fact as the "burden of proof" [115]. Certainly, the most elegant way to obtain definitive proof for a certain genetic variant underlying a QTL is transgenic complementation, by which one allele can be replaced with another through genetic modification. However, in many cases, circumstantial evidence, obtained by alternative experimental approaches may be sufficient to fortify one's claim of positional identification of a genetic variant responsible for a complex phenotype. Several strategies have been proposed and may be combined depending upon feasibility, whereby a list of possible experimental approaches include; the identification of polymorphisms controlling gene expression of a particular gene or alterations of the primary structure of the protein by amino acid substitutions, proof of concept by identification of gene function that is related to a particular trait, functional studies using in vitro cell culture systems, where effects of different alleles can be assessed or deficiency can be modelled by RNA interference, transgenesis with

bacterial, yeast artificial chromosomes or small interfering RNAs acting dominant over resident host alleles or leading to suppression of gene expression of the host allele; gene targeting and knock out technology, which recapitulate findings in original QTL mapping studies, screens of artificially induced mutations of mouse mutant archives and their respective impact on a particular phenotype and homology searches and comparative genomics as polymorphisms underlying QTLs may be conserved over species barriers [115,116].

RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is regarded a complex autoimmune disease involving chronic inflammation of arthrodial joints, mostly affecting the synovial lining eventually leading to pain and discomfort, joint stiffness and further progressing to cartilage destruction and bone erosion. It affects approximately 0.5 to 1% of the human population worldwide, with its peak onset dating approximately to the fourth decade of life [117]. Thereby, gender has a strong influence on RA development illustrated by the fact that women have an increased risk of developing the disease [118]. RA is a systemic autoimmune disease with extra-articular manifestations that include systemic inflammation of the sclera, the pleura of the lung and the pericardium as well as anemia, vasculitis and rheumatoid nodule formation. It is considered to be rather a complex syndrome than a single disease entity and clinical symptoms as well as disease course vary greatly between patients further complicating exact diagnosis. Therefore, the American College of Rheumatology (ACR) listed seven criteria, of which at least four have to be fulfilled in order to qualify the diagnosis of RA [119]. The symptoms include morning stiffness, arthritis of 3 or more joint areas, arthritis of hand joints, symmetric arthritis, rheumatoid nodule formation, serum rheumatoid factor (RF) and radiographic changes. With the introduction of novel, highly specific biomarkers for RA namely, antibodies against citrullinated protein antigens (ACPA), RA diagnosis criteria itself were recently been expanded by the European League against Rheumatism (EULAR) and the ACR [120].

GENETIC HETEROGENEITY

Despite decades of research the aetiology of RA has yet to be determined. First clues that genetic predisposition plays an important role for disease susceptibility has arisen from studies investigating the disease prevalence in mono- and dizygotic twins and siblings and thereby, it has been estimated that a genetic contribution of approximately 60% to overall disease susceptibility exists [121,122]. The identification of the first genetic factor that was linked to RA development followed the discovery of the MHC in the late 60s. Mixed lymphocyte cultures showed that the disease is associated with certain MHC alleles, in humans designated human leukocyte antigen (HLA), namely HLA-DR4 (HLA-DRB1*04) [123]. Following this initial finding several other HLA-DRB1 alleles were associated with RA and depending on the study it has been estimated that the HLA region accounts for 30 to 50% of the genetic association making it by far the strongest factor in RA susceptibility. In the late 80s it was anticipated by Gregersen and colleagues that all disease associated alleles share a common amino acid motif in the hypervariable region of the HLA-DRß1 chain comprising the peptide binding cleft, which led to the formulation of the “shared epitope hypothesis”, involving the preferential presentation of arthritogenic peptides consequently leading to the loss of immunological tolerance [124]. Though, GWAS studies investigating the complex nature of RA genetics consequently yielded highest association in the HLA region with the HLA-DRB1 polymorphisms being most associated, an ongoing debate persists about the nature of additional effects that could explain discrepancies in association of different HLA-DRB1 alleles [125]. Recently, Raychaudhuri and colleagues provided new clues with an extensive analysis of the

genetic contribution of the HLA associated alleles in human RA cohorts claiming that association with amino acid polymorphisms in three HLA proteins (HLA-DRB1, HLA-B, HLA-DPB1) explains most of the MHC association in ACPA positive RA [126].
A major step forward in complex disease genetics and the identification of non-MHC loci for RA, was the introduction of affordable genotyping methodology for analysis of large patient- and control cohorts using a hypothesis free approach, making association studies possible. This led to an explosion of data concerning genetic risk factors not only for RA, but also for other complex diseases. One of the strongest associations was found to a non-synonymous coding SNP (R620W) in the *PTPN22* gene encoding for the lymphoid specific phosphatase LYP. This association was initially identified in a candidate gene study in a T1D cohort and thereafter confirmed in North American RA and SLE cohorts of Caucasian origin [127,128,129]. Initially, the disease-associated variant of *PTPN22* was described as a gain of function mutant, which was difficult to join with the fact that PTPN22 constitutes a major negative regulator of early T cell activation via T cell receptor signalling [130]. This led to the hypothesis that the disease-associated allele may alter the threshold for negative selection in the thymus promoting escape of autoreactive T cells or leads to a decreased activity of T regulatory cells in the periphery [131]. Recent data involving the generation of humanized transgenic mice expressing, the disease-associated variant indicated that the Lyp620W variant is a target for rapid degradation by the cysteine protease calpain leading to overall reduced levels of Lyp in lymphocytes. In these mice hyperreactivity in the lymphoid and dendritic cell compartment was observed providing a more concise explanation for the role of PTPN22 as a negative regulator of TCR signalling and susceptibility factor in human autoimmunity [132]. Another gene that was associated with RA and SLE utilizing a candidate gene approach is the *STAT4* gene, encoding for a member of the signal transducer and activator of transcription family of proteins, further indicating a role for T cells in disease susceptibility [133]. Following this initial observation in Caucasians, it has been found that *STAT4* constitutes a genetic risk factor for development of SLE across different human populations [134,135,136]. The STAT4 protein is part of a signalling cascade downstream of the IL-12 receptor, involved in TH1 differentiation facilitating IFNγ production in $CD4^+$ T cells [137,138]. A general autoimmunity susceptibility factor that is not only associated with RA, but also other autoimmune diseases like T1D is the negative regulator of T cell costimulation CTLA-4 (cytotoxic T lymphocyte antigen), further outlining the role of T cells in RA pathogenesis [139,140]. An example of a genetic risk factor that has been associated in population specific manner is the (peptidyl arginine deiminase) *PADI4* gene. The association has only been found in Asian RA cohorts, whereas no association was observed in Caucasians of different geographical origin despite similar allelic frequencies of the associated SNP strongly arguing for the presence of additional, yet to be defined susceptibility alleles in the Asian population that interact with PADI4 [141,142,143]. It encodes for an enzyme that catalyses the deimination of arginine to citrulline, believed to be an important pathway that leads to the formation of ACPA, however clear experimental evidence demonstrating this link is so far lacking. Recent studies in HSNPT mice using KBxN serum transfer identified PADI4 as a candidate gene determining clinical disease severity in an antibody induced model of arthritis [93].
Another risk factor for RA that was initially identified by a Swedish- American consortium is the *TRAF1-C5* locus on human chromosome 9 [144,145]. Interestingly,

the C5 pathway has also been implicated as one of the major regulators of different experimental arthritis models in mice, and is a major candidate gene for the *Cia2* locus identified in a NOD x B10.Q cross [146,147,148,149]. Another gene that has been associated with susceptibility to systemic autoimmune diseases like RA and SLE in Caucasians is the ubiquitin-editing enzyme *TNFAIP3/A20*, which is located on human chromosome 6p23 [150,151,152,153]. Recently, mouse models that involved conditional ablation of *A20* expression in the myeloid lineage developed spontaneous arthritis like syndrome, accompanied by an inflammatory bowel disease (IBD) like syndrome [154,155]. Last but not least, with particular importance to this thesis is an association that has been found to a SNP close to the *CD2* gene, as we identified this gene as a major candidate gene for the *Cia21* locus in paper 4 [156].

ENVIRONMENTAL FACTORS

During the last couple of decades there have been enormous efforts in the characterization of environmental factors that initiate RA or influence the course of the disease. A brief selection of environmental factors that have been implicated in RA susceptibility is presented in following.

Sex hormones

As previously mentioned gender has a strong influence on disease susceptibility and female preponderance of about three women to every man affected by RA aligns with data concerning gender effect on other autoimmune diseases, with articular symptoms such as SLE [157,158]. Though, sexual dimorphism in autoimmune susceptibility has been anticipated for several decades, the exact aetiology of this phenomenon has yet to be elucidated. The importance of sex hormones in RA as well as other autoimmune diseases is best illustrated by the fact that pregnancy itself characterized by high levels of oestrogen and progesterone leads to an alleviation of clinical symptoms first reported in case reports by Hench more than 70 years ago [159]. Conversely, female RA patients often suffer a relapse within 6 months post partum, when oestrogen and progesterone levels drop during the lactation period [160]. Besides gonadal hormone related effects on the immune system, sex chromosomal specific effects as well as other pregnancy related phenomena like mother fetus MHC disparity may play a role, but are difficult to study on a human populational level and with definitive evidence for either of these hypotheses lacking, a general consensus on the matter has not been reached [161,162]. Studies, which tried to investigate the issue of sexual dimorphism of the immune response in humans at baseline, indicated that there are indeed differences in the immune response between the sexes. Females compared to males seem to have an altered cytokine profile, more biased towards a TH1 response, elevated CD4 cell counts and generally a higher antibody response towards vaccination [118,163]

Smoking

Another major environmental risk factor that has repeatedly been associated with RA is smoking. Thereby, both the amount of smoking and the duration of cigarette consumption seem to influence RA susceptibility [164]. Epidemiologic studies systematically investigating the connection between cigarette smoking and genetic risk

factors trying to establish a mechanistic link in the human population found an increase in risk for the development of ACPA positive RA in smokers that were positive for the shared epitope alleles [165]. This data outlines the notion that smoking triggers specific MHC dependent immune reactions possibly through the presentation of altered immunogenic peptides that contribute to the breach of tolerance and subsequently the development of RA [166]. Interestingly, a coding non-synonymous polymorphism in the protein tyrosine phosphatase *PTPN22*, that has been shown to be associated with the development of RA, further increased the risk of developing the disease, in conjunction with shared epitope alleles and seropositivity for ACPA [167]. Very recently, a citrullinated α-enolase epitope has been implicated as a specific citrullinated autoantigen in a subset of ACPA positive patients that links smoking and well established genetic risk factors like the HLA and the *PTPN22* risk allele providing a candidate for an altered peptide involved in breach of tolerance that may be causatively linked to autoimmune reaction preceding RA onset in these patients [168].

Infections

So far, studies aiming to associate human RA with a specific infection, proposing a certain infectious organism as a bona fide environmental trigger for the disease leading to breach of tolerance to self and subsequent autoimmunity, have been unsuccessful. Some examples of pathogens that have been suggested to play a role in RA susceptibility include bacteria like *Mycobacterium tuberculosis*, *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae* as well as viruses like the *Epstein Barr* virus or *Cytomegalovirus* [169]. Epidemiological studies elucidated that patients with long standing RA also have an increased risk of developing periodontal disease [170]. The anaerobic, gram-negative bacterium *Porphyromonas gingivalis* a member of the Bacteriodetes phylum that is found in the oral cavity is associated with peridontitis [171]. Of note is that *Porphyromonas gingivalis* is currently the only bacterium known to expresses peptidyl arginine deiminase enzymes that are able to citrullinate a wide variety of proteins [172]. Interesting in this context is that even non-RA patients with history of this infection were in some studies found to have antibodies against citrullinated proteins (ACPA) [173]. However, other reports examining a direct correlation between ACPA titres and *Porphyromonas gingivalis* infection in new onset, never treated RA patients yielded conflicting results and found no correlation [174].

CURRENT TREATMENTS

Similarly to other autoimmune disease entities there is no definitive cure for RA, and current therapies rather aim to alleviate its symptoms and reduce pain and discomfort caused by the disease. The first class of drugs used for RA treatment is DMARDs (disease modifying anti rheumatic agents) like methotrexate and others of lesser importance like gold salts, leflunomide, chloroquinine and other anti malarial drugs [175]. Another class of drugs used to manage RA is called NSAIDs (non- steroidal anti-inflammatory drugs) like aspirin, ibuprofen or diclofenac [175]. At last, the most successful agents in terms of efficacy currently available for RA are the so-called “biologicals”, which target cytokines or cell surface molecules expressed on lymphocytes. Thereby, for antagonistic therapies using monoclonal antibodies or soluble receptors directed against pathogenic cytokines like TNFα (etanercept), IL-1

(anakinra) and IL-6 (tocilizumab) have been developed and marketed [176]. A monoclonal antibody targeting CD20 (rituximab), depleting B cells initially produced for treatment of B cell lymphomas has also been successfully introduced into the clinic for RA underscoring the pathogenic role of these cells as key players in the arthritogenic process [176]. At last, a soluble receptor for CTLA-4 (CTLA-4-Ig, abatacept) preventing the binding of B7 family costimulatory molecules on T cells with their counterparts CD80 and CD86 on APCs, inhibiting positive T cell costimulation has also been approved for RA treatment [176].

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a complex neurologic disease involving chronic inflammation of the central nervous system (CNS), leading to progressive demyelination of the axons of neurons. It has been initially described by the French physician Jean Martin Charcot in 1868 as “sclérose en plaques” detected post mortem in histological sections of CNS tissue of MS patients [177]. In Sweden it affects approximately 0.2 per cent of the population with increasing risk for MS development with northern latitude whereas lower disease prevalence was recorded in developing countries in Africa, Asia, South America and the Caribbean [178,179,180]. The typical symptoms of MS include balance and motoric disturbances, numbness and paralysis of extremities as well as vision and sensory disorders. Similar to RA, there is a sexual dimorphism in susceptibility to the disease with a moderate female preponderance of two women to every man affected by MS and increasing prevalence of MS in women has been described in longitudinal studies in Canada [181,182].

GENETIC HETEROGENEITY

In line with what has been observed in RA, there is strong evidence for a heritable component governing MS susceptibility, exemplified by increased prevalence of MS in twins and first-degree siblings [183,184]. Though, very recently Baranzini and coworkers closely investigated genetic, epigenetic and transcriptomic differences of a twin pair discordant for MS, for which they found no major alterations that could explain the disease discordance [185]. Mixed lymphocyte cultures provided the first clues for the identification of the HLA complex as the major genetic risk factor for MS in humans [186]. In line with the initial observations, the serotype HLA-DR2 has been associated in linkage analysis in a dose dependent manner of MS and homozygosity has been reported to increase risk as well as clinical outcome of the disease [187,188]. As DR2 constitutes a common serotype, systematic genetic reanalysis of the HLA association in humans identified the DRB1*1501 allele as the major genetic risk factor for MS development across Northern European high risk- and African populations [189,190]. In addition to the class II association, HLA class I alleles have been described to influence MS susceptibility, whereby HLA-A*0301 constitutes the major risk allele [191].

In line with what has already been outlined for RA genetics, GWAS studies in patient cohorts and controls provided a major step forward for the identification of non-HLA susceptibility factors in MS. Thereby, the receptor for the cytokine IL-7 has been pinpointed as an important genetic risk factor for MS development [192,193]. Interestingly, the IL-7R has also been a major candidate for *Eae2* locus on murine chromosome 15, identifying syntenic regions in humans and mice controlling similar neurological syndrome [83,194]. Noteworthy, with regard to this thesis is the identification of an intronic SNP in the *CD58* gene, the human ligand for CD2 encoded on chromosome 1, identifying the CD2 costimulatory pathway as a potential target in MS susceptibility. Several, independent studies by North American, Swedish and Australian groups as well as large international collaborative efforts on MS genetics convincingly showed that the SNP rs2300747 within the *CD58* gene, is associated with increased risk of developing MS [195,196,197,198,199]. Thereby, it seems that the

protective allele rs2300747G increases the expression levels of CD58 mRNA in lymphoblastic cell lines and peripheral mononuclear cells of MS patients coinciding with an enhanced function of certain regulatory T cell subsets in vitro [200]. In addition, CD2 costimulation of regulatory T cell subsets revealed decreased suppressive activity in patients with MS [201]. A genetic factor, that is also associated with MS development, underscoring the role of T cells in disease pathogenesis, is the IL-2 receptor alpha chain gene [195,202]. In addition, IL-2Rα has been associated with development of T1D, an organ specific autoimmune disease involving destruction of insulin producing β cells in pancreatic islets [203]. The exact contribution of the different alleles of IL-2Rα to disease susceptibility in T1D and MS is complex [204]. Concordantly, the *IL2* gene on murine chromosome 3 has been identified as a candidate gene in murine models of T1D and MS in a NOD x C57BL/6 cross [205,206].

ENVIRONMENTAL FACTORS

Smoking

Similarly to RA, factors like gonadal hormones, and smoking in conjunction with disease susceptible HLA alleles seem to play a role in MS gene environment interactions and were proposed as environmental risk factors for MS development in Northern European population [207]. Epidemiologic studies evaluating the risk of smoking and use of oral snuff showed that only smoking, but not oral use of moist tobacco increase the risk of developing the disease arguing that inhaled non-nicotinic compounds triggering lung irritation, but not nicotin itself, constitute a risk factor for CNS autoimmunity [208,209]. With respect to these epidemiological findings, new data obtained in the EAE model in Lewis rats, might be of importance, which indicated that autoaggressive T cell blasts are obliged to migrate via the lung in order to acquire the capacity to cross the blood brain barrier, thereby creating a proinflammatory environment predisposing for a secondary autoimmune attack [210].

Infections

Similar results obtained in epidemiologic studies of RA, a definitive proof of concept for one specific environmental trigger in MS leading to breach of immunological tolerance and breakdown of the blood brain barrier is so far lacking. However, circumstantial evidence suggests that infections may play a role in disease susceptibility of MS. In humans, T cells reactive against a myelin basic protein peptide (MBP93-105) cross react and get subsequently activated by an artificial peptide that shares residues with human herpes virus 6 (HHV6) [211]. On the other hand there is evidence in the literature suggesting that systemic infections might increase the risk of relapses in ongoing MS [212].

CURRENT TREATMENTS

Like RA, in MS corticosteroids are used to manage acute relapses of the disease. Secondly, the immunosuppressive agent named mitoxantrone otherwise used for chemotherapy of cancer is used to treat certain forms of MS, but its use is limited for high cardiotoxicity and risk of acute leukaemia [213]. The sphingosine phosphate

receptor antagonist fingolimod is used to reduce relapses and delay disabilities in patients with relapsing forms of MS. A recently approved immunomodulator that marked a major step forward in MS therapy was the introduction of interferon β (IFNβ), which is used in substitution therapy [213]. Also available for MS treatment is a non-steroidal, non-interferon, antigen specific therapy with glatiramer acetate, a scrambled peptide of four amino acids (Ala, Lys, Glu and Tyr) that are present in myelin basic protein (MBP) [213]. A humanized monoclonal antibody directed against α4 integrin (natalizumab) is available in the clinic, but currently only used for treatment refractory forms of MS, as there is a risk of developing fatal John Cunningham virus (JCV) induced progressive multifocal leukoencephalopathy (PML) [213]. A promising humanized monoclonal antibody, which entered phase III clinical trials, is daclizumab, which is directed against the α chain of the IL-2R gene (CD25) [214].

ANIMAL MODELS

In order to understand the aetiology of common complex diseases, where onset, severity and pathology are determined by interactions of multiple factors, it is absolutely necessary to study these diseases on organismal level. However, many scientific experiments are for ethical reasons not allowed in humans. Therefore, animal models, closely resembling human disease, provide a unique opportunity to identify and characterize disease-associated pathways and make therapeutic intervention possible. An advantage is that they can be induced by standardized protocols under defined environmental conditions with complete control of the genetic setup of the investigated model organism. There are numerous animal models for autoimmune diseases in rodents, which probably mimic different aspects of the human pathology in patient subsets. Naturally, it is absolutely necessary to carefully characterize and compare the existing models with regard to human pathology and establish novel ones to fully cover the pathogenesis of human disease.

EXPERIMENTAL ARTHRITIS IN RODENTS

Arthritis models in rodents can grossly be distinguished into two distinct categories. Firstly, spontaneous models of arthritis arise from genetic manipulation or spontaneous mutation of genes regulating key pathways controlling arthritis susceptibility. In contrast, arthritis resembling diseases can also be induced in genetically susceptible rodents by immunization with various substances or passive transfer of arthritogenic antibodies.

Spontaneous arthritis models

SKG mutant mouse

The SKG mouse model of RA has originally been identified by Shimon Sakaguchi's research group as a spontaneous mutation in the *Zap70* gene that arose in a colony of BALB/C mice [215]. ZAP70 constitutes a key signal transduction molecule in T cells and a point mutation altering amino acid 163 from tryptophane to cysteine subsequently attenuates TCR signalling, altering T cell selection in the thymus promoting the escape of otherwise negatively selected autoreactive, arthritogenic T cells [216]. Interestingly, the penetrance of this model is strongly dependent on the microbial status of the host, as arthritis is not observed in germ free mice [217]. Thereby, yeast derived molecular pattern recognition molecules like β glucans (zymosan) and α mannan can trigger or augment joint autoimmunity in SKG mice, whereas blockade of Dectin-1, which is one of the major receptors for yeast derived molecular patterns suppresses the disease [217,218,219]. The arthropathy in SKG mice is cytokine dependent, as arthritis promoting effects for proinflammatory cytokines like IL-1, IL-6 and TNFα were observed, whereas IL-10 acts as a strong disease suppressant [220].

Abnormal limb (ALI) mutant mice

Large phenotypic screens for abnormal limb phenotypes in mice using a forward genetic approach with ENU mutagenesis led to the identification of a point mutation,

resulting in a gain of function mutant of the phospholipase Cγ2 (*Plcg2*) gene. These mice displayed a severe arthropathy and autoimmunity characterized by B cell dependent, but T cell independent, autoantibody production based on hyperreactivity in the humoral compartment through excessive calcium entry in B cells [221]. A transient expansion of the innate immune cell compartment contributing to progressive joint inflammation was also observed [221]. An additional independently identified mutation (ALI14) in the *Plcg2* gene was shown to promote arthritis and alterations of the immune system, but also displayed metabolic and fertility abnormalities [222]. Both gain of function mutations seem to compromise the autoinhibition of enzymatic activity of PLCG2 accompanied by increased membrane interactions of the protein in this hyperactive state [223].

Ncf1 mutated mouse

Though NCF1 has originally been identified as an arthritis regulator across rodent species in induced models of arthritis, it also promotes a spontaneously occurring arthritis like syndrome in ageing mice and post partum in female mice [31,35,224]. Recent data indicated that the spontaneous arthritis development might be environmentally triggered as *Ncf1* mutated and knock out animals are hypersusceptible to infection with otherwise commensal *Staphylococcus aureus* and *xylosus* species [225].

TNFα transgenic mouse

Another spontaneous murine model for RA is based on transgenic overexpression of a modified human TNFα gene or of a mutated murine transmembrane form of this cytokine, resulting in a progressive polyarthritis with 100% penetrance [226,227]. The severity of the disease is dependent on the genetic background as DBA/1 mice are most susceptible [228]. It is strictly independent of the adaptive arm of immune system as neither deficiency in B and T cells nor reciprocal bone marrow reconstitution influences the disease development [227,229]. Instead arthritis pathogenesis in TNFα transgenics seems to critically depend on synovial fibroblasts [229]. Crucial cytokines for arthritis pathogenesis are TNFα and IL-1, but not IL-6, demonstrated by the fact that the respective antagonistic treatments or genetically engineered deficiency of the first two, but not the latter cytokine, block the development of the disease [226,230,231].

IL-1R knockout mouse

In the year 2000, Horai and colleagues reported spontaneous arthritis involving deficiency in the IL-1R antagonist and in line with previously described models the arthropathy in these mice strongly depends on the genetic background outlining the importance of genetic interactions [232,233]. The adaptive arm of the immune system is critically linked to clinical disease development, indicated by arthritis resistance in RAG and TCRα deficient mice and the fact that autoantibodies could be observed [232,234]. In line with the genetic data, it was shown that T cell differentiation induced via co-stimulatory molecules CD134, CD154 and CD28 play a prominent role for disease initiation [234]. Experiments, concerning the cytokine dependency of this disease, elucidated the importance of TNFα and T cell derived IL-17, but not IL-6, governing the initiation and progression of joint pathology [235,236]. In contrast,

TNFα, but not IL-6 or IL-17, seems to be crucial for T cell independent psoriasis like disease in these mice [237].

IL-6R transgenic mouse

Following identification of the IL-6/IL-6R axis as a crucial player in CIA, a spontaneous arthritis model involving the IL-6 receptor was described [231,238,239]. These mice bear a single point mutation in the src homology 2 domain-bearing protein tyrosine phosphatase (SHP)-2 binding site of gp130, a subunit of the IL-6R receptor, mutating tyrosine 759 to phenylalanine, which results in excess activation of STAT3 and a RA like disease in aging mice [240]. Disease development in these mice was dependent on lymphocytes, accompanied by autoantibody production and accumulation of myeloid cells and activated T cells that expand under homeostatic conditions in IL-7 dependent manner [241]. Besides, the proinflammatory cytokine IL-17, exacerbated autoimmunity and arthritis in gp130(759F/F) mice, by a positive feedback loop, involving IL-6 secretion [242].

KRN transgenic mouse

An immune complex mediated, spontaneous arthritis was accidently identified, when KRN TCR transgenic mice (recognizing the bovine RNase in H2-Ak context) on C57Bl6 background were crossed to H2-Ag7 bearing NOD mice (K/BxN) [243]. These F1 progeny develop a polyarthritis from 3 weeks of age and autoimmunity in this model is directed against the ubiquitously expressed glucose 6 phosphate isomerase (GPI) enzyme, which catalyses the second step of the glycolysis following initial phosphorylation at the C6 atom, thereby mediating isomerisation of glucose 6 phosphate to fructose 6 phosphate [244]. Antigen specific T cells in the periphery precede the onset of arthritis in these mice, but B cells are also critically required as high transient levels of arthritogenic, anti-GPI autoantibodies perpetuate arthritis in K/BxN mice [244]. Surprisingly, anti-TNFα treatment at the time of disease onset does not block the arthritis development, whereas IL-4 dependency has been indicated as reduced disease is observed in the respective knock out mice as well as those undergoing anti-IL-4 treatment [245,246]. The importance of loss of tolerance to GPI in the arthritogenic process is illustratively shown by the fact that immunization with the both human GPI protein (hGPI) and immunodominant peptides induced arthritis like disease in genetically susceptible mice [247,248,249]. Despite, the ubiquitous expression of the bona fide autoantigen, pathology in these mice is surprisingly limited to inflammation to the joint and the heart valve resulting in progressive endocarditis [250].

Induced arthritis models

Besides spontaneous models, arthritis resembling pathologies can be triggered in rats and few mouse strains by intradermal immunization with mineral oil based adjuvants like pristane or incomplete Freud's adjuvant (IFA) as well as long carbon chain triterpenes (squalene) or alkanes like (hexadecane). Other forms of arthritis can be induced by infection of rodents with *Staphylococcus*, *Mycoplasma* and *Borrelia* as well as the passive immunization with their respective cell wall components. Arthritis in rodents can also be elicited by immunization with ubiquitously expressed antigens like hGPI emulsified in an adjuvant. Immunization with cartilage components, like type IX

collagen, aggrecan, cartilage oligomeric protein and proteoglycan in IFA or complete Freud's adjuvant (CFA), stably induces acute and sometimes chronic forms of joint inflammation. A model, which was extensively used in this thesis is based on the immunization with collagen type II (CII), the major protein component of articular cartilage emulsified in a mineral oil based adjuvant, whereas another class of induced arthritis models is based on passive transfer of antibodies either by transfer of arthritogenic serum or purified monoclonal antibodies.

Pristane induced arthritis (PIA)

Pristane is a saturated polyisoprenoid alkane (2,6,10,14 tetramethylpentadecane) that can be isolated from the liver of sharks [251]. The monosaturated diterpenyl alcohol phytol occurring as an ester in chlorophyll is the likely ubiquitous source for pristane in nature [252]. Interestingly, phytol has been found to increase oxidative burst in vivo and thereby corrected the genetic effect on PIA of a loss of function mutation in Ncf1 in the dark agouti (DA) rat [253]. A single intradermal injection of pristane induces a severe polyarthritis approximately 10 to 14 days after initial immunization in genetically susceptible DA rats that shares many macroscopic and histological features with human RA [254]. The peak of the acute arthropathy is usually reached after 3 weeks, and clinical disease subsides thereafter. Eventually, rats will progress into a chronic phase of arthritis that may last for several months. Though, the pathological mechanisms that govern PIA are far from being understood it has been suggested that T cells play a prominent role in susceptibility to the disease. This is illustrated by the fact that both depletion of αβ T cells or the TH1 associated proinflammatory cytokines TNFα or IFNγ ameliorate clinical disease in preventive and therapeutic treatment regimen [254,255]. Additional evidence for the prominent role of T cells in PIA pathogenesis stems from the fact the disease can be adoptively transferred using adjuvant primed, concavalin A (ConA) restimulated CD4, but not CD8 αβ T cells that were isolated from spleen or lymph nodes [255,256]. In line with what has been found in PIA, there is a strong dependency for TH1 cytokines secreted by donor CD4 αβ T cells, as antagonistic treatment with TNFα or IFNγ neutralizing antibodies in the recipients alleviates clinical disease [255].

Accompanying evidence for the role of T cells and the adaptive immune system in PIA arose from various mapping studies using DAxE3 crosses, which consequently yielded high association signals in the MHC region on rat chromosome 20 [257,258]. This result is somewhat surprising as in PIA as well as in other adjuvant induced arthritis models elicited by immunization with squalene, IFA or avridine, no administration of an antigen is involved [259]. Blocking experiments with antibodies directed against rat MHCII molecules confirmed the notion that adjuvant primed, ConA stimulated T cells critically depend on MHCII mediated activation in order to retain their arthritogenic potential [255]. Forward genetic studies in the laboratory rat identified an aforementioned coding non-synonymous polymorphism in the *Ncf1* gene underlying the *Pia4* susceptibility locus that is believed to govern the development of arthritogenic T cells [31].

Despite several years of research the nature of the postulated autoantigens for PIA still remains elusive, though recently the heterogeneous nuclear riboprotein (hnRNP)-A2 as well as collagen type XI (CXI) have been proposed as candidates for different phases of the disease [260,261]. The autoantigenicity of hnRNPs seems to critically depend on the ribonucleic acid component as nuclease treatment abrogates adoptive arthritis

transfer with hnRNP restimulated splenocytes [262]. Even though autoantibodies like rheumatoid factor (RF) can be found during PIA, it is generally not considered an arthritis model that is majorly governed by autoaggressive B cells, as neither transfer of serum, nor purified immunoglobulins (Ig) from arthritic rats, do elicit similar disease in naïve hosts [254,256]. In paper 1, we have used PIA in order to investigate the influence of the genetic background on the ability to detect the strongest described non-MHC loci in the laboratory rat.

Collagen induced arthritis (CIA)

With regard to this thesis the most important arthritis model has been CIA. Despite the fact that this model can be induced with immunization of autologous collagen (CII) in some genetically susceptible rodent strains that are sensitive to arthritis, it is mostly elicited by intradermal immunization with heterologous collagen emulsified in CFA or IFA [263]. CIA was initially described 35 years ago by Trentham et al. and can be elicited across species not only in rodents like rats, mice and guinea pigs, but also in non-human primates [264]. During disease development in rodents the initial immunization is followed by a severe reactive polyarthritis 3 to 5 weeks, which may resolve after three to four months or depending on the genetic background can become chronic and even show relapses similar to the human situation. Clinical CIA closely resembles human RA, exhibiting erythema, synovial hyperplasia, influx of inflammatory cells like macrophages and neutrophils, pannus formation, cartilage destruction as well as bone erosions. In line with what has been observed in human RA the major genetic factor determining susceptibility to CIA is the MHCII. In mice, H2-q and H2-r and possibly H2-b haplotypes confer susceptibility to CIA, induced with heterologous collagen emulsified in adjuvant [265,266]. A significant advance for the understanding of the aetiology of the disease marked the positional identification of the *Aβq* gene that is responsible for arthritis susceptibility in CIA, whereas mouse strains expressing the closely related *Aβp* gene are completely resistant to arthritis induction [267]. Proof of principle has been obtained by transgenic complementation studies that altered the critical amino acid in Aβp resembling Aβq, which in turn rendered these mice susceptible to CIA induction [268]. One of the possible explanations is a higher affinity for the immunodominant T cell epitope of collagen (CII256-270) of Aβq compared to Aβp [269]. Rat collagen differs in a single amino acid (Asp266Glu) from the mouse peptide (CII256-270), and differences in the affinity between these two peptides may explain the increase in arthritis susceptibility after induction of CIA with rat compared to mouse CII in H2-q bearing mice [270]. Interestingly, experiments using humanized HLA-DR4 expressing transgenic mice identified the glycosylated immunodominant epitope CII263-270 that is also recognized in human RA patients [271]. Consequently, various humanized transgenic mouse models bearing human HLA-DR1, HLA-DR4 and HLA-DQ8 susceptibility display increased CIA susceptibility upon CII immunization, whereas transgenics for human resistance MHC alleles conferred protection in genetically susceptible mouse strains [272,273,274].

CIA is generally considered an autoimmune model that depends on the cooperation of B and αβ T cells as the respective knock out models proved to be resistant to its induction [275,276]. The role of T cells is further outlined by the fact that knock out mice for T cell associated costimulatory molecules like CD28 or ICOS are also resistant to CIA development [277,278]. In line with this notion, knock out mice deficient in murine B cell coreceptors CD19/CD21 also fail to develop CIA, even

though they mount comparable antibody responses to heterologous and autologous CII, indicating diminished B cell help for autoreactive T cells [279]. The cytokine response during CIA has been carefully characterized in numerous studies, which laid the foundation for the development of antagonistic cytokine treatment for human RA. Neutralization of TNFα using antibodies was shown to reduce joint inflammation and cartilage destruction in murine CIA, marking a significant milestone for the establishment of anti TNFα therapy [280]. Classically, CIA has been considered a TH1 type of disease even though therapeutic targeting of the key cytokine INFγ by antibodies yielded conflicting results, depending on route of administration and time points during disease, when treatment was initiated [281,282,283]. In addition, genetic ablation of IFNγ or its receptors resulted in partially opposite phenotypes, and both genetic background related effects as well as the protocols used for initiation of CIA might have contributed to the discrepancies in the data [284,285,286,287].
A novel proinflammatory T cell subset, that has been recently discovered and seems to play an important role in CIA susceptibility, are TH17 cells illustrated by the fact that neutralization of IL-17 as well as the respective knock out mice prove to display reduced CIA severity [288,289]. On the other hand TH2 cytokine ablation by the aforementioned methods exacerbated disease in case of IL-10, whereas supplementation therapy with exogenous recombinant IL-10 ameliorated the disease [290,291]. Experiments using both the CIA and the EAE model, evaluating the role of the humoral part of adaptive immunity proposed a regulatory role for IL-10 producing B cells in these diseases [292,293]. The role of IL-4 in CIA remains controversial with probably divergent possibly antagonistic roles for this cytokine during the course of the disease [294,295,296].
Furthermore, B cells as source of autoantibodies are an important player in CIA. A lack of negative selection of B cells can be inferred from the robust, specific immunoglobulin G (IgG) response directed against CII, which is readily detectable prior to disease onset and maintained during CIA development [297]. Moreover, the triple helical structure of CII seems to play a crucial role for CIA induction, as denatured CII fails to induce a sufficient antibody response to cause CIA [298].

Collagen antibody induced arthritis (CAIA)
In CIA, anti-CII antibodies are a driving force in disease pathogenesis, illustrated by the fact that passive transfer of anti-CII antibodies from CIA immunised mice into naïve hosts elicits a similar disease [299,300]. Markedly, the same results have been obtained across species after transfer of anti-CII antibody containing Ig fraction of a RA patient into mice [301]. The pathogenic B cell response during CIA development was carefully characterized and B cell hybridomas against six major epitopes (M2139-J1, CIIC1-$C1^{III}$, UL1-U1, CIIC2-D3, CIIF4-F4, CIIE8-E8) were described, of which some were used for transfer experiments presented in paper 4 and 5 [302]. These epitopes are also recognized in human RA patients, reacting against modified cartilage, further underscoring the relevance of CIA as a relevant model for human RA [303,304]. Monoclonal antibodies derived from these hybridomas were carefully characterized for their arthritogenicity and found to induce arthritis upon single injection as well as in different combinations [305]. Several studies demonstrated that pathogenic antibodies against CII alter cartilage ultrastructure leading to loss of fibrillious CII and induce chondrocyte death in vitro, probably explaining why antibody transfer alone is able to elicit arthritis [306,307].

A few days after initial antibody transfer the disease is usually enhanced with an adjuvant like lipopolysaccharide (LPS) boosting the innate immune system leading to an acute form of self limiting arthritis, resembling actively induced CIA [302]. Studies, undertaken to evaluate the importance of TLRs in this process, yielded that TLR4 signalling plays a prominent role in mediating LPS induced aggravation of CAIA [308]. On the other hand ROS dependent exacerbation of CAIA by LPS seems to rather operate via TLR2 [309]. With regard to this thesis the downstream events after application of different environmental triggers, the genetic factors involved and their consequences for disease pathology are subject of paper number 5. The adaptive immune system seems to be dispensable for CAIA illustrated by the fact that the MHC region did not exert any influence on CAIA susceptibility and mice deficient in either the B or the T cell compartment do not show any significant differences in clinical disease [299,302,310]. However, double deficient mice develop less clinical CAIA arguing that either component of the adaptive immune system may exert a regulatory role in this scenario [310].

It is generally accepted that the innate arm of the immune system is the major player determining the pathogenesis of CAIA. The importance of granulocytic cells such as neutrophils is underscored by the fact that mice with genetic deficiencies affecting neutrophil numbers as well as treatment with a neutrophil depleting antibody protect from CAIA development [302,311]. Of importance, for the induction of CAIA, are Fc receptors, initially hypothesized after common γ chain knock out mice have been found to be resistant to arthritis induction with anti-CII antibodies [312]. Several studies using forward and reverse genetic approaches confirmed that the Fc gene cluster on murine chromosome 1 is involved in arthritis susceptibility in both CIA and CAIA [149,312,313]. However, the exact contribution of both activating- and the inhibitory Fc receptors to autoantibody mediated pathologies in the mouse remain not entirely understood, as early studies involving gene targeted mice for particular Fc receptors were hampered by the use of genetically impure, chimeric mice, leading to confusion within the literature [314,315]. Pathogenic antibodies against CII do not only crosslink Fc receptors on immune cells, thereby contributing to joint inflammation, but are also able to fix complement, depending on their isotype. The disease modulating effect of the complement system in immune complex mediated diseases such as CAIA may happen at different levels, for example by promotion of phagocytosis of opsonized particles, facilitation of immune complex clearance, lysis of target (host) cells via the membrane attack complex and attraction of inflammatory cells with anaphylatoxins [316].

K/BxN serum transfer induced arthritis

As previously outlined K/BxN transgenic mice, that recognize GPI develop a spontaneous arthritis like syndrome, characterized by high titres of anti-GPI antibodies of predominantly γ1 isotype. The pathogenicity of these autoantibodies is illustrated by the fact that both serum transfer from K/BxN mice as well as transfer of purified anti-GPI antibodies elicits arthritis in naïve hosts [147]. This disease proved to be largely independent of the adaptive immune system as neither the MHC is a susceptibility factor, nor are B and T cells an absolute requirement for disease development, based on experiments in immunodeficient RAG1 knock out mice [317]. However, a regulatory role of proinflammatory TH17 cells was proposed by the Mathis/Benoist group, who initially identified this model [318]. An environmental effect on disease susceptibility

in this setting cannot be excluded, as microbial colonization of mice with TH17 promoting segmented filamentous bacteria promotes the disease [319].
More established is the role of the innate arm of the immune system in the development of K/BxN serum transfer arthritis. Both neutrophil- and macrophage depletion with anti-GR1 (RB6) antibodies or clodronate liposomes respectively, render mice resistant to K/BxN serum induced arthritis, whereas reconstitution experiments in the latter case restored the disease susceptibility [320,321,322]. Additional evidence for the role of macrophages comes from K/BxN serum induced arthritis resistant osteopetrotic op/op mice, which carry a spontaneous nonsense mutation in the *Csf1* gene and therefore lack cells of the monocyte/macrophage lineage [323,324]. A series of experiments suggested the importance of mast cells for disease initiation, as mast cell deficient mice with mutations in either *Kit* or its receptor, prove to be resistant to disease induction, which in turn can be broken by mast cell engraftment [325]. However, this notion was challenged with the introduction of highly specific CRE mediated ablation of mast cells in mice, which did not display altered disease susceptibility upon K/BxN serum transfer [326]. Additionally, a disease-modifying role for platelet microparticles has been suggested [327].
On the cytokine level, quintessential roles for IL-1β and TNFα in disease development were proposed based on studies in knock out mice for the respective cytokines, their receptors as well as conventional QTL mapping and comparative analysis of cytokine secretion in different low and high responder mouse strains [328,329,330]. On the other hand the cytokines IL-6 and IL-4 prove to be dispensable, as the respective gene targeted mice did not show altered disease susceptibility [246,328]. Sequential analysis of the events that lead to neutrophil infiltration during K/BxN serum transfer stressed the importance of Fc receptor genes, complement factor C5a and neutrophil adhesion molecules for the development of this disease [321]. The importance of Fc receptors is further outlined by resistance to K/BxN arthritis in knock out mice for the common γ chain [331]. Thereby, the activating Fc gamma receptor III seems to play a prominent role, whereas reports for the solely inhibitory Fc gamma receptor IIB remain controversial [331,332]. Studies with different deficiencies in complement pathways outlined the importance of the alternative, but not the classical or lectin binding pathway of complement activation, with particular importance for the anaphylatoxin C5a for the recruitment of neutrophils [331,333]. A hypothesis free approach mapping clinical disease severity of K/BxN serum transfer, using HS mice, confirmed a QTL at the *Hc* gene locus and identified *Ptgs1* (*Cox1*) as a likely candidate gene involved in severity of joint disease [93].

EXPERIMENTAL MULTIPLE SCLEROSIS MODELS

As for arthritis, animal models for MS can be distinguished into spontaneous and induced ones. A distinct category of MS mimicking animal models in rodents is mediated by adoptive transfer of pathogenic T cells. In addition there have been descriptions of mouse models like quaking viable (*qkv*) mice that display neurodegeneration and dysmyelination, but the phenotype did not appear to be lymphocyte dependent [334,335].

Spontaneous multiple sclerosis models

Spontaneous EAE like disease has been observed in several transgenic mouse strains, which bear transgenic TCRs specific for immunodominant epitopes of brain constituent proteins like myelin basic protein (MBP) or proteolipid protein (PLP) [336,337,338]. However, the spontaneous disease development is clearly linked to the environment, since it does not develop in specific pathogen free environment. Thereby, the activation state of APCs determined by the exposure to microbial products is critical for initiation of CNS pathology in these transgenic mice [339]. Another transgenic mouse line bearing a TCR specific for myelin oligodendrocyte glycoprotein (MOG) amino acid 35-55 peptide spontaneously developed optic neuritis with an incidence of 30%, but did not progress to full blown encephalomyelitis like disease [340]. Significant excitement arose, when two groups back to back reported the generation of a spontaneous mouse model for neurodegenerative disease. As they crossed these MOG35-55 specific TCR transgenic mice to transgenic mice bearing Ig heavy chain knock in replacing the J region by the recombined heavy chain of a monoclonal antibody binding to a conformational epitope on MOG in the context of I-Ab, more than half of these double transgenic mice developed distinct inflammatory lesions in the spinal cord and optical nerves, but not in the brain, closely resembling Devic disease in humans [341,342]. In addition, the development of a distinct MOG specific TCR recognizing peptide 92-106 in the context of I-As and the previously outlined heavy chain knock in produced a spontaneous relapsing remitting EAE like syndrome, when this double transgenic mouse line was backcrossed to the SJL/J background [343]. Spontaneous EAE like syndrome was also observed with varying incidence in case of a few lines transgenic for human HLA-DR2 MS associated alleles in conjunction with transgenic TCRs recognizing MBP, axillary molecules facilitating MHC/TCR interaction and in some cases mutations preventing receptor rearrangement [344,345,346].

Multiple sclerosis models based on adoptive cell transfer

A major difference to CIA induced with articular CII is that EAE can be elicited by adoptive transfer of encephalitogenic T cells into naïve hosts. Initially, this model was established by transfer of lymph node cells from rats immunized with spinal cord homogenate [347]. Later it was shown that it relies on transfer of MHCII restricted T cells [348,349]. The most common models involve transfer of $CD4^+$ T cells, however transfer of MBP specific $CD8^+$ T cells from *shiverer* mice, genetically deficient in MBP, was also proposed to elicit clinical EAE [350,351]. Similarly, what has been found for actively induced EAE, passively transferred disease was initially perceived as a TH1 like disease, since cytokine profiling of transferred T cells indicated IFNγ as the majorly produced cytokine and the main pathogenic driver CNS pathology [352]. However, with the identification of a novel proinflammatory T cell subset, IL-17 producing TH17 cells, which were also shown to regulate experimental neuroinflammation, this concept has been partially reevaluated [353,354,355]. Thereby, both the TH1 and TH17 differentiated T cells seem to be encephalitogenic and thereby may complement each other in the pathogenic process to facilitate lesion development in distinct regions of the CNS [356].

Induced multiple sclerosis models

The most commonly used animal model for multiple sclerosis is experimentally induced autoimmune encephalomyelitis (EAE), which was accidently discovered by Louis Pasteur in 1893 as a complication in his search for an antirabies vaccine, where immunization of volunteers with dried brain preparations of rabies infected rabbits led to severe paralysis symptoms and incidence estimates reached from 1 per 3000 to 35000 vaccinations for this complication [357]. Only in the 1930s it has been recognized by Rivers and coworkers that minor myelin proteins included in central nervous tissue preparations of virus infected encephalitis patients and not the virus per se transferred encephalitis like disease to non human primates [357].
A demyelinating disease in genetically susceptible rodents can be induced with both major as well as minor protein components of the myelin sheath like proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) or their immunodominant epitopes emulsified in IFA or CFA. A surprising result came when one report clearly indicated induction of MOG induced EAE, in MOG deficient mice expressing a transgenic TCR specific for MOG 35-55, attributing it to cross reactivity to a neurofilament M peptide that shared essential TCR contact points leading to an autoimmune attack on the CNS [358]. Generally, pertussis toxin (PTX) is given as an additional environmental stimulus during EAE induction believed to enhance the breakdown of the blood brain barrier (BBB) initiating disease pathology, though an immunomodulatory role altering the immune response to CNS autoantigens has also been proposed [359,360]. Depending on the genetic background of the mice and the immunization protocol used, the disease course may vary and both acute forms of EAE and more chronic relapsing pathogenesis were described [361]. With regard to this thesis the most important model is the MBP peptide 89-101 induced EAE, which produces a chronic progressing and/or relapsing disease in B10.RIII mice starting seven to sixteen days after initial immunization with 250 µg myelin basic protein peptide emulsified in *Mycobacterium tuberculosis* H37RA strain containing CFA [362].
EAE is classically considered a T cell mediated autoimmune model and similarly to CIA knock out mice for T cell associated costimulatory molecule CD28 are relatively protected from EAE induction and progression of the disease [363,364]. However, ICOS deficiency renders mice particularly sensitive to EAE and CD28 independent EAE induction has also been reported [278,365]. EAE has been perceived as a TH1 type of disease and genetic ablation of INFγ or its receptors and neutralization of IFNγ promoted the disease in murine models [366,367,368]. Moreover, anti-TNFα treatment with antagonistic antibodies found protective in animal models of demyelination [369]. However, discordant results in human clinical trials with supplementation of IFNγ and neutralizing TNF agents, led to a rigorous scientific debate about the usefulness of EAE as a relevant animal model in humans [370,371]. Additional confusion in the literature arose from reports that clearly showed that genetic ablation of IL-12p35 did not abrogate clinical EAE even though IL-12 is considered a signature cytokine for TH1 T cell differentiation [372]. The discovery of a novel T cell subset termed TH17, due to production of IL-17a and initially derived by in vitro differentiation using IL-6 and TGFβ, provided novel clues for the understanding of EAE pathogenesis as these cells were shown to be critical for initiation of EAE elicited by T cell transfer [373,374,375]. The identification of the heterologous cytokine IL-23, a member of the IL-12 cytokine family, sharing the IL-12p40 subunit with the eponymous cytokine provided a key for

the understanding of the aforementioned discrepancies in CNS pathology during EAE development associated with IL-12 deficiency [376,377]. Paradoxically, therapeutic targeting of IL-17 with antagonistic antibodies as well as transgenic overexpression does not alter susceptibility to murine EAE dramatically, arguing for redundant roles of this cytokine in the disease process [378,379]. Instead, Codarri and colleagues proposed GM-CSF as a non-redundant cytokine mediating CNS pathology in the EAE model [380].

Although, EAE is generally not considered to be an autoimmune model that is majorly governed by autoantibodies, a role for B cells in disease susceptibility has been suggested. In fact, EAE experiments by Janeway and others using B cell deficient mice first indicated a regulatory role for these cells in the disease process [381]. Additional circumstantial evidence for a role of B cells in MS stems from the fact that oligoclonal bands of immunoglobulins are a diagnostic criterium for MS in humans and transfer of anti-MOG serum has been shown to lead to an exacerbation of MOG induced EAE in mice [382]. Very recent experiments indicated B cell derived IL-6 as an important pathogenic player, by which B lymphocytes drive EAE pathogenesis [383].

PRESENT INVESTIGATIONS

PAPER I

In this study we assessed the influence of the genetic background on the ability to detect the strongest previously described non-MHC loci for PIA. To do so we transferred congenic fragments containing three non-MHC QTL in rat experimental arthritis from the susceptible DA rat into the disease-resistant E3 strain. We found that a disease promoting, DA derived, congenic fragment containing the arthritis regulating *Ncf1* gene did not break resistance in the E3 genetic background, but enhanced autoimmune B cell responses. The introgression of the disease promoting loci *Pia5* and *Pia7*, also located on rat chromosome 4, ultimately broke the resistance and led to arthritis development in the E3 rat. To assess the genetic effect of the MHC, an E3 derived MHC congenic strain was transferred into the susceptible DA background and various F(1) and F(2) hybrids were generated and monitored for arthritis susceptibility. Our results show that the E3 derived MHC locus introgressed onto DA background enhanced arthritis only when it interacted with E3 genes on a mixed background. We also assessed epigenetic effects using F1 hybrids using yin yang crosses. We found a small but significant effect on arthritis susceptibility in progeny of DA mothers, however since the effect was not transient over the course of the experiment, we concluded that epigenetic effects mediated by X chromosomes and mitochondria do not play a major role in PIA susceptibility in this setting. Similarly, we did not detect a major influence of the Y chromosome on the development of PIA, as male rats derived from our experimental cross showed almost identical arthritis. The findings in these congenic lines confirm the existence of 3 major QTLs that regulate the severity of arthritis and are sufficient to induce the transformation of a completely arthritis-resistant rat strain into an arthritis-susceptible strain. This study also reveals a dramatic difference in the arthritis-regulatory potential of the rat MHC depending on genetic background, suggesting that strong epistatic interactions occur between MHC and non-MHC genes.

PAPER II

Precise definition of the pathways involved in disease pathogenesis posts a major obstacle for therapeutic intervention in common complex autoimmune diseases. Identification of genetic factors in model organisms holds significant promise for an increased understanding of disease pathology and thereby may aid rationale drug development. In the present study we aimed to utilize HS mice, a novel tool for genome wide mapping in rodents, in order to map CIA, the most frequently used animal model for RA in humans. We used mice derived from the Northport stock with genomic contributions from eight different mouse strains, and crossed them with the arthritis susceptible C57BL10/Q (BQ) strain. We selected H2-q homozygotes from the F2 generation and further intercrossed them for an additional generation, to generate a large F3 population that could be used to study the genetic architecture of CIA. Overall, we immunized and phenotyped a cohort of 1764 mice for arthritis development. In order to gain maximum information of our collaborative effort, we additionally analyzed approximately 100 biochemical, metabolical and haematological phenotypes.

All mice were genotyped and for the bioinformatic analysis we used the HAPPY algorithm. We thereby, found 26 loci for CIA, of which 18 were novel. We fine mapped a number of previously described loci, with particular importance for the *Cia2* (C5) locus on murine chromosome 2 and the Fc gamma receptor locus (Fcgr) on chromosome 1. Our study outlines the usefulness of heterogeneous stock derived mouse cohorts for mapping of quantitative traits. We provided one of the most comprehensive genetic analyses of CIA, which serves as an excellent resource for further fine mapping of existing CIA loci.

PAPER III

In this study expanded our previous genome wide analysis in a HS derived mouse cohort, to also analyse QTLs controlling autoantibodies towards CII, ACPA and RF a hallmark of human RA. All antibody concentrations were measured by standard ELISA, and linkage analysis was performed using a linear regression based method. We thereby set out to identify loci controlling formation of anti-CII antibodies of different IgG isotypes (IgG1, IgG3) and antibodies to major CII epitopes (C1, J1, U1), which are also recognized in a subset of RA patients strengthening the role of CIA as a relevant animal model for RA. Additionally, we investigated the humoral immune response towards a citrullinated CII peptide (CitC1) and RF. Both ACPA and RF are part of the revised ACR and EULAR criteria to classify RA diagnosis in humans. We found that the anti-CII antibody-, ACPA- and RF responses were all controlled by distinct genes, with particular importance for the immunoglobulin heavy chain (Ig_vH) locus, the Fc gamma receptor (Fcgr) locus and the complement component 1 (C1q) locus. Our study demonstrated that not only anti-CII antibodies, but interestingly also ACPA and RF are associated with arthritis development in mice. We reported the to date most comprehensive genetic analysis of clinically relevant antibody response during CIA development using a HS derived cohort of mice. Our data indicates complex genetic control anti-CII antibodies of different isotypes, ACPA and RF, which are all governed by unique and few shared loci. Clearly, our results argue in favour of the idea that antibody specificity is also determined by non-MHC genes, with particular importance for the Ig_vH locus. In conclusion, our study demonstrates the potential of HS stock mice for identification of genes associated with complex phenotypes like antibody formation.

PAPER IV

We have previously identified a locus (*Cia21*) on murine chromosome 3 that predominantly affects CIA severity in female mice. Gender differences in incidence of human autoimmune diseases such as MS and RA are well documented in the literature, whereas the aetiology of this phenomenon remains largely elusive. Therefore, we decided to systematically investigate the role of sexual dimorphism in a congenic strain harbouring the *Cia21* locus. To isolate the underlying chromosomal region, we performed traditional congenic mapping down to a single gene congenic mouse line that revealed relative protection in T cell mediated models of autoimmunity exclusively in female mice. In order to sought out the influence of female gonadal hormones we used castrated mice, which showed a complete ablation of the protective effect of *Cia21* on EAE susceptibility, arguing for a direct role of female sex hormones in

susceptibility to autoimmune disease in our model. Thereafter, we demonstrated that the costimulatory molecule CD2, which is located outside of the original congenic fragment, is differentially expressed in vivo in the thymus between congenic and wild type animals. We then carefully characterized of estrogen receptor related (ERR) binding sites encoded within the congenic fragment and indicated by previous chromatin immunoprecipitation experiments. Targeted resequencing revealed several SNPs between the B10.RIII and RIIIS/J derived congenic mouse strain that alter these transcription factor binding sites, one of which in fact led to an alteration of a oestrogen response element (ERE). With our study, we successfully positionally identified a region that controls genetic susceptibility to complex autoimmunity. Our study for the first time identifies CD2, a protein involved in T cell costimulation, as a risk factor for sexual dimorphism in joint and CNS tissue autoimmunity in mice. At last these results underscore the importance of transcription factor binding sites, enhancer and silencer elements that are not directly associated with a particular gene for the control of expression QTLs that may prove essential for our understanding of complex trait biology.

PAPER V

Infections with pathogens have long been suspected to initiate or perpetuate the autoimmune process in complex disease pathologies. So far, epidemiological studies aiming to associate human RA with a specific infection, proposing a certain infectious organism as an environmental trigger for the disease leading to breach of tolerance to self and subsequent autoimmunity, have been unsuccessful. We investigated this issue using the previously described partial advanced intercross between *Eae3* and *Eae2* bicongenic mice, by which several sub QTLs of the *Cia5* locus on murine chromosome 3 have been identified; one of them (*Cia22*) affects disease severity during the late phase of CIA. We dissected the original *Cia22* region using recombination assisted breeding protocols and found reduced CIA development in a 2.38 Mb RIIIS/J derived fragment encompassing the murine chitinase like gene locus. Moreover, this congenic showed a clear adjuvant specific protective effect on CAIA susceptibility, indicating distinct pathways by which bacterial and yeast derived adjuvant perpetuate chronic inflammation of the joints. Experiments using mice deficient in the *Ncf1* gene, highlighted the importance of NOX2 derived ROS producing cells being critical for the arthritoprotective effect on CAIA development mediated by *Cia22*. By combining exome sequencing and gene expression analysis of closely related murine chitinase like genes, we identified the *Chi3l3* (*Ym1*) gene as a likely candidate for *Cia22*, with its expression virtually absent in RIIIS/J derived congenic mice. Targeted resequencing of the promoter of *Chi3l3* identified several candidate SNPs that may influence mRNA and subsequently protein expression by alteration of essential transcription factor or RNA polymerase binding sites. Overall, our study identified CHI3l3 as a novel adjuvant specific regulator of experimental arthritis in mice, thereby indicating a previously unrecognized immunomodulatory mechanism of pathogen derived pattern recognition molecules altering susceptibility to chronic inflammatory joint disease mediated by ROS producing granulocytic cells. It underscores the importance of careful characterization of inflammatory pathways involving different pattern recognition receptors that in turn may hold significant promise for future for therapeutic intervention in chronic inflammatory diseases.

CONCLUDING REMARKS

With the work presented in this thesis, we investigated the genetic control of autoimmunity in experimental rodent models of RA and MS. Animal models that can be run under stable environmental conditions with potentially unlimited cohort size provide a unique opportunity to unravel genetic factors controlling the heritability of complex autoimmune traits. We examined the genetic effects of previously identified PIA QTLs in an otherwise resistant genetic background of the laboratory rat, thereby outlining the importance of genetic interactions for the susceptibility to experimental artritis. We secondly introduced HS mice as a novel tool for improved QTL mapping for CIA and the associated autoantibody response. Lastly, we identified *Cd2* and *Chi3l3* as novel candidate genes for the arthritis regulating QTLs *Cia21* and *Cia22* respectively. Despite these apparent successes there are several open questions that yet remain unresolved as the hypothesis free, positional cloning of polymorphisms underlying autoimmune QTLs using either association studies in HS mice or locus based congenic dissection prove more difficult than initially anticipated.

Unfortunately, our HS study using an inbred outbred rodent population did not yield conclusive results on the positional identification of previously implicated polymorphisms in candidate genes like *Hc* (*C5*). This may be explained by either an over estimation of genetic effect of a given mutation on a complex phenotype like arthritis, an underestimation of the complex interactions between a certain mutation and the genetic background with many different alleles segregating in our HS cohort or thirdly the low frequency of susceptibility allele. The introduction of affordable genotyping technology and the availability of large patient cohorts for autoimmune disease, which resulted in an explosion of data concerning the identification of genetic factors for common complex autoimmune disease involving chronic inflammation, authors argued against the necessity for gene identification in model systems. However, one should keep in mind that the GWAS approach faces problems and therefore enormous sample sizes for the identification of statistically significant polymorphisms are needed. In addition, these studies often yield the most significant hits in intergenic regions that are not associated with any particular gene or they identify a series of significantly associated SNPs that are in linkage disequilibrium and stretch over several genes. Thus, GWAS rarely provide mechanistic understanding of how a particular polymorphism invokes on a pathway that ultimately changes genetic susceptibility to a particular disease. We face a similar problem in our *Cia21* and *Cia22* project, but with the mouse genome being completely accessible to genomic manipulation it is theoretically possible to experimentally validate our candidate genes. Therefore, the dissection of the complex genetics of quantitative traits in model organisms will continue to aid our understanding of the genetic heritability of these traits in humans. It also underscores the importance for the continuous development of new animal models to accurately mimicking the disease to identify novel genes and pathways associated with complex autoimmunity in humans and ultimately improve therapeutic options for affected patients.

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