FROM DISEASE TO THE GENE - Identification of arthritis-regulating loci in rats

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FROM DISEASE TO THE GENE – IDENTIFICATION OF ARTHRITIS-REGULATING LOCI IN RATS

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LUND UNIVERSITY
Faculty of Medicine

The dissertation will be held on Friday the 13th of February 2009, 13.00
at the Biomedical Center, Rune Grubb lecture hall.

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Abstract

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The first part of this thesis is summarizing two major problems we have faced in the past years. In the first study we investigated the genetic setup and the response towards various arthritis models of two DA rat substrains. We detected several genetic and phenotypic differences, suggesting that one of the substrains had been genetically contaminated from another rat strain. The second study is based on the observation that a spontaneous mutation in our DA rat colony results in decreased arthritis susceptibility in the DA rats. We subsequently isolated the mutation in a new substrain of DA rats, called DACP, and using genetic linkage analysis we located the mutation and identified a new quantitative trait locus (QTL) for pristane-induced arthritis (PIA) at chromosome 9, Pia27. In the second part of this thesis, we were utilizing the traditional congenic rat strain strategy in the identification and characterization of arthritis regulating loci. The third paper investigated the influence of different genetic backgrounds on the detection of previously reported loci for PIA. We found that the arthritis-regulating gene Ncf1 as well as the major histo-compatibility complex (MHC) are silent in certain genetic backgrounds, while they can be detected in other genetic setups. The fourth study describes the positional cloning of the immunoglobulin lambda light chain (Igl) locus as one locus controlling rheumatoid factor (RF) production in rats. In addition, evidence suggests that this genetic region may be associated with Ovalbumin-induced airway inflammation, an animal model for allergic bronchitis or asthma. Identification of genes involved in complex disorders such as RA will be extremely valuable in understanding disease regulating mechanisms as well as improve diagnosis and identification of specific targets for therapeutic drugs. However, the findings in this thesis demonstrate that mapping those genes is a complex and challenging process and involving various problems, such as genetic variability and complex genetic interactions.

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Identification of genes involved in complex disorders such as RA will be extremely valuable in understanding disease regulating mechanisms as well as improve diagnosis and identification of specific targets for therapeutic drugs. However, the findings in this thesis demonstrate that mapping those genes is a complex and challenging process and involving various problems, such as genetic variability and complex genetic interactions.

LIST OF PUBLICATIONS

This thesis is based on the following articles that are referred to in the text by their Roman numerals

I. **DA rats from two colonies differ genetically and in their arthritis susceptibility**

Carola Rintisch, Rikard Holmdahl

Mamm Genome. 2008 Jun;19(6):420-8

II. **A spontaneous mutation at rat chromosome 9 protects DACP rats from experimentally induced arthritis**

Carola Rintisch*, Jonatan Tuncel*, Ulrika Norin, Rikard Holmdahl

Manuscript

*These authors contributed equally to this work

III. **Detection of arthritis susceptibility loci *Ncf1* and the major histocompatibility complex region in rats depends on the genetic background**

Rintisch C, Förster M, Holmdahl R.

Manuscript accepted in Arthritis & Rheumatism

IV. **Positional cloning of the Igl genes controlling rheumatoid factor production and allergic bronchitis in rats**

Rintisch C, Ameri J, Olofsson P, Luthman H, Holmdahl R.

Proc Natl Acad Sci U S A. 2008 Sep 16;105(37):14005-10.

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LIST OF ABBREVIATIONS

AIL	Advanced intercross line
APC	Antigen presenting cell
CII	Type II collagen
CCP	Cyclic citrullinated peptide
CIA	Collagen-induced arthritis
Con A	Concanavalin A
cM	Centi Morgan
HLA	Human leukocyte antigen
HS	Heterogeneous stock
IFA	Incomplete Freund's adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LOD	Logarithm of odds
Mb	Megabase (1 million nucleotides)
MHC	Major histocompatibility complex
NADPH	Nicotinamide adenine dinucleotide phosphate
OIA	Oil-induced arthritis
PIA	Pristane-induced arthritis
QTL	Quantitative trait locus
RA	Rheumatoid arthritis
RI	Recombinant inbred
RF	Rheumatoid factors
RNO	Rattus norvegicus
SE	Shared epitope
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SSLP	Simple sequence length polymorphism
TCR	T cell receptor
Th	T helper cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell

INTRODUCTION

THE IMMUNE SYSTEM

Every day we come into contact with a large variety of microorganisms such as viruses, bacteria and fungi. Many of these, known as pathogens, are capable of causing diseases. Our immune system is the collection of natural defence mechanisms to fight back those foreign invaders. The immune system consists of a complex network of specialized cells and molecules, each having specific tasks assigned to them.

The innate immune system is regarded as the first line of defence against invading pathogens. It consists of cells and molecules that defend the host through a series of non-specific mechanisms, such as surface barriers (skin and mucous membranes), the complement system and phagocytic cells [1]. These cells bind to antigens using pattern-recognition receptors (PRRs), such as the Toll-like receptor family of proteins, which are specific to certain characteristics of broad classes of infectious organisms [2]. PRRs recognize patterns of bacterial and viral products such as lipopolysaccharides, petidoglycan, unmethylated CpG motifs of bacterial DNA, and double-stranded RNA, and mediate the uptake of microbes by phagocytes. A subset of those cells also functions as antigen presenting cells (APCs) by digesting the swallowed pathogen into smaller fragments and presenting them to specialized cells of the adaptive immune system [3].

The main cells of the adaptive immune system are the B and T lymphocytes. Antibody-producing B cells mature in the bone marrow and oversee the humoral immune response. T cells are also produced in the bone marrow but are educated in the thymus and constitute the basis of cell-mediated immunity. Both cell types share the ability to somatically rearrange their variable (V), diversity (D), and joining (J) region gene segments and thus, generating millions of highly diverse lymphocyte receptors.

IMMUNOLOGICAL TOLERANCE

Every day new lymphocytes are produced and because the gene rearrangement is a random process there is a great risk that many of the receptors may also bind to self-molecules. To prevent these self reactive lymphocytes from mounting an immune response there are a number of mechanisms that enables the immune system to discriminate between self and non-self antigens. This is known as immunological tolerance. Tolerance is classically divided into central and peripheral tolerance, depending on whether the control mechanisms operate in the central lymphoid organs (thymus, bone marrow) or the peripheral organs (lymph nodes, spleen, etc.).

Central Tolerance

There are two main mechanisms by which central B cell tolerance may occur. Immature B lymphocytes that encounter the native antigen in the bone marrow

during their process of maturation undergo apoptotic cell death or, alternatively, change their receptor specificity through receptor editing [4].

The mechanisms responsible for central T cell tolerance and T cell maturation in the thymus are more complex and not yet completely understood. However, it is undisputed that T cell precursors emerging from the bone marrow are recruited to the thymus. The first step of T cell selection, known as positive selection, occurs in the thymic cortex where thymocytes showing no affinity for peptide-MHC presented by cortical thymic epithelial cells (cTECs) die of neglect [5].

Only lymphocytes showing a productive rearrangement of their T cell receptor (TCR) and a sufficient affinity for the peptid-MHC complex are positively selected. After positive selection they migrate to the medullary areas of the thymus, where they differentiate into single positive CD4+ or CD8+ T cells [6]. Obviously, this cell pool contains all possible T cell clones, including not only those potentially able to recognise exogenous peptides but also those reactive towards self-peptides. The following process, which results in the elimination of the great majority of thymocytes able to recognise self-peptides, is known as negative selection. Negative selection occurs, because thymocytes showing high affinity for a self-peptide in the thymus undergo apoptotic cell death following self-peptide interaction [7]. However, some of the T cells receiving a relatively strong signal, yet not strong enough for negative selection, can develop immune-suppressive functions and negatively regulate responses of other T cells [6].

The main question puzzling researchers for years is how the T cells can encounter such a high number of self-peptides in the thymus, especially since many of these peptides are thought to be tissue- or organ-specific. It is now known that medullary thymic epithelial cells (mTECs) play a major role in the presentation of self-peptides and the process of negative selection. mTECs are capable of so called promiscuous gene expression, meaning that they can express peptides that are otherwise exclusively expressed in specific tissues or organs [8]. This promiscuous expression is regulated by a protein named “autoimmune regulator” (AIRE) [9]. AIRE is a transcription factor that drives the expression of certain organ-specific genes. Through this mechanism, diverse tissues and organs such as epidermis, liver, brain, pancreas and intestine can be represented by mTEC. More than a 100 different self-antigens have been identified [10]. At the end of the process of negative selection, only single-positive CD4+ or CD8+ thymocytes equipped with TCRs not showing high affinity for self-peptides will survive. They migrate into the periphery and colonise secondary lymphoid organs, where they will encounter exogenous peptides that are captured and presented by dendritic cells.

Peripheral Tolerance

Central Tolerance is the most important mechanism in the deletion of autoreactive T cells. However, negative selection in the thymus is incomplete and not all self-reactive cells are deleted. To prevent that these cells may elicit an autoimmune response, there are additional tolerance mechanisms in the periphery, which induce unresponsiveness in mature lymphocytes. Peripheral tolerance for mature B cells can occur when the B cell encounters a specific antigen in the absence of specific T helper (Th) cells [11, 12]. This leads to that the B cell not only remains inactive, but it also becomes incapable of activation, even if it would re-encounter the same antigen under appropriate conditions. The second possibility of tolerance induction is a partial

activation of the mature B cell. Under this condition the B cell is excluded from lymphoid follicles (follicular exclusion) [13].

The mechanisms for peripheral T cell tolerance are more complex. Four main mechanisms of peripheral tolerance play an important role in maintaining self-tolerance and prevention of autoimmunity: deletion, anergy, ignorance, and regulatory T cells.

Deletion is a mechanism of peripheral tolerance of mature T cells, which is based on their apoptotic cell death. Deletion usually occurs when T cells encounter high antigen concentrations or if they are heavily activated. This process is known as "activation-induced cell death" (AICD) and is mediated through the high expression of surface molecules known as Fas (CD95), as well as its ligand (FasL or CD95L) [14, 15].

Anergy occurs when a T cell encounters its proper peptide under suboptimal stimulation conditions. In the absence of a co-stimulatory signal, provided through the interaction of CD80 or CD86 with CD28, T cells stay inactive, even if they would re-encounter the same peptide on the surface of professional APCs equipped with co-stimulatory molecules [16]. Another method to generate an anergic stage is if peptide recognition by the Th cell is accompanied by interaction with the suppressive cytotoxic T lymphocyte associated antigen (CTLA)-4 molecule instead of with the activating CD28 [17]. Under both conditions, the Th cell does not die and persists in the body, but becomes functionally unresponsive.

Ignorance refers to the common occurrence of low numbers of low affinity autoreactive T cells existing in the presence of low levels of presented self-peptides without becoming activated. It has been estimated that each APC expresses a maximum of 10^5 MHC molecules per cell. T cells must bind at least 10-100 identical peptides on an APC to become activated. Most peptides presented on APCs would therefore be below the threshold for T cell detection [1]. In addition, potentially autoreactive T cells may never encounter their antigen because the antigen is expressed in a so-called immune-privileged site, such as the brain or the eye, which is characterized by specific physical and immunological structures limiting the immune systems ability to enter this site.

The fourth mechanism of peripheral tolerance is represented by regulatory T cells. The existence of suppressor cells was suggested already 30 years ago, but it is only in the past decade that the existence of T cells capable of suppressing the immune response has been conclusively demonstrated. These cells have been named T regulatory (Treg) cells. Tregs represent a heterogeneous subset of T cells, which have a variety of characterizations and functions. Perhaps one of the best described and most extensively studied population of regulatory T cells are the thymus derived, naturally occurring CD4+CD25+ Tregs, for which the expression of Foxp3 has been shown to be critical for the development of their unique regulatory phenotype [18]. CD4+CD25+ Tregs do not produce cytokines and seem to exert their suppressive activation via a cell contact-dependent mechanism [19]. Several other populations, such as the Tr1, Th3, NKT, γδ T and CD8+ T cells have also been described to possess regulatory activity, mainly via the production of cytokines, such as IL-10 or TGF-β [20].

AUTOIMMUNITY

In some individuals the immune system fails to properly distinguish between self and non-self and instead launches an inappropriate response against cells and molecules of its own host. This phenomenon is known as autoimmunity and the diseases it causes are called autoimmune disorders. Approximately 5% of the human population are affected by autoimmune diseases [21] and although the sex distribution varies between different diseases it is evident that women are generally more often affected than men [22].

The reasons why the immunological tolerance is broken in some individuals are still not well understood, but it is commonly believed that a combination of genetic, environmental and hormonal factors plays together in the genesis of autoimmunity. It is hypothesised that certain mechanisms may trigger autoimmune responses. For example, antigens, which are normally restricted to one part of the body, and therefore not usually exposed to the immune system, are released into other areas, where they can be encountered by the immune system. So can for example in sympathetic ophthalmia, the damage to one eye result in an autoimmune response to eye proteins that then damages the uninjured eye [23]. Second, exogenous antigens may share structural similarities with certain host antigens, so-called molecular mimicry [24]. Cross-reactive antibodies are generated and bind to the host antigens, amplifying an immune response. It is known, that Lyme disease is caused by a tick-borne spirochetal bacterium, known as *Borrelia burgdorferi* that causes inflammation of the joints. Although arthritis resolves with antibiotic treatment, about 10% of patients with Lyme arthritis develop persistent synovitis, which can last for several years [25, 26]. Molecular mimicry and its involvement in autoimmune processes have been discussed for many decades, but it still unresolved whether or not the infectious agents are only bystanders or true trigger of the autoimmune response [27]. Another possible cause of autoimmunity is that molecules in the body are altered by drugs, infections, tissue damage or other environmental factors, resulting in the creation of so-called neo-epitopes. These neo-epitopes are no longer recognized as self by the immune system and hence, an autoimmune response is mounted. Fourth, the immune system itself may be dysfunctional. For instance a genetic mutation in the *AIRE* gene, which leads to the impaired elimination of autoreactive T cells in the thymus, results in the manifestation of a rare autoimmune syndrome known as autoimmune polyendocrinopathy syndrome type 1 (APS1) [28]. Another example is the rare but fatal disorder called immunodys-regulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrom, which is caused by mutations in the *FOPXP3* gene that results in the defective development of CD25+ CD4+ regulatory T cells [29]. Both APS1 and IPEX are examples of monogenic diseases where a single mutation in a gene that plays a central role in immune regulation and tolerance is causing severe autoimmune disorders.

Autoimmune diseases can be divided into two broad categories: organ-specific and systemic autoimmune diseases [3]. In organ-specific autoimmune diseases, the immune response is directed towards a target antigen unique to a single organ or gland, so that the manifestations are largely limited to that organ. The cells of the target organ may be damaged directly by humoral or cellular-mediated effector mechanisms. In insulin-dependent diabetes mellitus (IDMM), for example, the autoimmune attack is directed against specialized insulin-producing cells that are located in the pancreas. Another organ specific autoimmune disease is multiple sclerosis (MS), which affects the central nervous system (CNS: brain and spinal cord).

In MS, autoreactive T cells participate in the formation of inflammatory lesions along the myelin sheaths of nerve fibers, destroying the myelin and thus, interfering with the transmission of nerve impulses. Alternatively, autoantibodies may overstimulate or block the normal function of the target organ. In myasthenia gravis, autoantibodies bind to the acetylcholine receptors on the motor end-plates of muscles and thus, block the normal binding of acetylcholine and also induce complement-mediated cell lysis. The result is a progressive weakening of the skeletal muscles.

In systemic autoimmune diseases, the response is directed towards a broad range of target antigens and involves a number of organs and tissues. Tissue damage is widespread. One example of a systemic autoimmune disease is systemic lupus erythematosus (SLE), in which autoantibodies to a vast array of tissue antigens, such as DNA, RNA, histones, platelets, red blood cells and clotting factors, are produced.

RHEUMATOID ARTHRITIS

Another example of a systemic autoimmune disease is rheumatoid arthritis (RA) that primarily affects the synovial joints. In the joints inflammatory cells attack and eventually destroy bone and cartilage structures. The symptoms that distinguish RA from other forms of arthritis are the inflammation of many joints at the same time (polyarthritis) and that the pain of RA is usually worse in the morning ("morning stiffness") compared to osteoarthritis (OA) where the pain worsens over the day.

Table 1. The 1987 revised criteria for the classification of RA*

Criterion
1. Morning stiffness
2. Arthritis of 3 or more joint areas
3. Arthritis of hand joints
4. Symmetric arthritis
5. Rheumatoid nodules
6. Serum rheumatoid factor
7. Radiographic changes

* For classification purposes, a patient shall be said to have RA if he/she has satisfied at least 4 or these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks.

Usually, the joints are affected in a symmetrical fashion. Extra-articular manifestations, such as rheumatoid nodules in the skin, pulmonary fibrosis, vasculitis and glomerulonephritis occur in about 40% of individuals with RA and this also distinguishes the disease from osteoarthritis [30]. Also constitutional symptoms of appetite and weight loss, fever and fatigue are common in RA. In 1987 the American Rheumatism Association established a list of seven diagnostic criteria that since then are widely used for the classification of RA (Table 1) [31]. Rheumatoid arthritis is one of the most common autoimmune diseases and affects approximately 0.5-1% of the population worldwide [32]. It has been estimated that the peak age of onset of RA is in the fifth decade of life [32]. However, RA may occur as early as in the second decade of life and in fact the seropositive, polyarticular form of juvenile rheumatoid arthritis occurs predominantly in adolescent girls [33].

Pathology

RA is characterized by a chronic synovial inflammation, which eventually leads to cartilage destruction and bone erosion, causing deformity and loss of function of the

joint. RA is not a single disease entity but rather a heterogeneous syndrome caused by different pathways involving B cells and autoantibodies, T cells, the cytokine network as well as fibroblasts and many other cell types [34].

T cells and the cytokine milieu. Although the pathogenesis of this disease is far from completely understood, T helper cells and the cytokine milieu seem to play a central role in its autoimmune manifestation. The vast majority of cellular infiltrates in the synovial tissue of RA patients consist of CD4+ αβ T lymphocytes [35, 36], nearly all expressing the memory phenotype marker CD45RO and carrying a distinct profile of activation markers, such as CD69, CD44, CCR4, CCR5 and CXCR3 [37, 38]. It is assumed that arthritogenic peptides from foreign or self-proteins are presented to T cells by professional APCs such as dendritic cells, macrophages, or activated B cells. This process involves binding of peptides to MHC class II molecules, and interestingly, about 50-80% of the patients with RA carry the so-called shared epitope (SE) of the HLA-DRB1 cluster [39, 40]. These alleles share a highly homologous amino acid sequence (QKRAA, QRRAA, RRRAA) at the third hypervariable region of the HLA-DR β chain, which confers binding of specific peptides and thus affects antigen presentation to TCRs [41]. Despite the fact that this hypothesis formulated by Gregersen and colleagues in 1987 is probably the most popular in the field of HLA genetics in RA, more than 20 years of extensive search for the arthritis-inducing peptide(s) remain inconclusive. Nevertheless, once the T cells recognize these hypothetical antigens in the articular tissues, the lymphocytes are retained in the synovium and the persistence of inflammation is ensured.

Within the synovial environment, the T cell phenotype is altered. It is known that T helper cells can be divided into different subsets based on their cytokine profiles [42]. Th1 cells produce IFNγ, IL-2, but not IL-4, IL-5, IL-10, or IL-13; Th2 cells express the opposite profile. Th1 cells generally regulate cell-mediated immunity against viruses or intracellular pathogens and phagocyte-dependent inflammation, while Th2 cells evoke a strong humoral response, including isotype switching to IgE and favour eosinophil differentiation and activation to control helminth infections [43]. In addition, a new subset of T helper cells, denoted Th17 cells, has been identified recently. These cells are characterized as preferential producers of IL-17, IL-21 and IL-22, which mediate host defensive mechanisms to various infections, especially extracellular bacteria infections [44, 45]. Furthermore, Th1, Th2 and Th17 cells seem to be mutually antagonistic, and each can suppress the activity of the other.

A vast number of studies have established that T cells in RA are primarily biased towards Th1 cells, and are thus triggered to secrete IFNγ and IL-2, as well as to supply help to other cell populations [46-48]. These include B lymphocytes in follicle-like structures of the synovium, which through cell-cell contact are activated and prompted to produce opsonizing and complement-fixing antibodies [49]. Moreover, the cell contact from T cells also induces the activation of monocytes/macrophages, as well as of resident synovial cells [50, 51].

Whereas it has been clearly demonstrated that IFNγ producing Th1 cells can be found in increased numbers in synovium from RA patients, there is still inconsistency on whether or not the Th17 cytokine IL-17 is also present in synovial samples of RA patients [52-54]. However, it seems to be established that IL-17 is present at high levels and a driving force in a number of animal models for human RA. It may reflect one of the pathogenic pathways leading to chronic inflammation and destruction in

the joint [55-57]. It has many of the same functions as IL-1 and TNFα and can enhance cytokine and metalloproteinase production by fibroblast-like synoviocytes as well as osteoclastogenesis [53, 58].

B cells and monocytes. Most recently B cells came back into the spotlight. Under the appropriate conditions, autoreactive B cells will produce antibodies, and in the last decades various autoantibodies have been identified in sera of RA patients. Perhaps the most extensively studied autoantibodies in RA are the rheumatoid factors (RF), which bind to the Fc region of IgG and therefore contribute to the formation of immune complexes. This may lead to further activation of the monocyte lineage by triggering signalling cascades after Fc-receptor engagement [59, 60]. But most important for the flare of interest in B cells was the discovery of antibodies against citrullinated proteins, for example cyclic citrullinated peptide (CCP) and their high specificity for RA [61]. Only shortly after the discovery of anti-CCP antibodies, clinicians reported the first positive therapeutic results using a B cell depleting antibody [62]. B cells are usually involved in the adaptive immune response by producing specific antibodies, but once they are activated, they can also efficiently take up and present antigens to T cells and thus, a vicious cycle of events can be stimulated [63].

Th1 cells produce a variety of cytokines, including IFNγ and IL-2. The latter, originally described as a T cell growth factor, is a powerful activator of monocytes/ macrophages [64]. Once activated, monocytes/macrophages respond to IL-2 with production of growth factors, microbicidal activities and secretion of various pro-inflammatory cytokines, among them TNFα, IL-1 and IL-6 [65]. Additionally, IL-12 produced by synovial macrophages, has been detected in RA and is known to bias T cell differentiation towards the Th1 phenotype. IL-18, which is a member of the IL-1 family and is also present in the RA joint, can synergize with IL-12 and enhance the Th1 cell differentiation [66].

All these cytokines are pivotally involved in the generation of inflammatory as well as destructive responses. In addition, many of the cytokines may come from various cell types and thus, support each other's activity. So it is notable, that the high amount of TNFα for example, which is a key molecule in the cytokine world, may also come from several sources other than activated monocytes/macrophages. In fact, it has been shown that both T cells as well as B cells can produce significant amounts of TNFα [67-69].

Resident synovial cells and osteoclasts. The establishment of RA involves a number of events and cellular interactions. In the recent years it has become evident that resident synovial cells are not just innocent bystander cells but may be actively engaged in the joint inflammation and destruction. Most interestingly, it is these resident cells and not the migratory inflammatory cells that are usually found at the site of destruction [51]. When isolated from RA patients and implanted into SCID mice, fibroblast-like cells show an invasive behaviour, which may partly be explained through the upregulation of cellular proto-oncogens and anti-apoptotic molecules [70-72]. Except the resident cells, basically all effector cells are recruited from the blood into the synovium via chemotactic factors and therefore chemokines are considered key elements in the pathogenesis of RA. Fibroblast-like synovial cells produce chemokines, such as CCL19 and CXCL12, which attract monocytes and

lymphocytes into the joint [73, 74]. Furthermore, they constitutively express CXCL5, a ligand for CXCR2 mainly located on neutrophils, which further can be upregulated by stimulation with TNFα [75]. With respect to cartilage damage, fibroblast-like cells are thought to play an activate role through the release of matrix-metalloproteinases (MMPs: MMP1 and MMP3) [76].

While all inflammatory responses induced by proinflammatory cytokines will directly translate into synovial inflammation with pain, swelling including effusion, and stiffness, bone destruction is primarily mediated by activation of osteoclasts via the RANK-RANKL receptor system [77, 78]. RANK is expressed on osteoclasts and fibroblasts, whereas its ligand RANKL is found on stromal cells but, equally important in the context of inflammation-mediated osteoclast activation, also on activated T lymphocytes [79]. Additionally, TNFα has a pivotal role in the pathogenesis of inflammatory osteolysis. While the cytokine alone does not induce osteoclastogenesis, it does so by directly targeting macrophages within a stromal environment that expresses permissive levels of RANKL. Thus, TNFα synergizes with minuscule amounts of RANKL, which promotes osteoclastogenesis and inflammatory osteolysis [77]. Like TNFα, IL-1 on its own is capable of osteoclast recruitment. Both cytokines act independent of each other, shown by blockage of either TNFα or IL-1. Separate inhibition of either of the cytokines could not completely arrest the periarticular damage of inflammatory arthritis, whereas inhibition of both is substantially more effective [80].

Treatment

Despite large research efforts, there is still no cure for RA. However, in the last decades many different types of treatments have been developed to alleviate symptoms and modify the disease process [81]. Disease modifying anti-rheumatic drugs (DMARDs: methotrexate, anti-malaria medication – hydroxy-chloroquine, sulfasalazine, leflunomide, gold salts, etc.) are used to suppress the immune system, decrease pain and reduce or prevent joint damage. DMARDs are so called "slow-acting anti-rheumatic drugs" and it takes weeks or months before they have an effect. Thus, to provide faster relief of ongoing symptoms DMARDs are usually combined with non-steroidal anti-inflammatory drugs (NSAIDs: aspirin, ibuprofen, diclofenac), which reduce pain and minor inflammation by inhibition of cyclooxygenases (COX-1 and COX-2) and thus, inhibition of prostaglandin production. Alternatively, DMARDs are prescribed in combination with steroids (glucocorticoids: prednisone, prednisolone), which have a strong anti-inflammatory effect and quickly decrease pain and joint swelling, but have only a modest ability to reduce cartilage damage and bone erosion. A recent advance in the past 10 years in the management of RA is the use of biological agents, which block certain key molecules involved in the pathogenesis of the disease [82, 83]. They include TNFα blocking agents, the anti-CD20 antibody (depletion of B cells), CTLA4-Ig (inhibition of costimulatory molecules CD80 and CD86) and anti-IL-1 agents. In addition new biological agents that are in development include anti-IL6 receptor antibody and anti-CD22. Unlike DMARDs, biological agents work rapidly. Biological agents may be used alone or in combination with other drugs, but because of their extensive cost and their way of administration (injection) they are often reserved for patients who have not sufficiently responded to traditional DMARDs and for those who cannot tolerate DMARDs in doses large enough to control inflammation.

Environmental Risk Factors

Although the cause of RA remains unknown, the general consensus is that both genetic and environmental factors contribute to its occurrence. While the identification of genetic risk factors has been rapidly progressing the past 5 years, the success defining the environmental risk factors has been limited. This is largely due to the fact that environmental factors important in RA may act many years before the first clinical symptoms become apparent and thus, are difficult to trace back. Several retrospective studies have shown that already up to 10 years before the onset of RA, an increased production of RF as well as anti-CCP antibodies could be detected in a large group of patients [84, 85]. The long interval of subclinical progression of disease hampers the identification of environmental triggers for RA immensely. Despite these challenges, studies have identified numerous candidates, including cigarette smoking, hormonal factors, stress, and various infectious diseases.

Hormones and pregnancy. Approximatelly 0.5-1% of the population worldwide suffers from RA; 60-75% of those affected are women [22, 32]. This raises, of course, the question of why there is such an increased prevalence of autoimmunity in women. It has been shown that basic immune responses differ between females and males, where women have an increased antibody response after vaccination, a higher absolute number of CD4 lymphocytes and an increased production of Th1 cytokines [22, 86]. Furthermore, it is suggested that this sexual dimorphism of the immune response is mediated through sex hormones, mainly oestrogen, progesterone and testosterone. Perhaps the most striking evidence comes from studies of pregnant women with RA, of whom 75% have a decreased disease activity throughout pregnancy, when oestrogen and progesterone concentrations are highest. This is often followed by a relapse of disease within the first 6 month of the post-partum period, when oestrogen and progesterone concentrations fall [87]. This fluctuation of disease activity has been explained by the shift of Th1/Th2 cytokine responses during and after pregnancy due to the hormonal environment. At high levels, oestrogen and progesterone seems to favour cytokines of the Th2-type responses like IL-4 and IL-10. At the same time Th1 cytokines IFNγ, IL-2 and TNFα, which contribute to synovitis and joint destruction in RA, are suppressed [88]. Thus, the modulatory effects of oestrogen seem to be quite different between normal immune responses in non-pregnant women and immune responses in pregnant women with RA. This dilemma was partly resolved with the realization that oestrogen shows biphasic dose effects. While lower, non-pregnant levels facilitate cellular immune responses and stimulate IFNγ production, higher doses, as they occur in pregnancy, suppress T cell functions and have discrete regulatory effects on the Th1/Th2 balance [89, 90].

Infections. Potential microbial candidates implicated in the development of RA include *Mycobacterium tuberculosis*, *Escherichia coli* and *Proteus mirabilis*. But also viral infections with Epstein-Barr virus, retroviruses and parvovirus B19 have been suggested [32, 91]. A number of potential mechanisms have been proposed by which infections may alter the immune system and thereby elicit autoimmune responses. Potential mechanisms include the selection of the B and T cell repertoire, and the change of relative levels of Th1 and Th2 cytokines [92]. However, until now there is no consistent finding of a single infectious agent explaining the aetiology of RA.

Stress. Stress may also be an important risk factor in the pathogenesis of RA, considering that the activation of the stress response results in the release of neurotransmitters, hormones and activation of immune cells. It has been shown that disease flare-ups are linked to a higher number of minor stressors few days prior to the visit of a clinician. In addition, a study over a period of 5 years showed that RA patients with a higher daily stress level had a poorer outcome and significantly more bone erosion after 5 years [92].

Diet. Several epidemiological studies suggest a potential protective effect of lifelong consumption of fish, olive oil and cooked vegetables [93]. Mediterranean diet as a whole has also been reported as a factor reducing the risk or having a beneficial effect on the development of RA [94, 95]. Another study found that persons with a high level of red meat consumption and total protein intake were at an increased risk for developing inflammatory polyarthritis [96].

Cigarette smoking. A very early recognized risk factor for RA is smoking. Numerous studies have by now confirmed the association between cigarette smoking and development of RA [97-99]. But only most recently, smoking has been studied in a more mechanistic context. Several research groups have demonstrated that smoking is a risk factor in RF and anti-CCP positive RA, and that the risk is greatly enhanced in individuals carrying one of the disease-associated allele of HLA-DRB1 (SE) [100, 101]. These data suggest, that smoking might trigger some rather specific immune reactions, for example the immunity towards citrullinated proteins in the presence of certain genes [102].

Mineral oil exposure. Worth mentioning are the results from a Swedish case-control study, which analyzed the association between occupational exposure to mineral oil and RA. It was found that among men, exposure to any mineral oil was associated with a 30% increased relative risk of developing RA [103]. This finding is of particular interest with regard to this thesis, since the same mineral oils can induce polyarthritis in rats.

Heterogeneity and Genetic Influence

RA is not a single disease entity with a well-defined disease mechanism. Rather, it is a group of heterogeneous diseases of unknown aetiology, which are manifested by chronic joint inflammation. This is also reflected in the genetic heterogeneity of RA. RA is a so-called complex disease in which various genes are contributing to the pathology. Studies of twin concordances have estimated that the shared genetic effect explains as much as 60% of disease susceptibility [104]. However, finding the underlying genes is not simple and exactly how complex the genetics of RA can be, is demonstrated promptly with the first example, the association with the human MHC region.

Already 30 years ago the association to the human leukocyte antigen HLA-DR4 (HLA-DRB1*04) was detected [105]. This is the strongest RA associated gene and it has since its discovery been confirmed in many populations across the world.

Subsequent studies showed that not one but several HLA-DRB1 alleles are associated with disease [106-108]. Based on those findings, Gregersen *et al.* formulated the shared epitope hypothesis, which proposed that all RA-associated HLA-DRB1 alleles share a conserved motif of amino acid residues (QRRAA, QKRAA, RRRAA) in the third hypervariable region of the DRβ1 molecule [41]. According to this theory, binding of certain arthritis-inducing peptides to the identical antigen binding pockets of the HLA molecules may then trigger an immune response of autoreactive T cells. Although the predisposing effect of the SE-encoding HLA-DRB1 alleles is generally accepted, the hypothesis of the shared epitope has been challenged in recent years. It has become increasingly clear that it is not simply the SE alleles conferring an increased risk of disease. Several reports have shown that the risk for RA associated with different but SE-identical DRB1 alleles varies considerably, for example HLA-DRB1*0404 is a much stronger susceptible factor than HLA DRB1*0101 [109]. In addition, protective HLA-DRB1 alleles carrying another amino acid sequence (DERAA) instead of the SE sequence confer dominant protection against RA both in the presence and absence of SE susceptibility alleles [110, 111]. It is however, still a matter of debate whether this protection is mediated by regulatory T cells. Furthermore, several reports have postulated a number of other important associations to polymorphisms in this very gene rich area of the genome, which may be in linkage disequilibrium with HLA-DRB1. These include certain DQ-DR haplotypes, while others have implicated additional loci telomeric to the HLA, for example the TNFα locus [110, 112, 113]. Regardless on whether all genetic contributions to RA arising from the HLA locus is accounted for by the HLA-DRB1-SE association or not, in total the HLA region contributes with about 30-50% to the genetic component of RA.

From the discovery of the HLA association in the 1970's until 5 years ago, basically no other gene could be conclusively linked to the development of RA. However, this changed rapidly with the completion of the Phase I HAPMAP project in 2005, which identified over 1 million single nucleotide polymorphisms (SNPs) in 269 individuals from four populations [114]. In addition, advances in SNP genotyping technologies have reduced the time and costs and improved the accuracy to such levels that genotyping of extremely large numbers of genotypes are now feasible. As an example, chips containing 500,000 to 1,000,000 SNPs have been developed recently. At the same time large patient and control cohorts have been generated, and the exchange of data and collaboration of research groups has been intensified.

These are the key factors leading to the considerable progress that has been made in the identification of non-MHC genes implicated in the susceptibility to RA. Among these genes is the *PTPN22* gene, which was first discovered as a type 1 diabetes susceptibility gene four years ago [115]. Shortly after, Begovich and colleagues confirmed the association of the minor T-allele of the non-synonymous SNP (rs2476601, R620W) with RA [116]. The *PTPN22* gene encodes the lymphoid-specific phosphatase (Lyp), an important negative regulator of T cell activation. Paradoxically, the disease-associated variant has been described to result in a gain-of-function form of the enzyme, leading to stronger suppression of the early T cell activation process. This might lead to a failure in deleting autoreactive T cells during thymic selection or a decrease in the activity of regulatory T cells [117]. Interestingly, *PTPN22* appears to be a common autoimmune regulator, as it is not only associated to type 1 diabetes and RA, but also to SLE, juvenile idiopathic arthritis, Graves' disease and generalized vitiligo [118]. Notably, the variant confers the second largest genetic risk to development of RA and the susceptible TT and TC genotypes are

strongly associated with RF positive disease but were not linked to the shared epitope [116, 119].

Three more genetic associations have been reported and successfully replicated in numerous studies in the past two years. The first is the disease association to *STAT4*, which was identified in a North American and a Swedish cohort [120] and was later on confirmed in a Korean [121], Colombian [122], Japanese [123] and 4 more European populations [124, 125]. *STAT4* encodes a transcription factor that transmits signals induced by several key cytokines, including IL-12 and IL-23, and stimulates the transcription of specific genes, including IFNγ. Therefore, STAT4-dependent signalling by IL-12 receptor plays a critical role in the development of a Th1-type T cell response [126]. No correlation between the risk allele and the presence of RF or anti-CCP has been observed. Unlike several other risk genes for RA, the Odds ratios observed in the Asian population were quite similar to those in the Caucasian population, indicating that *STAT4* is a common genetic risk factor for RA, with similar strength across major racial groups. Additionally, *STAT4* was also found to be associated to SLE [122, 123].

The second locus found to be associated with the development of RA was identified in an intergenic region between *TRAF1* and *C5* [127]. Later studies replicated the finding from the British cohort in the North American, Swedish, Dutch [127-129], another British [130] and Greek populations [124]. The disease-associated SNP is located within a region encoding a putative binding site for the immune and inflammatory response related CCAAT/enhancer binding protein, which may be disrupted by the minor T allele of the associated variant [131]. In addition, the SNP was most strongly associated with an increased risk for RA in the anti-CCP positive and RF positive subgroups respectively [127, 129]. Interestingly, there appears to be a strong interaction between *TRAF1-C5*, *HLA-DRB1* and *PTPN22* and when combining the three highest risk variants together (two alleles of the SE, TT or TC genotype at *PTPN22* and GCG/GCG genotype at *TRAF1-C5*), they generate a more than 45-fold increased relative risk for developing RA [129].

The third genetic region that has been implicated to harbour an autoimmune disease-regulating gene is located at chromosome 6q23. In two independent genome-wide association scans from Caucasian cohorts of RA patients and additional case-control samples an association was detected near the *TNFAIP3* gene [132, 133]. In fact, two statistically independent SNPs have been discovered in this region, one conferring a protective haplotype and the other a risk haplotype, suggesting the existence of two independent susceptibility alleles. Interestingly, the identified SNPs are located in a 63kb region of linkage disequilibrium that falls outside of any coding sequence; the nearest genes, TNFAIP3 and OLIG3, are ~185kb away. In both studies, the disease conferring SNPs were strongly associated in the RF positive and anti-CCP positive subgroups. As for *PTPN22* and *STAT4*, also this region was associated with SLE susceptibility and appears to be a regulator of various autoimmune conditions even though more studies are needed in order to find the disease-underlying mechanism [134].

There is considerable variation of the disease frequency among different populations. Some ethnic groups, for example native American-Indian populations have an increased prevalence of RA, while other countries, for example developing countries or China and Japan have a relative low occurrence of RA [135]. Even in Europe scientists have detected a clear North-South divide, where south European countries

have lower incidence and prevalence rates than North European and North American countries [136]. These discrepancies might have several causes, which could be of either environmental (diet, infections) or genetic nature. Thus, one must assume that genetic associations to certain disease alleles will only be detected in distinct ethnic groups. One example is the disease association to the *PTPN22* gene, which was highly reproduced in various Caucasian populations but could not be detected in a Japanese cohort [137]. On the other hand, the *PADI4* gene with significant associations in Asian populations cannot be linked to RA patients with Caucasian origin. The association to *PADI4*, which is involved in the citrullination pathway and thus, is thought to play an important role in the pathogenesis of RA, was originally detected in a Japanese cohort [138]. Later it could be replicated in another Japanese and Korean population, but it does not seem to have any effect in European populations [139-142].

Despite the recent advances in gene identification, many susceptibility genes are yet to be discovered. Among the difficulties involved in gene identification in humans are factors inherent to this complex trait such as variable penetrance, variable and most important low relative risk associated to a single disease allele and epistatic interactions between various disease alleles. But also the genetic heterogeneity of the human population and complex interactions between environmental and genetic factors hamper the identification of genes associated with human RA [143]. Thus, it is not surprising that only a minor fraction of disease-associated genes have been conclusively identified so far. The challenge now is to identify the remaining, probably smaller, genetic effects and explore how these variants interact with each other as well as environmental factors to induce development of RA. Therefore, animal models are very attractive tools because use of such models not only overcomes these genetic complications, but also permits studies under stable environmental conditions. Being able to model the genetic background and adjust it to the specific demands of each study, as well as changing one or two genetic and environmental factors at the time will be an advantage of animal models in the future.

RAT AS A MODEL ORGANISM FOR HUMAN RA

The Laboratory Rat

The Norway rat, brown rat or *Rattus norvegicus*, is a mammal in the order Rodentia. It originated in Asia and then spread over the world in close association with human. It may have reached Europe around 1700 and then largely supplanted the smaller *Rattus rattus* (black rat). All laboratory rat strains are derived from the *Rattus norvegicus* species. It is commonly believed that the domestication of wild rats began in the eighteenth and nineteenth centuries, originally in Europe and later in America, when rats where trapped and sold for food or used for rat-baiting contests. In this era albino, nonagouti black and hooded coloured rats were preferentially captured or selected from offspring of captive rats due to their distinct and attractive appearance. Because behavioural traits, such as calmness and docility, are associated with black and hooded fur colour, the process of domestication may therefore have increased the incidence of these particular rats in the domesticated rat population [144, 145]. Albino and mutant rats were brought to the laboratories as early as in the nineteenth century to serve as objects of physiological and behavioural studies. It is safe to state that the *R. norvegicus* was the first animal species to be domesticated strictly for scientific purposes [146]. The Wistar Institute in Philadelphia is one of the first

laboratory animal breeding and research facilities and one of the most popular strains used for research, the Wistar rat strain as well as most other laboratory strains today, are descended from a colony of rats established at this institute. Today there are more than 200 inbred strains and substrains and the list of congenic, consomic, recombinant inbred and transgenic strains is constantly growing (http://rgd.mcw.edu/). The laboratory rat and the laboratory mouse are by far the most commonly used experimental animals in many fields of medical and biological research. The ease of breeding combined with short generation times have contributed to the widespread use of these species as experimental mammals. The rat is a high valuable model organism for the analysis of many complex areas of biomedical research, such as cardiovascular diseases, metabolic disorders (diabetes mellitus), neurological disorders and behaviour (learning, epilepsy research), autoimmune diseases (arthritis, experimental allergic encephalomyelitis), cancer and renal diseases. Of essence for the genetic research was the sequencing of the rat genome, whose first draft was published in 2004, shortly after the human and mouse genomes [147]. This and other recent advances in rat genetics will hopefully provide the tools of successful gene-identification for complex traits in the laboratory rat.

The DA rat strain and substrains. Although in the literature the name DA rat is often wrongly designated as "dark agouti", it was, in fact, named because it expressed the "d" blood group allele of Joy Palm, and it is "A" agouti in color (http://rgd.mcw.edu/). It was developed by Dr. T.T. Odell, Jr. at the Oak Ridge National Laboratory, Tennessee until filial generation F11, and then completed by Dr. Darcy Wilson at the Wistar Institute in about 1965 [148]. Its origin is unknown, and for a long time assumed to be closely related to COP, however, recent genetic studies suggest a close relation to ACI instead. After 20 generations of brother/sister matings a strain is considered to be inbred and shortly after this was achieved, the DA rat was brought to many institutes around the world. Inbreeding was continued, and consequently various substrains have been established. Currently, there are 11 different DA substrains listed at the rat genome database (RGD), among them DA/OlaHsd and DA/ZtmRhd, but it must be assumed that there are many more since not all of them have been reported to RGD. As all the other rat strains, also the DA rat has many strain specific physiological, biochemical and immunological characteristics, but what makes the DA rat to such an exceptional rat strain in arthritis research is its susceptibility to a vast variety of arthritis models.

Experimental Models

Unlike in the mouse, there is no spontaneous model for arthritis in rats. However, there exists a wide spectrum of inducible arthritis models that can be used in these rodents. In principle, rat models can be divided into three general groups. In the first group, arthritis is induced by immunization with cartilage-derived antigens such as collagen type II (CII), collagen type XI or cartilage oligomeric matrix protein in incomplete Freund's adjuvant (IFA) [149-151]. The second group is induced by intradermal injection of various oil-based adjuvants, for example pristane and IFA alone [152, 153]. But also exogenous chemicals such as hexadecane or endogenous lipids such as squalene can induce arthritis [154, 155]. The third group of rat models is induced with various forms of yeast and bacterial cell wall products, for example

peptidoglycan-polysaccharide fragments from group A streptococci [156, 157]. Each of the rat models has clinical features that resemble RA in humans. The models, however, also differ among themselves and in comparison to RA with respect to disease onset, severity of the joint inflammation, patterns of involved joint, and various additional clinical and systemic manifestations. Although none of the models mirrors all features of human RA, each of these models might provide unique insights into certain pathogenic pathways leading to RA.

Collagen-induced Arthritis (CIA)

The CIA model in rats was first described by Trentham *et al.* in 1977 [149], and has since then become one of the most common rat models for human RA. There are substantial variations in the induction of CIA in respect to the origin and preparation of the collagen, which is also reflected in the disease outcome. CIA is typically induced by a single intradermal injection of either heterologous (non-rat) or homologous (rat) CII in IFA. While heterologous collagen is highly arthritogenic in a broad number of rat strains (BB(DR), DA, LEW, LOU, SD, W, WF), homologous collagen is only arthritogenic in DA and LEW rats. Interestingly, Larsson and colleagues described a remarkable difference between those strains in the latter model. LEW rats showed disease onset characterized by involvement of only the ankle and knee joints with a self-limiting, acute disease course, whereas DA rats showed disease onset characterized by symmetric involvement of the interphalangeal joints with a chronic relapsing disease course, suggesting that clinical features of arthritis induced with identical stimuli are different depending on the genetic background [158]. Further studies have shown that the DA strain is the only rat strain susceptible to CIA when induced with lathyritic (pepsin-free) collagen II. The susceptibility to homologous CII in LEW rats is strongly dependent on contaminating pepsin derived from porcin, which is known to form stable complexes with collagen and induce a strong immune response [159]. It is apparent that the clinical features of homologous collagen-induced arthritis in DA rat are more similar to those seen in RA than those observed in other variant of collagen-induced arthritis, therefore a majority of studies are focused on this particular rat strain.

After immunization of DA rats with rat CII, rats develop a symmetric and severe polyarthritis within 14-18 days (Figure 1). Both autoreactive T cells as well as B cells, which produce arthritogenic antibodies against CII, play a critical role in disease progression. The involvement of pathogenic antibodies was demonstrated by Stuart *et al.* who transferred antibodies from immunized rats into naïve recipient and thus, provoked development of arthritis within 1-3 days, displaying all of the major histopathological characteristics of the early lesion in immunized rats [160]. However, the disease was transient, the lesions less severe and without pannus formation. It was further shown, that development of arthritis after passive transfer of anti-CII antibodies is dependent on complement activation, as rats depleted of complement factor 3 were protected and did not show any accumulation of neutrophils in the joints or erosion of cartilage [161]. Besides B cells and production of pathogenic antibodies, also T cells are crucial in the establishment of CIA. While

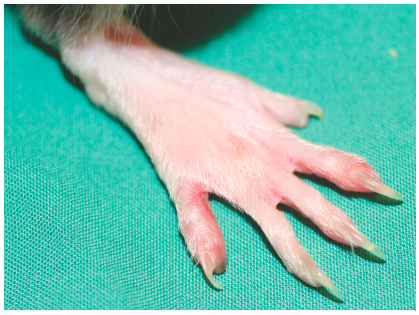

Figure 1. Arthritic hind paw from a DA rat 15 days (day of onset) after collagen injection.

normal rats develop severe arthritis after collagen-injection with T lymphocytes, contributing significantly to the cellular infiltrates, nude rats of the same strain did not develop any signs of arthritis [162]. In addition, CIA can be reduced by depletion of the αβT cells before immunization, before onset or after disease development, however, it is not as effective when the disease is already established [163, 164]. As in human RA, it is the pro-inflammatory cytokines TNFα and IL-1 driving the disease progression and joint destruction [165-167]. Furthermore, there appears to be an altered Th1/Th2 balance towards Th1 cytokine profile accompanied by IL-2 and IFNγ expression [168].

Apart from the pathological features similar between CIA in rats and RA in humans, also the genetic contribution appears to be regulated in similar ways. The disease is genetically controlled by MHC and non-MHC genes [169]. Whereas the MHC association appears to be very broad in CIA induced with heterologous collagen ($RT1^{av1}$, $RT1^{u}$, $RT1^{l}$, $RT1^{a}$, $RT1^{e}$), its MHC association is very limited in homologous collagen-induced arthritis ($RT1^{av1}$, $RT1^{i}$, $RT1^{a}$) [169-173]. A large number of crosses, with DA or BB(DR) as susceptible and F344, ACI, and BN as resistant strains, have been studied in order to identify genetic regions associated with the disease [174-177]. In each cross a selected set of genes seems to regulate disease susceptibility and severity. These studies show that CIA is indeed complex and polygenic.

Pristane-induced Arthritis (PIA)

Pristane is a saturated polyisoprenoid alkane (2,6,10,14-tetramethylpentadecane) and can be found in substantial amounts in the liver of sharks and other marine animals, which is the source of pristane used in our studies [178]. It is known to be a degradation product of chlorophyll and therefore omnipresent in the diet. However, intradermal injection of small volumes of pristane (50-150µl) at the base of the tail induces severe arthritis in susceptible rats (DA, LEW) [152]. Although it does not exactly mimic human RA, PIA, nevertheless, shares many clinical, histological, serological and genetic features with RA. Another main advantage of PIA is its highly predictable disease course (Figure 2). Using the PIA model in DA rats, arthritis develops suddenly 10-14 days after immunization followed by an episode of severe and destructive arthritis in the peripheral joints. The peak of disease severity is typically reached around 3 weeks after pristane injection and gradually subsides another 2 weeks later. After the acute phase of the disease, a chronic relapsing disease develops, which may persist for many months.

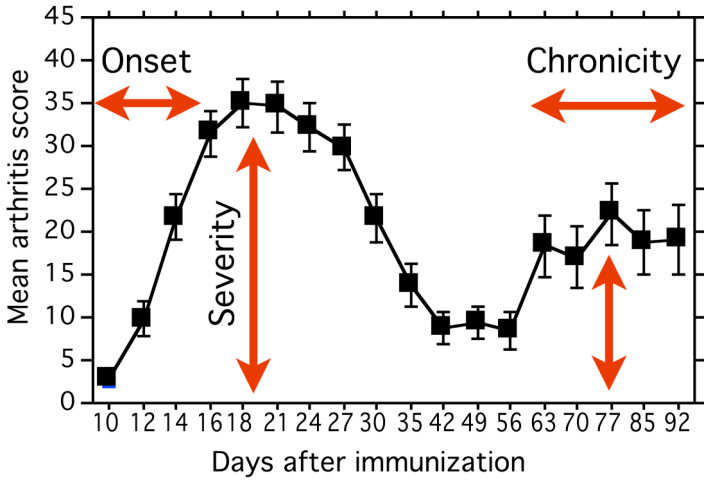

Figure 2. Arthritis curve after pristane injection in DA rats. In addition to the daily disease score, parameters such as day of onset, maximum severity and chronicity can be assessed.

Although the pathological mechanisms leading to the development are still

unclear, it is proposed that during the induction phase the T cell population is polyclonally activated, including already primed self-reactive T cells [179]. These cells, which were held in balance by various tolerance mechanisms may then expand and drive the disease. There is substantial evidence for the role of T cells in the induction and development of PIA. For example, depletion of αβT cells in the induction and in the chronic stage of PIA reduces the incidence as well as the severity of arthritis [152]. But also blocking of Th1 cytokines IFNγ and TNFα ameliorates the disease [180]. Six days after pristane injection a polyclonal expansion of B and T lymphocytes can be detected in the draining lymph nodes [180] and the expansion continues until 12 days post immunization. A few days after onset, large pannus formations are evident in the ankle joints, with cartilage destruction and bone erosion [152]. Inflamed joints contain several inflammatory cell types, including macrophages and neutrophils. In addition, a vast number of highly differentiated CD4+ αβT cells can be detected in the synovial tissue [180]. Sera from affected rats show elevated IL-6 cytokine levels as well as acute phase protein levels during the acute stage of disease, suggesting the presence of an acute systemic inflammation [181]. In the acute and chronic phase of arthritis, cartilage oligomeric matrix protein (COMP) is elevated in blood reflecting the degree of cartilage degradation [182]. In contrast to CIA, pathogenic antibodies against type II collagen are not produced in PIA, and neither in PIA nor in other oil-based arthritis models can passive transfer of serum or purified Igs provoke arthritis, suggesting a very limited role of arthritogenic B cells in this model [152, 183]. However, RF, which are found in about 60% of the patients with RA, are also produced in PIA [184].

Various crosses between DAxE3 rats have been analyzed in detail to describe the genetic basis of this model. The findings demonstrate that different phases of the disease (onset, acute, chronic) are associated with distinct sets of genes, but that there is substantial overlap with other forms of autoimmune disease [181, 184-189]. Although non-MHC genes play a more dominant role in the disease susceptibility to PIA, there is strong evidence for a MHC association. However, this association is clearly weaker than the one, which has been observed in CIA models [152, 185, 188, 190]. Nevertheless, the MHC association is surprising because there is no exogenous immunogen involved, nor has it been possible to isolate antigen-specific T cells in pristane-primed DA rats. However, recently Holmberg *et al.* demonstrated that pristane-primed T cells require activation through MHC II complexes, as treatment with blocking antibodies against RT1B (DQ) and RT1D (DR) molecules ameliorates arthritis development [180]. In regard to the MHC restriction, only syngenic and semi-allogenic, but not allogenic recipients, developed arthritis after transfer of pristane-primed donor cells. Moreover, studies of MHC congenic DA rats showed that *RT1*^{f} restricted, pristane-primed T cells restimulated in vitro with heterogenous nuclear ribonucleoprotein (hnRNP)-A2 produced large amounts of IFNγ and TNFα, suggesting that hnRNP-A2 may be one of the antigens involved in pristane-induced arthritis [191].

Adoptive spleen cell transfer

Pristane- and other oil-induced arthritis models are T cell mediated diseases. This is supported by the fact that concanavalin A (Con A)-stimulated, adjuvant-primed αβT cells derived from lymph nodes can transfer disease, whereas only Con A stimulated, but not adjuvant-primed cells, do not transfer disease [183]. Further studies have shown that only transfer of CD4+ but not CD8+ αβT cells transfer

arthritis to naïve donor rats [180]. Subsequently, it has been found that both spleen and lymph node derived, IFNγ and TNFα producing CD4+ αβT cells are able to transfer arthritis. In fact, donor T cells are dependent on their Th1 cytokine secretion in order to provoke arthritis, as the disease is ameliorated after treatment with blocking antibodies against IFNγ and TNFα in recipients [180]. Typically, rats develop arthritis within 5-7 days after adoptive transfer of primed lymphocytes, with the severity strongly depending on the amount of arthritogenic cells injected. The disease severity reaches its maximum score at around day 9-12, followed by a regression period of approximately 3-4 weeks. Arthritic lesions are histologically characterized by synovitis, consisting of hyperplasia of the synoviocytes and infiltration by numerous mononuclear cells. Twelve days after transfer, severe pannus formation in ankle joints has been observed, in which also the donor cells could be located. In opposite to PIA, only minimal bone and cartilage destruction is observed after transfer. Typically, adoptive transfer of pristane-primed spleen cells has a self-limiting acute disease course. However, Holmberg *et al.* has shown that a chronic relapsing disease can develop in irradiated recipients after transfer of lymph node, but not spleen cells (Holmberg *et al.*, 2004, Manuscript).

From Disease To The Gene

Identification of arthritis-regulating genes in rats is a complicated process and can be separated into two major stages; first, from phenotype and genotype to QTL, and second from QTL to the gene (Figure 3). The first step consists of correlating genetic markers with a phenotype in a segregating population, known as genetic linkage analysis. The second step involves narrowing down the chromosomal region that is correlated with the phenotype to an interval as small as possible, preferable

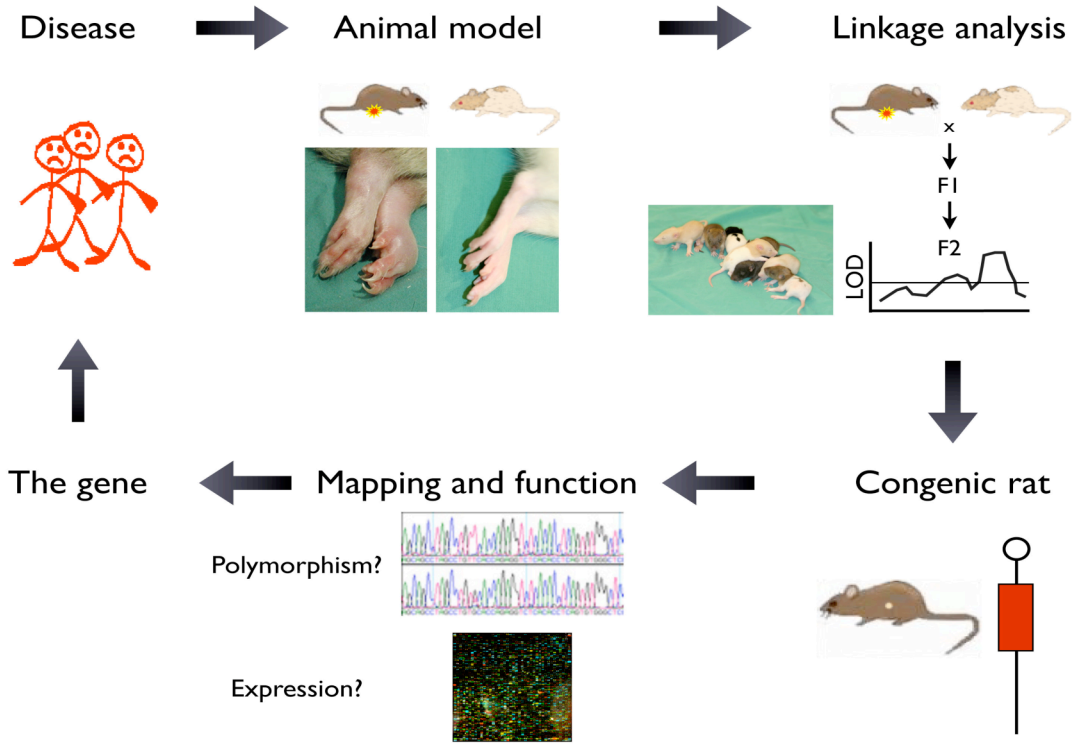

Figure 3. From disease to the gene. Positional cloning strategy of arthritis-regulating genes.

containing only one gene, which is known as positional cloning. Because this is usually not possible, one must assign the phenotype to a single gene by other means, for example functional assays, transgenics or knock-in animals, which is also known as positional candidate gene identification [192].

Mapping Suceptibility Genes

The first step in gene-identification is genetic linkage studies and QTL mapping. These are used to locate the chromosomal positions of arthritis-regulating genes, by the development of segregating crosses of rats. Usually, the segregating population is constructed by crossing two phenotypically distinct, for example arthritis-susceptible and arthritis resistant, and genetically different inbred strains of rats, to produce a first filial (F1) generation. All rats in the F1 population are identical, as they have inherited one chromosome from each parental strain. The F1 population is then either intercrossed to generate a second filial (F2) generation or backcrossed to one of the parental strains (N2 generation). Each rat in the F2 and N2 generation has a unique genotype, due to recombination events during meiosis. Therefore, every individual rat taking part in the linkage study needs to be both phenotypically evaluated for the particular disease trait and genotyped using genetic markers, evenly spaced along the chromosomes in order to detect their parental origin. Linkage analysis is then performed by computer software packages such as Mapmarker [193] or RQTL [194], which first construct a genetic linkage map and then detect the loci related to the phenotype. A measurement of the probability that two loci at the same chromosome are linked is the logarithm of the odds (LOD) score. A LOD score higher than 3 is generally accepted as evidence for linkage, when the whole genome is screened with 100 to 300 markers. Genetic linkage analysis is based on the observation that genes residing physically close on a chromosome remain linked during meiosis. Morgan and colleagues showed that the degree of linkage increases with physical proximity of the genes. For theses genes, the segregation ratio for the genotypes and phenotypes departs from the Mendelian independent assortment ratios. Therefore, if arthritis-regulating genes are often passed to the offspring along with specific marker alleles, then it can be concluded that the genes responsible for the disease are located close by on the chromosome of these markers. A quantitative trait locus (QTL) is defined as a chromosomal region that segregates with a phenotype in a cross between rat strains at a defined statistical significance [146].

Genetic linkage analyses using various arthritis models for RA, including adjuvant-induced arthritis, collagen-induced arthritis, oil-induced arthritis and pristane-induced arthritis, have led to the detection of more than 60 QTLs across the whole rat genome [195]. Nearly every autosome and the X and Y chromosomes, except Rattus norvegicus (RNO)11, 13 and 17, contain at least one arthritis QTL. Some of the QTLs appear to be model specific, for example *Cia7* on RNO2 and *Pia3* on RNO6 [175, 177, 185, 196]. However, a substantial number of QTLs overlap between various arthritis models, such as *Aia1*, *Cia1*, *Oia1* and *Pia1* on RNO20 (rat MHC locus) or *Aia3*, *Cia3*, *Pia5* and *Scwia1* on RNO4 [152, 174, 185, 197-199]. Also RNO1 (*Cia2*, *Pia8*) and RNO10 (*Cia5*, *Oia3*, *Pia10*) have a high number of model-overlapping QTLs [174, 186, 187, 200]. Interestingly, these are also the regions where the most RA-associated genes are located, suggesting that these chromosomes may play an important role in the regulation of arthritis. Overall, there are significantly more RA-associated genes in rat QTL regions as contrasted with non-QTL regions, indicating the relevance of the QTL mapping studies in rat [201].

Congenic Strains

The second step of gene identification is often more tedious and laborious than genetic linkage analyses, because it involves breeding and analysing of many rat generations. Once a QTL has been identified it is important to confirm its location and eventually dissect the locus into such a small region that it will be possible to positionally clone the gene. Traditionally, this is done using the congenic strain approach. Congenic strains are generated by transferring a specific genetic locus from a donor strain to a recipient inbred strain (Figure 4, right) [202]. The function of the donor strain is to provide the differential locus, whereas the recipient strain provides the new genetic background for the differential region. It involves two mating steps. First, the donor and recipient rat strains are outcrossed to introduce the differential locus to the recipient. However, this step also leads to the introduction of undesired donor strain alleles. Therefore, multiple backcrosses to the recipient strain are performed in order to replace the contaminating donor strain alleles with alleles from the recipient strain (Figure 5). In each backcross generation heterozygous carriers of the differential locus are selected for further breeding. With each backcross generation the contaminating donor alleles are statistically reduced by half. According to this equation, congenic animals crossed to the recipient inbred strain for 10 generations will harbour < 0.20% of heterozygous loci in those parts of their genomes not linked to the differential region. By definition, 10 crosses to the recipient inbred strain have to be performed in order to consider the resulting variant as congenic.

Since the generation time of rats is about 3 months, it will take about 3 years to generate such a congenic strain. This considerable time factor resulted in new strategies to create congenic strains in a shorter time by using additional selection criteria. By using marker associated selective breeding protocols congenic animals could be generated in significantly fewer generations (5-6) than with the conventional method. It is based on a genome-wide analysis of genetic polymorphisms. The selection at each backcross generation is based not only on the presence of the differential locus but also on the absence of contaminating donor alleles form other parts of the genome (Figure 4, left) [203, 204]. Lander and Schork introduced the term “speed congenics” in 1994 describing congenic strains developed using such methods [143]. Once a congenic strain has been generated and the QTL has been confirmed the genetic fragment needs to be reduced to a size as small as possible, preferable so it contains only one or a few genes. This is accomplished by the generation of subcongenic lines, which have been selected for recombinant fragments in the region of interest and tested for the disease-regulating phenotypes. Finally, once this locus has been successful reduced to a feasible size, yet still containing several genes, there are several methods in order to identify the disease-regulating gene. This may involve extensive DNA sequencing to identify all genetic polymorphisms, differential expression analyses of all genes in this region and functional assays, such as *in vitro* stimulation of various cell populations, transfection experiments or promotor assays. Also transgenic, deficiency-complementation tests in knock-out, as well as knock-in mice are well-established tools for the evaluation of potential candidate genes. However, the latter techniques are only of limited availability in rats and due to species differences, might not be possible to use for all identified genes. Further, proof of gene-identification may include the verification of those genes in humans when association studies would demonstrate a clear correlation between the functionally relevant allelic variant and the risk of disease.

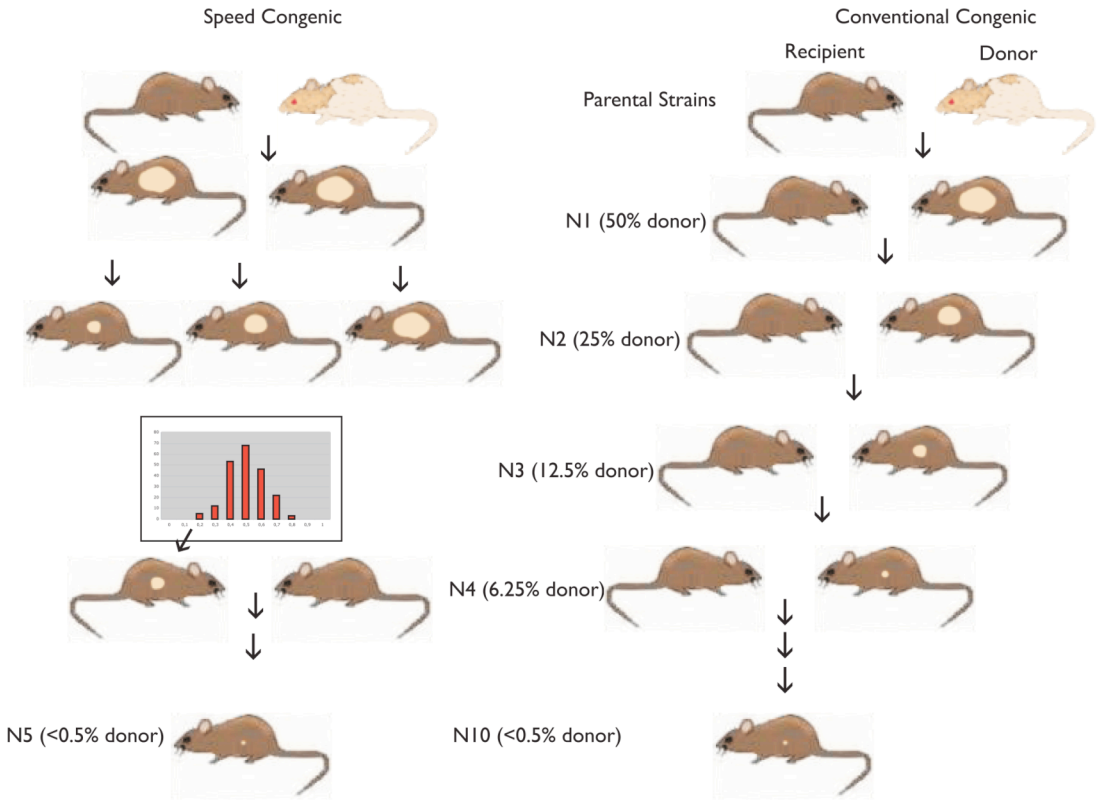

Figure 4. Generation of congenic rat strains strains through conventional backcross breeding (right) or marker associated selective breeding (left).

Although the first linkage studies for arthritis models in rats were reported more than 10 years ago, and more than 60 QTLs have been mapped since, only one gene (*Ncf1*) and one gene-complex (*APLEC*) have been linked to arthritis by positional candidate gene identification so far. The first gene to be identified was *Ncf1*, which was found to be polymorphic in the coding region, leading to an amino acid substitution in the encoding P47phox protein [205]. As part of the NADPH complex, it is involved in the production of radical oxygen species (ROS). Paradoxically, a high production of ROS leads to protection from experimentally induced arthritis, both in rats and in mice [206]. The second genes that have been associated with arthritis in rats are the antigen-presenting lectin-like receptor gene complex (*APLEC*), which encodes immunoregulatory C-type lectin-like receptors [207]. A large number of polymorphisms and many differentially expressed genes complicated the association to a single gene of this complex, and it is possible, that in fact more than one gene of this complex could be responsible for the arthritis-regulating effect.

The phenomenon that one QTL is caused by multiple linked genes, each with a small effect size, may be one explanation for why there has been only limited success in positional gene-identification so far. Actually, there are many examples of this phenomenon in different research areas, and also some of the arthritis-regulating QTLs have been dissected into sub-QTLs, for example *Cia5* (*Cia5*, *a*, *d*) on chromosome 10 in rat and *Cia5* (*Cia5*, *21*, *22*) on chromosome 3 in mouse [208, 209]. Notably, the only QTLs that have led to gene identification were caused by single genes and have had exceptionally large effect sizes [210]. For example, *Pia4*, the positionally cloned QTL harbouring the *Ncf1* gene, had an estimated effect size of 25%, whereas the effect sizes of most unresolved QTLs are believed to be much smaller than 5%.

Alternative Strategies

Several alternative strategies have been proposed to close the gap between QTL identification and gene identification. Many of them are based on recent developments, including new genomic resources (rat genome sequence, SNP database), animal resources (recombinant inbred lines, heterogeneous stock), and techniques (chip-based SNP typing, whole-genome expression studies). Whereas some of the strategies are aiming at detection of new and improved fine-mapping of already existing QTLs, other are targeting the process of gene-identification.

Advanced intercross lines (AIL) are crosses of two parental inbred strains, which have been outcrossed to produce an F1 generation and subsequently intercrossed to produces an F2, F3, etc., until F15 to F20 generation (Figure 5). Through repeatedly and pseudo-randomly intercrossing, the rats accumulate new recombinations in every generation. As for an F2 generation, every rat has a unique phenotype and genotype and thus, must be individually evaluated. Because many more recombination events occur in AIL, the markers for genotyping must be set much more densely than for an ordinary F2 intercross. However, a vast number of SNP data for more than a 100 rat inbred strains have recently become available. Simultaneously the costs for SNP genotyping continued to decrease. Both factors might make the AIL studies more and more popular. Although the generation of an appropriate AIL requires many years and at least 50 breeding couples, it is a valuable tool to map and fine-map QTLs with high resolution [211, 212].

Partial advanced intercrosses (PAI) combine the strategies of AIL and congenic strains and have been used, so far, for fine-mapping and interaction studies in arthritis models in mice [209, 213]. PAIs are advanced intercrosses of two congenic strains and thus, differ only at two loci whereas the rest of the genome is fixed. This limits the costs of genotyping and interactions with other loci and therefore increases the power for detecting the loci of interest. However, the PAI strategy is very time consuming as it first involves the establishment of the congenic strains and then the generation of the advanced intercross between them. With this in mind, it can be a valuable tool of studying interactions between two loci either on different or on the same chromosome, but regarding fine-mapping it is not superior to the conventional congenic approach.

Recombinant inbred (RI) lines are crosses between two inbred strains, which have been outcrossed to produce an F1 generation and then randomly intercrossed for a few generations to accumulate sufficient recombination events. Finally, approximately 100 breeding couples are repeatedly sibling mated for a sufficient number of generations to produce identical offspring (Figure 5). This results in a great number of inbred lines whose genome is a mosaic of the genomes of the parental strains. Once a set of RI lines has been genotyped, the marker data are available for all subsequent mapping experiments. One can also phenotype multiple individuals from the same line to reduce individual, environmental and measurement variability [214]. However, the generation and maintenance of such high number of animals is extremely time consuming and costly and has therefore not been used in many mapping studies. Currently, the largest sets of rat RI lines are derived from the BN and SHR rat strains (32 lines) as well as from F344 and LE (34 lines), which are used for QTL mapping, expression QTL (eQTL) mapping and gene-identification in metabolic and behavioural traits [215, 216].

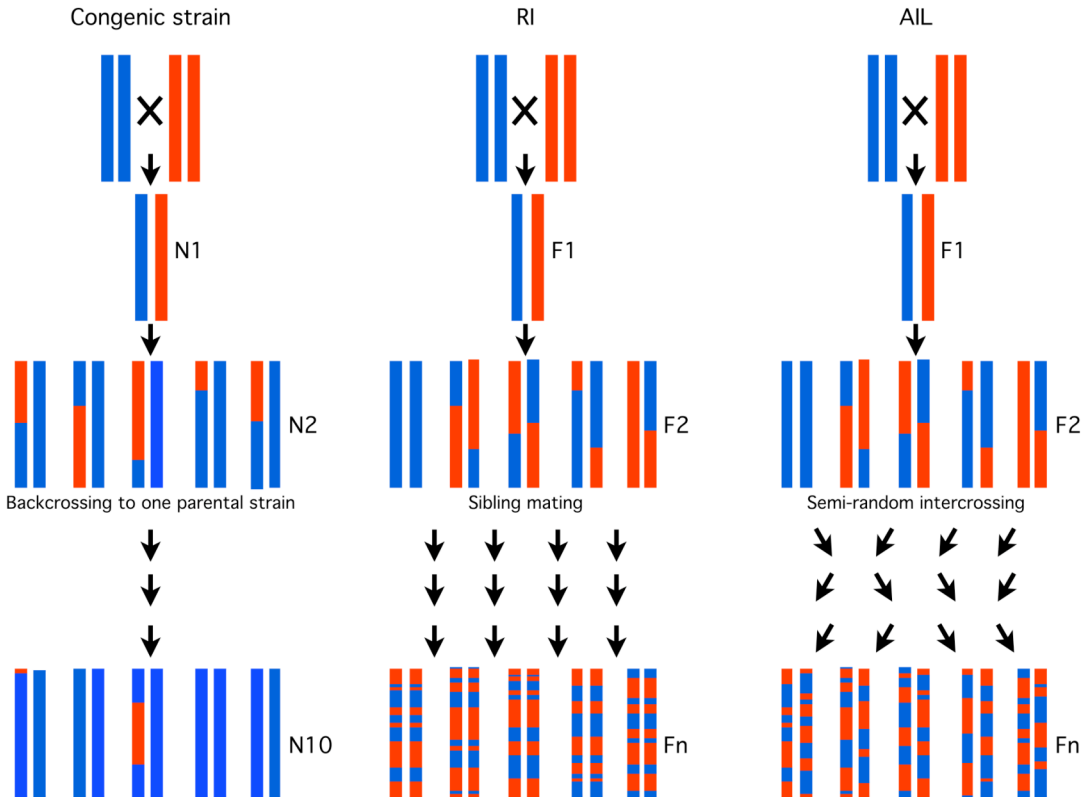

Figure 5. Breeding scheme and genetic setup of three experimental crosses used for gene-mapping.

Heterogeneous stocks are advanced intercross lines of more than two inbred strains. The existing HS stocks in mice and rats are derived from eight strains, but theoretically any number of initial strains can be used. Using a pseudo-random breeding protocol, which reduces the loss of genetic variations in the population, recombinations accumulate in the rats, which allow high-resolution mapping of QTLs with a confidence interval of less than 1 cM. The drawback is that the rats need to be individually phenotyped and genotyped with an even denser marker setting than for ordinary AILs. Genetic variations from many strains complicate the analysis and a special designed QTL mapping program (HAPPY), which estimate the probability that an allele descends from each progenitor strain, had to be developed [217]. The best characterized rat HS panel was established more than 20 years ago and is derived from eight genetically distinct and phenotypically diverse inbred strains (ACI, BN, BUF, F344, M520, MR, WKY and WN) [218]. Panels of these HS rats are maintained in USA and Europe and currently used for mapping studies of a wide variety of traits, including behavioural, metabolic and inflammatory traits. Although, arthritis is not one of them, other inflammatory traits may provide valuable knowledge that can be transferred to the arthritis research.

Knockout rats are still rare among all available rat strains. To date, it is still not possible to culture germline-competent rat embryonic stem cells, the basis for generating traditional knockouts. Yet the knockout strategy has been a basic tool for the proof of gene-identification so far. Some disease models however, will be species specific, and thus, mouse knockouts cannot be used in the process of gene-identification in rats. Nevertheless, in the past years, new strategies have been described to generate gene knockouts in rats. These involve random chemical

mutagenesis techniques or transposon insertional mutagenesis combined with improved high-throughput screening strategies [219]. Knockout rats can eventually be used for gene-identification per se or as a tool for gene-identification in complex traits, by studying QTL-knockout interactions [220].

Gene-expression profiling can help to identify genes when combined with other genetic mapping approaches. New technologies, such as a commercial rat microarray platform, made it possible to perform whole-genome expression analysis of up to 31,000 genes or 850,000 exons, which can be used for comparing inbred or congenic strains. Notably, even for large chromosomal fragments of genetically distant strains the number of differentially expressed genes is relatively small. The identification of *Cd36* as an insulin resistance gene was the first example of cloning a complex-trait gene using combined expression-microarray and linkage approach. In the last two years a significant number of genes have been positionally identified through the integrated use of genetic mapping and gene expression [219, 221].

Many databases focusing on rat genetics or having rat genetics integrated in their program are publicly available and continuously updated. The rat genome database (RGD), Ensembl, US National Center for Biotechnology (NCBI), University of California, Santa Cruz (UCSC) and Mouse Genome Informatics (MGI) are some of the most important sources for **data mining**. They include the rat gene catalogue (position, expression, function), all rat QTLs, simple sequence length polymorphism (SSLP) and SNP map locations and rat strain catalogues. By combining all possible information gathered so far it might be possible to fine-map QTLs and point out candidate genes. For example, when a QTL has been identified in different combinations of inbred crosses, the strain distribution pattern can be combined with mapping data to refine the region, which contains the functional variant. However, this assumes that genetic information from all strains are available and so far, this has not been the case. Currently, there is one additional inbred strain (SHR) to be sequenced but many more are needed in order to provide the tools for so called "in-silico mapping" strategies.

PRESENT INVESTIGATIONS

The first part of this thesis investigates the genetic setup and the phenotypical response towards various arthritis models of three DA rat substrains. The genetic study involved a whole-genome scan using SSLP marker, linkage analysis and the generation of congenic lines. In addition to the genetic experiments, three different models for RA have been tested in these rats. The study originated from our observation of increasing variation of arthritis susceptibility in our previously established DA/ZtmRhd rat colony, which after months of struggle forced us to establish a new DA colony from a different source (DA/OlaHsd). Although this crippled all intended investigations of previously generated DA.E3-*Pia* congenic rats, including QTL fine-mapping and positional cloning of disease-regulating genes, it led, however, to the identification of a new QTL regulating arthritis susceptibility and the characterization of three substrains of DA rats. We found all three substrains to be different in their genetic setup and in their phenotypical response towards various arthritis models.

PAPER I

DA rats from two colonies differ genetically and in their arthritis susceptibility

Aim. The goal of this study was to genetically compare two DA substrains, DA/ZtmRhd and DA/OlaHsd, which have been used in numerous linkage studies in arthritis research. At the same time we wanted to address whether they differ in their arthritis susceptibility or severity following pristane- and collagen-injection.

Results. We performed a whole genome scan using 248 SSLP markers; 35 of them were found to be polymorphic between DA/ZtmRhd and DA/OlaHsd. Using the initially typed markers, we identified seven regions on chromosome 2, 3, 5, 10, 13 and X with two or more consecutive polymorphic markers. After adding additional markers in close proximity to those initially typed, we found only one genetic region on chromosome 3, which contained a large contaminating fragment. Fine-mapping of this locus revealed a fragment size > 15 Mb, with 16 SSLPs and 2 SNPs between both strains. After conclusively establishing the genetic difference between the substrains, we investigated their phenotypic characteristics in two different arthritis models. In both models, DA/OlaHsd rats were found to be more prone to develop severe arthritis than DA/ZtmRhd rats. Because the large contaminating region on chromosome 3 colocalized with a previously reported QTL, *Cia11*, we generated partly congenic lines for this region, to investigate their arthritis susceptibility. Studies of these congenic rats, however, did not detect any arthritis difference between the three possible genotypes (homozygous DA/OlaHsd, homozygous DA/ZtmRhd or heterozygous), and we therefore ruled out this region to be responsible for the phenotypical difference observed in the parental DA substrains.

Discussion. Although we could not confirm the existence of other contaminating regions, we do not rule out this possibility. In fact, later available SNP data revealed several other polymorphic regions besides chromosome 3, including chromosome 1, 2, 7 and 13. Not mentioned in Paper I is the observation, that besides *Cia11* on chromosome 3, two additional *Cia* QTLs colocalized with contaminating fragments,

which were found to be polymorphic in the SNP genome scan. This is *Cia10* on chromosome 2, and *Cia4* on chromosome 7 [175, 222]. Both have been found in DA crosses, either with ACI (*Cia10*) or F344 (*Cia4*) and both have already been confirmed in congenic rats [190, 223, 224].

Future plans. Because the SNP data from the STAR consortium became available only after we had already performed our study, we did not systematically investigate all genetic variations found in that study [225]. However, with this in mind, an F2 intercross between both DA substrains would be an extraordinary opportunity to localize the arthritis QTL(s) that varies between them. Not only is the chance of detection of this/these QTL(s) high, due to a minimum of possible interactions with the background genome, but the contaminating fragments are also extremely small. This minimizes the number of markers to genotype and has a great advantage in proceeding with congenic rats. In addition, the generation of a speed congenic rat derived from these strains would only need 3-4 generations until the background genome is cleared from all other contaminations. Therefore, the proposed future studies building on the findings from paper I would be an excellent tool in fine-mapping and positionally clone arthritis-regulating gene(s).

PAPER II

A spontaneous mutation at rat chromosome 9 protects DACP rats from experimentally induced arthritis

Aim. This study was based on the observation that a spontaneous mutation in our DA/ZtmRhd rat colony resulted in decreased arthritis susceptibility. We aimed to isolate the mutation in a new substrain of DA rats, named DACP, to genetically localize it, and to phenotypically characterize the effects of this mutation.

Results. We performed genetic linkage analysis and identified a new QTL for PIA at chromosome 9, *Pia27*, harbouring the mutation. This QTL also regulates subphenotypes, such as the number of B and T cells, levels of Igs in serum, as well as expression levels of CD25 and number of CD25+ CD4 T cells. Studies from DA and DACP rats supported these findings. Furthermore, these studies established clearly that the mutation is involved in up regulating the number of T cells, which appear to be more activated than T cells in non-mutant DA rats. Controversially, these activated T cells from DACP rats are non-arthritogenic and are unable to provoke disease when transferred into DA rats. Furthermore, we detected no evidence that T cells from DACP rats have increased regulatory functions.

Discussion. Despite large efforts to identify the causative mutation, sequencing and expression analysis of various candidate genes at chromosome 9 did not reveal any difference between DA and DACP rats. In particular the *Vav1* gene, which has been strongly implicated in two different disease models [226](Maja Jagodic, personal communication), was found to be non-polymorphic and not differentially expressed, suggesting that this gene does not account for the actions of *Pia27*. We anticipate that the identification of the *Pia27* regulating gene will generate an important novel candidate gene or candidate pathway for inflammatory diseases. Not mentioned in paper II is the observation that we obtained extremely small confidence intervals for the QTLs found on chromosome 9. It should be mentioned, however, that the genetic map is based on the 51 animals from our cross and might therefore be different from other maps that have been published for chromosome 9. Nevertheless,

for arthritis incidence, for example, the 1.5-LOD support interval was found to be smaller than 7 cM. And for other subphenotypes, which were highly penetrant and appeared to be influenced only by this single QTL, we observed even smaller confidence intervals, for example < 2 cM for the number of B cells and levels of IgG_{2a}, and <3 cM for the number of CD25+ CD4 T cells. Taking advantage of these phenotypes will be extremely helpful in the generation of minimal congenic rats and positional cloning of the underlying gene. In fact, we have already begun to generate a DACP congenic rat harbouring a fragment from the E3 strain. By selective phenotyping of recombinant rats and breeding of only those animals that were found to be positive for the phenotypes, we have minimized the congenic fragment in the third backcross generation to a fragment size of less than 4 cM. However, as discussed in paper II, this region still suffers from incomplete sequencing and most of the SSLP markers used for genotyping are still not annotated or are clearly at the wrong position. This has hampered the development of a correct physical map of this region.

Future plans. Future experiments are therefore focused on testing additional SSLP as well as SNP markers in order to determine the physical size of the congenic fragment. It will also help to identify or rule out possible candidate genes. At the same time we are continuing to sequence already annotated genes in this region and performing gene expression analysis. Detected polymorphisms between DACP and E3 will be utilized in further narrowing down the congenic fragment, while a polymorphism between DACP and DA might be the relevant mutation we seek to identify. The positional identification of the gene regulating arthritis susceptibility and the further characterization of the disease-regulating mechanism will be the goal of this study.

PAPER III

Detection of arthritis susceptibility loci Ncf1 and the major histocompatibility complex region in rats depends on the genetic background

Aim. In paper III, we assessed the influence of the genetic background on the detection capacity of previously described arthritis regulating loci.

Results. We show that the disease enhancing DA allele of *Ncf1* did not break the arthritis resistance of the E3 rat and thus, was silent in this certain genetic background. On the other hand, the E3 allele of the MHC was found to be silent in a pure DA background but protected from arthritis in a mixed (DAxE3) background. In the last part of this study we examined epigenetic effects in F1 hybrids. Although we observed a significantly increased arthritis severity in female rats from a DA mother, the effect was very small and brief. Thus we concluded that epigenetic effects play no major role in the arthritis development in this genetic setup. Because male rats showed nearly no difference in arthritis severity, we exclude the Y-chromosome as a carrier of PIA-regulating genes in this genetic setup.

Discussion. This study reveals a dramatic difference of the arthritis-regulating potential of two previously identified loci in various genetic backgrounds. This needs to be considered in future investigations that involve the isolation of genes in a certain genetic setup or the transfer of those into different genetic backgrounds.

Future plans. A follow up study will focus on the identification of small effect size QTLs for PIA. In addition, we will aim to identify the genetic regions interacting with the MHC locus or other arthritis regulating loci. This involves a whole-genome analysis of the 650 animals from the previously described (E3.DA-*Pia457*xDA)F2 intercross. At the same time we are constructing an E3.DA-Pia1457 quadruple congenic rat, which will be tested for arthritis susceptibility and used for studying possible interactions of *Pia1*, *Pia4*, *Pia5* and *Pia7*.

PAPER IV

Positional cloning of the *Igl* genes controlling rheumatoid factor production and allergic bronchitis in rats

Aim. The aim of this study was to physically map the most prominent QTL for the production of RF (*Rf1*).

Results. Using a strategy that combined congenic mapping and high genetic resolution mapping in an advanced intercross line (AIL) we have positionally cloned the immunoglobulin lambda light chain (*Igl*) locus. This locus explains the previously described *Rf1* quantitative trait locus controlling RF levels in rats. The E3 allele of the *Igl* gene was associated with higher levels of both RF-IgG and RF-IgM in serum. In addition, there is suggestive evidence that the *Rf1* locus may control pathogenic inflammation as it was found to be associated with allergic eosinophilic bronchitis and increased IgE levels.

Discussion. Although RF are found in 70-90% of RA patients, they are not specific for the disease and found in many other chronic inflammatory conditions. However, until now the research has focused exclusively on biological and pathological roles of RF in RA. The findings of this study will allow a more precise dissection of the role of RF in specific pathological pathways occurring in a wide spectrum of inflammatory diseases, including asthma.

Future plans. The main goal in future studies will be the identification of the genetic polymorphism(s) underlying this trait and to conclusively associate the *Igl* locus with the severity of airway inflammation. This will involve the generation and phenotypic testing of recombinant rats with minimal congenic fragments and large-scale sequencing.

CONCLUDING REMARKS

The genetic dissection of complex traits is an intricate process, but when it is successful it will lead to the identification of disease-regulating genes, and elucidating molecular mechanisms involved in the pathogenesis of these traits. These discoveries provide the opportunity for more accurate diagnostics, development of new, more specific drugs and an individual treatment of each patient. For many years, animal models of RA were thought to be the basic tools of hypothesis free gene identification, but so far genetic studies using rats have had only limited success. In fact, researchers have encountered significant difficulties in the analysis of complex traits. This thesis is summarizing two major problems we have faced in the past years. Both problems concern the genetic variability of inbred animals, which were either found to be contaminated or mutated. Hence despite these hassles, we present future perspectives on how we could turn around these difficulties and use them in our favour. Genetic linkage studies of two DA rat strains that are genetically highly similar and yet differ in their arthritis susceptibility might be a fast way in QTL identification and congenic strain development. Positional cloning of a spontaneous mutation that regulates easy accessible and highly penetrant phenotypes will be relatively easy compared to cloning of low penetrant QTLs usually found in arthritis models. Although the exact molecular variant of this gene is highly unlikely to be relevant in human RA, there is the potential that another variant of this gene may be associated with human RA. In addition, the identification of this gene will be extremely valuable in understanding disease regulating mechanisms.

Recent genome-wide association studies in human populations have provided a direct approach of identifying genes in complex traits, hence raising the question about the continuing need of animal genetic studies. Despite the problems we encountered in this thesis, I share the beliefs of other researchers that rat models will be, in many ways, valuble tools in the gene discovery of complex traits [219]. First, genome-wide association studies in humans still have the problem to precisely identify the gene or their mechanisms of actions. For example, disease-associated SNPs are often found in regions with no genes or, alternatively, span several genes. Second, positionally cloned animal QTLs will be excellent tools for the characterization of molecular pathways and study of mechanisms involved in the pathogenesis of the particular disease. These detailed studies would be mostly impossible in humans. Third, the advantages of new technologies, cloning strategies and genomic resources, which are now available for animal studies and continuously develop, will potentially increase the success in gene discovery in rats. After a decade of QTL mapping, we are clearly just at the beginning of understanding the complex genetics in RA and other multifactorial diseases, but it will be exciting to see what in the next 10 or 15 years will be achieved in this area of research.

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