Immunogenetic and immunological aspects of rheumatoid arthritis : DERAA and anti-citrulline reactivity can make the difference

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Immunogenetic and Immunological aspects of Rheumatoid Arthritis

DERAA and anti-citrulline reactivity can make the difference

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Immunogenetic and Immunological aspects of Rheumatoid Arthritis

DERAA and anti-citrulline reactivity can make the difference

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
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prof. dr. J.J. van Rood
dr. A. Ioan-Facsinay
dr. W. van Eeden

Ieder schepsel, ieder wezen
Is als prentenboek te lezen
      En houdt ons een spiegel voor:
Ons bestaan en ons verscheiden,
Onze vreugde en ons lijden
      Geeft het ons in tekens door.

Dichter: Alanus Ab Unsulis

Ca. 12^{e} eeuw, Frankrijk

Vertaald uit het Latijn door Willem Wilmink

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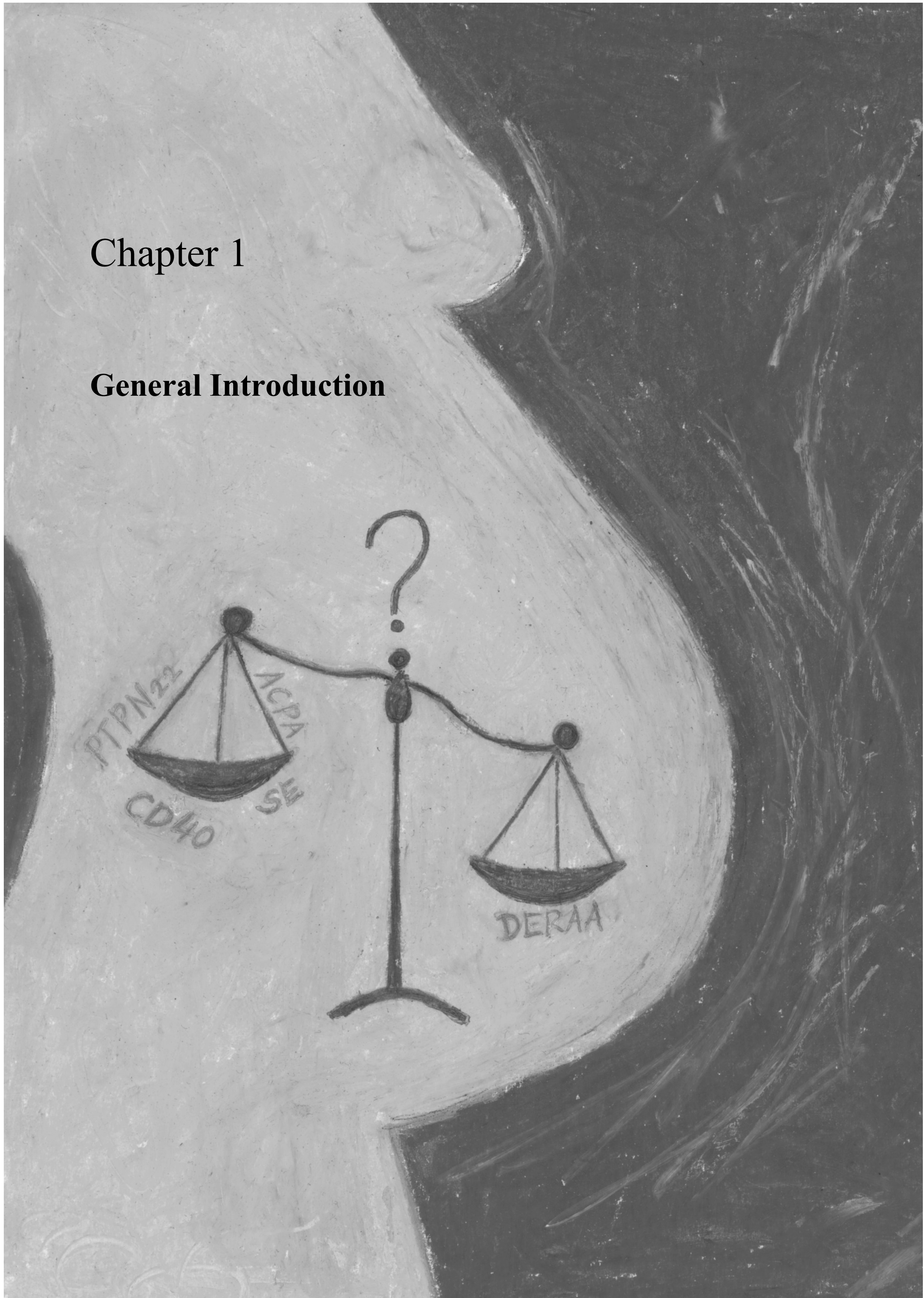
Chapter 1

General Introduction



Undifferentiated and Rheumatoid Arthritis

Arthritis is a group of conditions characterized by inflammation of the joints. This inflammation can lead to breakdown of the cartilage of the joints that can be caused by different mechanisms, e.g. autoimmunity, fractures, wearing, or infection. The different types of arthritis are diagnosed according to disease criteria, leaving cases that cannot be classified in one of the accepted categories of rheumatic diseases (usually referred to as 'undifferentiated arthritis' (UA)). The diagnosis of rheumatoid arthritis (RA), an inflammatory autoimmune disorder characterized by a chronic inflammation of the synovial tissue of several joints, is based on a list of seven criteria developed by the American College of Rheumatology (1). These criteria include clinical, radiological and laboratory findings; i.e. morning stiffness, arthritis of three or more joint areas, arthritis of hand joints, symmetric arthritis, serum rheumatoid factor, rheumatoid nodules, and radiographic changes. The RA patient population is clinically heterogeneous since only four of these seven ACR criteria have to be fulfilled for the diagnosis of RA. The occurrence of RA varies among countries and areas over the world, but has a prevalence of approximately 1% in Europe (2;3). In the Dutch population, women are affected by RA approximately two times more frequently than men (4).

In the Leiden Early Arthritis Clinic (EAC), which provides an inception cohort of patients with recent onset arthritis (5), 37% of the patients are diagnosed with UA and about 20% with RA at their first visit. After 1 year, 32% of the UA patients have qualified for the diagnosis of RA, indicating the complexity of a diagnosis at initial presentation.

RA patients can develop different kind of autoantibodies, amongst others against citrullinated proteins (anti-citrullinated protein antibodies (ACPA)). Citrullination is a post-translational conversion (deimination) of Arginine to Citrulline residues performed by the enzyme peptidylarginine deiminase (PAD) (*Figure 1)* that results in a small change in molecular mass (<1 Da) and the loss of one positive charge. Although citrullination is a common natural process, these ACPA are specific for RA, and can be measured already years before symptomatic disease (6;7). Recently, it has been shown that $ACPA^+$ and $ACPA^-$ RA patients show a different disease course, probably indicating that $ACPA^+$ and $ACPA^-$ RA reflect a totally different disease (8-10).

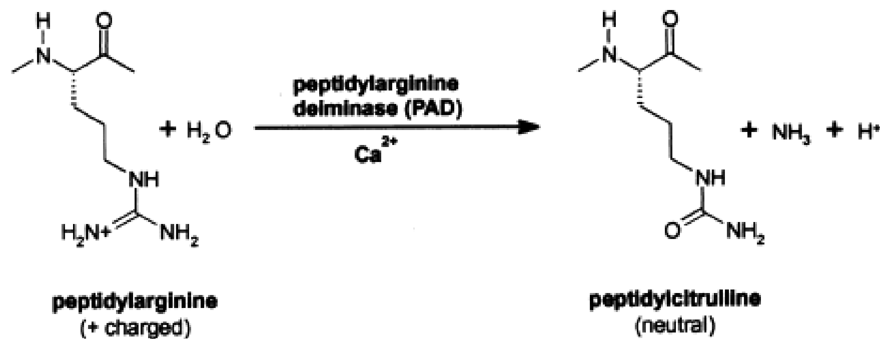


Figure 1. Citrullination. An Arginine residue is enzymatically converted by peptidylarginine deiminase (PAD) into a Citrulline residue in the presence of Ca^{2+}.

Progression and Severity scoring

RA is characterized by the proliferation of synovium and the destruction of cartilage and bone during the progression of the disease. Destruction of the cartilage is a consequence of pro-inflammatory cytokines and enzymes that are released during the chronic inflammation process inducing enhanced breakdown of cartilage matrix and reduced synthesis of matrix components by the articular chondrocytes (11;12), but joint erosion results more directly from osteoclasts (13). The formation of pannus, which results from the proliferating synovium (14-16), will eventually lead to joint space narrowing and joint erosions. This is, at least in part, mediated by fibroblast-like synoviocytes from the synovium (17).

Radiographic joint damage is an important outcome measure in RA, in addition to assessments of physical function and disease activity, which all associate with each other (18). It reflects cumulative disease activity and is related to overall disability (19;20). Therefore, progression rates are also influenced by the current therapy, i.e. disease modifying anti-rheumatic drugs (DMARDs) (21;22). Several scoring methods for the assessment of radiographic joint damage exist, from which the most well known are the Larsen (23) and Sharp-van der Heijde method (24;25). Both methods score the individual joints of the extremities but the Sharp-van der Heijde method scores hands and feet for the amount and severity of erosions and joint space narrowing separately (25). The Sharp-van der Heijde method is sensitive to detect changes over time and shows reliable results since it has a low measurement error (26). Analyses of radiographic progression can be analyzed on the individual patient or a patient group level. For both applies that the radiological progression is linear in the first

(approximately) five years but further in time, the curve levels off to a plateau (18;27-29).
Because nowadays, RA patients are seen in an earlier phase of the disease, before the appearance of well established indicators of poor prognosis such as erosions and nodules, markers which have a good predictive value on radiographic damage in an early phase of the disease will become more important.

Genetic Risk Factors for RA

The pathogenesis of RA is, as in many other autoimmune diseases, complex and largely unknown. It is generally accepted that both genetic and environmental factors contribute and probably also interact with each other. It has been described that genetic factors contribute for about 2/3 to the development of RA (30;31). The contributing risk factors can differ for the susceptibility to, and the progression of RA.
The strongest genetic risk factor, both for susceptibility and severity, has been mapped to the HLA-class II region, most probably DRB1. HLA-class II molecules consist of an α- and β-chain which both have constant and variable regions. The variable regions constitute the binding groove for the peptide to be presented by HLA molecules to T cells of the immune system (*Figure 2*). A particular part of the binding groove, the third hypervariable region, is involved in the susceptibility to RA development. At position 70 to 74 in this third hypervariable region, different variants of amino acid sequences are present. Certain HLA-DRB1 alleles share common epitopes at this position. Regarding the risk for RA development, three variants can be discriminated; either amino acids of the so-called shared epitope (SE), the sequence “DERAA”, or ‘neutral’ amino acids are present. Compared to the ‘neutral’ HLA-DRB1 alleles, carriership of HLA-DRB1 alleles with the SE increases the risk for RA development, and “DERAA”-containing HLA-DRB1 alleles decrease the risk. Both the SE and the “DERAA”-containing HLA-DRB1 allele effects will be discussed in more detail below.

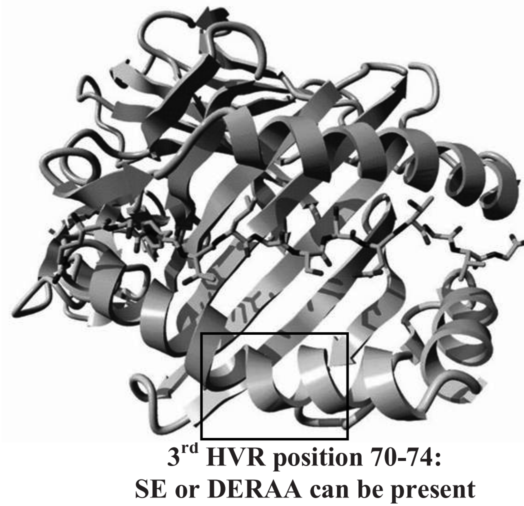


Figure 2. HLA class II molecule (adapted from Boots et al. (32)). The variable regions of the α- and β-chain build up the peptide binding groove. The rectangle indicated in the figure shows the position of the third hypervariable region (HVR) where the shared epitope (SE) or “DERAA”-sequence can be present.

Shared Epitope and ACPA

HLA-DRB1 molecules containing the amino acid sequence R(Q)K(R)RAA (i.e. the amino acids Arginine, (Glutamine), Lysine, (Arginine), Arginine, Alanine, Alanine) at position 70-74 in the third hypervariable region are belonging to the Shared Epitope (SE) alleles. This epitope is present in the HLA-DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001 and *1402 alleles. The SE is associated with an increased risk (about 2.5 times (33)) to develop RA and a more severe disease course. It is postulated that this SE sequence, which is present in the binding cleft of the HLA-DR molecule, is directly related to the binding of RA inducing peptides to the SE-containing HLA-DR molecules. These peptides are then presented to T cells thought to play an important role in the pathogenesis of RA. Since there is high linkage disequilibrium between HLA-DRB1 and HLA-DQB1 alleles, it is also hypothesized that the HLA-DQB1 alleles are associated with RA susceptibility, although these associations cannot be distinguished (34-36). RA-inducing peptides are not identified yet, but several findings indicate new directions for epitope discovery. Recently it has been shown that SE-containing HLA-DRB1 alleles do not confer risk to the development of RA itself, but predispose to the development of anti-citrullinated protein antibodies (ACPA). These antibodies are highly predictive for RA development as discussed in a previous section (6;7). ACPA^{+} UA Patients have approximately

fifteen times higher odds to develop RA within one year compared to ACPA$^-$ UA patients. These ACPA are commonly measured in the IgG isotype, but are also present in the IgM and IgA isotype (37).
The presence of IgG ACPA indicates the presence of T cell help. One of the proteins described to be citrullinated in vivo and present in the synovial fluid of RA patients is the cytoskeletal protein vimentin (38;39). We have recently shown that 90% of ACPA$^+$ RA patients recognizing a citrullinated peptide derived from vimentin carry one or two SE-containing HLA-DRB1 alleles (40), suggesting the involvement of helper T cells recognizing a citrullinated epitope from vimentin in the context of the SE-containing HLA-DRB1 alleles. The identification of citrullinated vimentin-derived T cell epitopes recognized by HLA-DR4 positive individuals is described in **Chapter 4** of this thesis.

"DERAA"

"DERAA" stands for the amino acid sequence of Aspartic acid-Glutamic acid-Arginine- Alanine-Alanine which is present in the HLA-DRB1 alleles of the subtype HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304. These alleles are present in 29% of the population (33;41-43).It has been shown by several groups that the frequency of "DERAA"-containing HLA-DRB1 alleles is reduced in RA patients compared to healthy controls and that the risk to develop RA is reduced by about 40% in DERAA positive individuals (33). Since the "DERAA" sequence is positioned at the same location as the SE in the HLA-DRB1 molecule, the effect of "DERAA" has to be evaluated after stratification for presence of the SE-containing HLA-DRB1 alleles. In this way, it has been shown that the effect of "DERAA"-containing HLA-DRB1 alleles is independent of the SE-containing HLA-DRB1 alleles (33).

"DERAA"-containing HLA-DRB1 alleles can be inherited, but can also be conferred as non-inherited maternal antigens (NIMA). NIMA can be conferred from the mother during and/or shortly after the pregnancy since the immune systems of mother and child are in close contact and cell trafficking will occur (44-47). The phenomenon of NIMA was described for the first time in 1954 for the RhD antigen (48) and is illustrated in *Figure 3*. The terminology is oriented from the point of view of the child in a family, since most studies are coming from the transplantation field. It has been described that haplo-identical NIMA-mismatched sibling transplants have a graft survival similar to that of HLA-identical siblings in contrast to NIPA-mismatched siblings, indicating tolerance for the HLA-mismatch from the mother (49-51).

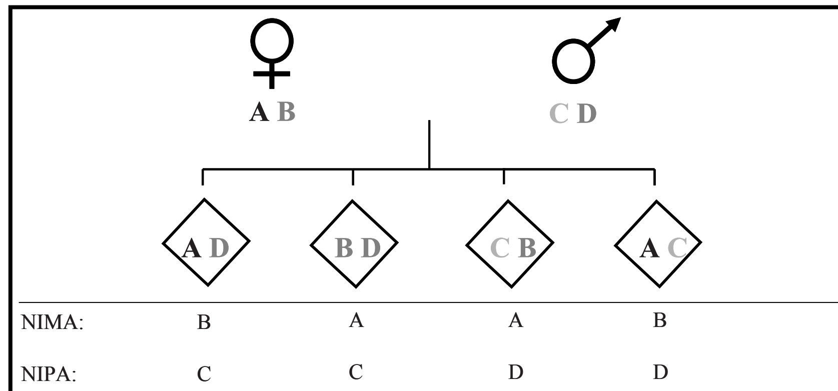


Figure 3. Terminology of non-inherited maternal antigen (NIMA) and non-inherited paternal antigen (NIPA). The terminology is orientated from the point of view of the child. ◊ gender can be male or female.

The phenomenon of protection against RA by "DERAA"-containing HLA-DRB1 alleles as NIMA is studied in **Chapter 2** of this thesis and discussed in comparison with the inherited effect in **Chapter 3**.

Next to HLA-DRB1 there are several other genes involved in the risk for RA development. Below, only the additional risk alleles studied in the context of this thesis are discussed.

PTPN22

The protein Tyrosine phosphatase named Lyp is encoded by the protein Tyrosine phosphatase, non-receptor type 22 (PTPN22) gene and is expressed by many cell types present in haematopoietic tissues, like T cells, B cells, NK cells, monocytes, dendritic cells and neutrophils (52).

Genes can differ in their nucleotide sequence between different individuals in a population. When the frequency of a single nucleotide change is equal or higher than 1%, this is called a single nucleotide polymorphism (SNP). The most studied SNP of the PTPN22 gene, the C1858T missense single-nucleotide polymorphism, is associated with RA, UA (52-56) and several other autoimmune diseases (57-59). Upto now, it seems that the SNP does not have an effect on the severity of RA, only on the susceptibility (53;60).

There is a large variation in allele frequency among different ethnic populations among the world of the T-variant of the allele, with a variation only in Europe from 2-

3% in Italians to 15% in Finnish people (61). There are several articles describing the association of the PTPN22 T allele of the C1858T polymorphism with the development of $ACPA^{+}$ RA, therefore implicating a correlation of this SNP with the production of ACPA (62-64).

We investigated in **Chapter 5** whether the C1858T polymorphism of the PTPN22 gene can give additive value to the prediction of progression from UA to RA when it is combined with presence of ACPA.

Genome wide association studies (GWAS)

In the past few years several genome wide association studies (GWAS) have been performed to scan the entire genome for common polymorphisms associating with different autoimmune diseases, including RA (65-68). Since thousands of patients and controls from different populations are studied in the GWAS, these studies may identify risk factors with modest effects on the risk for RA development, and also allow one to study subpopulations of patients for specific effects.

From all genes identified to be associated with RA, we studied based on a recent GWAS (67) six newly identified SNPs for their influence on the severity of RA (**Chapter 6**). These SNPs are located around genes that are either involved in activation of the immune response (CD40, TNFRSF14, CCL21 and PRKCQ) or play a role in intracellular processes involving cell cycle and homeostatis (MMEL1, KIF5A and CDK6).

Statistical modelling

Several statistical models may be applied to the analysis of genetic associations. The kind of statistical model appropriate for the analysis is dependent on the correlation between the variables and measurement groups, the distribution of the data and the type of study performed. Below, two models are discussed that are used for the different genetic association chapters described in this thesis.

Theory of Bayes

To study the effect of numerous factors on the development of a disease, modelling of the risk factors studied is performed to fit the observed data with the expected frequencies. Modelling of all these risk factors preferably results in prediction of the outcome. A theory that calculates the probability that a hypothesis is true based on the available information is the theory of Bayes, which is used for the studies performed in

Chapter 2 and 5 of this thesis. With this theory, a prior probability (e.g. to develop RA), based on the risk of an individual in a certain population, is converted into a posterior probability, calculated on the basis of extra information derived from the risk factors studied. The prior probability is most often the prevalence of e.g. a certain disease (or symptom) in the population (69-71).

Linear Mixed Model

A mixed model is a multiple variance analysis often used to compare groups of individuals including multiple measurements from one individual e.g. in time. The data of every individual are plotted in a linear way, and combined for the whole group, thereby intrapolating missing values (72). In a mixed model both random and fixed effects are included. Fixed effects are categorical variables from which all levels are fixed and known, whereas random effects are variables where only a random sample of possible values is measured. A Gaussian distribution is assumed for the variables studied. Since all individual measurements are taken into account in one analysis, it is a powerful method with low intra-individual variability and therefore smaller group sizes are required (73). A linear mixed model was used in Chapter 6 of this thesis to study the effect of different SNPs on the severity of RA.

Outline of the thesis

Chapter 2 reports a family study in which we studied whether "DERAA"-containing HLA-DRB1 alleles protect against the development of rheumatoid arthritis not only when the alleles are inherited but also when they are present as a non-inherited maternal antigen (NIMA).

Chapter 3 is a review that summarizes the epidemiological findings about "DERAA"-containing HLA-DRB1 alleles and RA and the observation we made in Chapter 2. Both associations are compared and possible explanations for the protective phenomenon are described.

On the same location in the HLA-DRB1 molecule as the "DERAA" epitope, the so-called shared epitope (SE) can be present. These SE-containing HLA-DRB1 alleles predispose to the formation of ACPA and therefore the development of RA. Since ACPA-producing B cells are helped by T cells, we studied in **Chapter 4** whether we

could identify possible T cell epitopes from an important ACPA target antigen, namely the citrullinated vimentin protein.
Both for ACPA and the C1858T polymorphism of the PTPN22 gene associations with RA development have been described. We studied the individual contribution of ACPA and a PTPN22 SNP on the prediction of RA development from UA in **Chapter 5**. We also studied the influence of the PTPN22 SNP on the level of ACPA.

Genes can be involved both in the susceptibility and severity of RA. This is the case e.g. for the SE-containing HLA-DRB1 alleles. Therefore, we studied in **Chapter 6** the contribution of several SNPs that were newly identified as risk factors for the development of RA and the severity of the disease.

The results obtained in the chapters 2-6 of this thesis are summarized and discussed in **Chapter 7**.

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Chapter 2

Protective effect of non-inherited maternal HLA-DR antigens on Rheumatoid Arthritis development

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Abstract

Rheumatoid arthritis (RA) is a complex genetic disorder in which the HLA-region contributes most to the genetic risk. HLA-DRB1-molecules containing the amino-acid sequence "DERAA" (i.e. HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304) are associated with protection from RA. It has been proposed that not only inherited but also non-inherited HLA-antigens from the mother (NIMA) can influence RA-susceptibility. Up to now, no protective NIMAs were described. Here, we studied whether "DERAA"-containing HLA-DRB1-alleles as NIMA are associated with a protective effect.

Hundred-seventy-nine families were studied, 88 from the Netherlands and 91 from the UK. The frequency of "DERAA"-containing HLA-DRB1-alleles of the Dutch mothers (16.1%), but not of the fathers (26.2%), was lower compared to the general Dutch population (29.3%; p=0.02). This was replicated in the English set of patients and controls (p=0.01). Further, of all families, 45 contained at least one "DERAA"-negative child with RA and at least one "DERAA"-positive parent. The odds for the "DERAA"-negative RA patients of having a "DERAA"-positive mother was significantly lower as compared to having a "DERAA"-positive father (OR 0.25; p=0.003).

These data show a protective NIMA-effect in a human autoimmune disease and indicate that a "DERAA"-positive mother can transfer protection against RA to her "DERAA"-negative child.

Introduction

Rheumatoid arthritis (RA) is a complex genetic disorder in which the HLA-region contributes most to the genetic risk. Especially HLA-DRB1 molecules sharing a common epitope, R(Q)K(R)RAA,(i.e. the amino acids Arginine, (Glutamine), Lysine, (Arginine), Arginine, Alanine, Alanine) at position 70-74, the so-called shared epitope (SE), are associated with both susceptibility to and severity of RA (1-4). At the same position of the HLA-DRB1 molecules as the SE, the amino acids "DERAA" (i.e. the amino acids Aspartic acid, Glutamic acid, Arginine, Alanine, Alanine) can be present. Individuals carrying HLA-DRB1 alleles that express this "DERAA"-sequence ("DERAA"-positive individuals) ("DERAA" is present in HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304) have a lower susceptibility to develop RA and less severe disease compared to individuals with 'neutral' (SE- and "DERAA"-negative) HLA-DRB1 alleles. "DERAA"-containing HLA-DRB1 alleles protect in both SE-negative and SE-positive individuals and therefore this effect is independent of the effect of SE-alleles (5).

It has been proposed that not only inherited but also non-inherited HLA-antigens from the mother (NIMA) as opposed to those from the father (NIPA) can influence the immune reactivity of an individual with implications for tissue transplant survival and susceptibility to autoimmune disease (6-8). During pregnancy the immune systems of mother and child are in close contact and trafficking of cells, antibodies and/or antigens can occur. Confrontation of the fetal/newborn immune system with the NIMA may have a lifelong influence on the immune response of the child. It has been shown in transplantation studies, that haplo-identical NIMA-mismatched sibling transplants had a graft survival similar to that of HLA-identical siblings, whereas NIPA-mismatched sibling transplants did as poorly as did recipients of maternal and paternal grafts (9).

We have described that HLA-DR4 or SE NIMA but not HLA-DR4 or SE NIPA are associated with susceptibility to RA, because HLA-DR4 or SE-negative RA patients have more often a HLA-DR4 or SE-positive mother compared to a HLA-DR4 or SE-positive father (10, 11). This observation was confirmed in one study (8) while in two other studies there was a non-significant trend in the same direction (12, 13). When the studies were combined a significant HLA-DR4 and SE NIMA effect in DR4 or SE negative patients was observed (8). This is not or less clearly the case for HLA-DR4 or SE-positive RA patients (10, 11, 14). The strongest genetic risk factors for type I

diabetes, HLA-DR3-DQ2 and HLA-DR4-DQ8, are also more frequent in mothers as compared to fathers of patients negative for one or both of these antigens (7).
As there is so far no evidence for a protective effect in human autoimmune disease for NIMA we were interested to study whether "DERAA"-containing HLA-DRB1 alleles as NIMA are associated with a protection against RA.
To answer this question, 88 Dutch and 91 English families were typed for HLA-DRB1. Families in which the RA patient did not carry a HLA-DRB1 allele containing "DERAA" and either the father, the mother or both carried "DERAA"-containing HLA-DRB1 alleles, were analyzed for the presence of a NIMA effect mediated by "DERAA"-containing HLA-DRB1 loci.

Patients and Methods

Dutch RA families: 88 consecutive patients with RA fulfilling the 1987 ACR criteria were recruited in 1996 in two outpatient clinics: 37 from the Leiden University Medical Centre, Leiden, and 51 from the Jan van Breemen institute, Amsterdam. At time of inclusion, both parents of the patient had to be alive. Blood samples were drawn from patients and their parents to perform HLA-DRB1 typing.

Dutch Controls: A randomly selected panel of 423 healthy unrelated Dutch individuals served as control population for the Dutch HLA-DRB1 allele frequencies (5).

Dutch control families: HLA-DRB1 typings of 208 healthy mothers and child pairs were analyzed to control for the specificity of a possible NIMA effect of "DERAA"-containing HLA-DRB1 alleles in the RA families. These families were collected from a database (36) that includes deliveries that took place in of the Obstetric Department of the Leiden University Medical Centre.

English families: Potential multi-case RA families were notified from a number of sources including consultant rheumatologists, routine questioning of patients in clinics and direct approaches via the media. Especially families with sibling pairs or extended affected pedigrees were identified (37). The diagnosis was confirmed by a trained rheumatologist. Diagnostic classification was based on the modified 1987 ARA Criteria (38). Blood samples of all individuals were taken for HLA-DRB1 typing. For the NIMA analysis, all "DERAA"-negative children with RA of each family were taken into account.

English controls: An English Caucasian study population from the Allele Frequency Database consisting of 177 individuals was used as control population for the English HLA-DRB1 allele frequencies (39).

HLA genotyping

HLA-DRB1 alleles were determined in all RA patients, their parents, brothers, sisters and controls. In the English families, seven typings of the HLA-DRB1 alleles of either the mother or the father were deduced from the alleles present in the other family members.

HLA-DRB1 typing for the Dutch individuals was performed as described previously (11). In England HLA-DRB1 typing was performed by polymerase chain reaction, using specific primers and hybridization with sequence-specific biotin labeled oligonucleotides (Dynal kit, Dynal Biotech, Wirral, UK). In four of the 88 fathers and one of the 88 mothers no definitive HLA-DRB1 allele could be assigned. Therefore, these individuals were excluded from the analysis.

The following HLA-DRB1 alleles were classified as containing the "DERAA" epitope: HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304.

Statistics

The patient characteristics of the Dutch and English patients were compared with either a Chi-square (dichotomous variables) or independent T-test (continuous variables). For the patient groups of table 3 (<30 individuals per group), the patient characteristics were compared with the Fischer exact and Mann-Whitney tests.

The "DERAA" frequencies of the mothers and the fathers of both the Dutch and English RA patients were compared separately to the "DERAA" frequency in the Dutch and English healthy control populations, respectively, by using a Chi-square test. In the Dutch healthy control population, the frequency of "DERAA"-containing HLA-DRB1 alleles in women and men was also compared.

An association between the presence and absence of the "DERAA"-containing HLA-DRB1 alleles as a NIMA or a NIPA was calculated using odds ratios with 95% confidence intervals combined with a Chi-square test. The observed frequency of "DERAA"-positive mothers was compared to the expected frequency using a binomial test. The expected frequency was calculated with the method of Bayes and a comparable distribution of the English and Dutch families contributing to this analysis was taken into account for the calculation of the expected frequency. These analyses

were performed for parents of patients not carrying a “DERAA”-containing HLA-DRB1 allele.
The Chi-square, independent T-tests and the Binomial test were performed using SPSS_12.0 Software (Chicago, IL, USA). The odds ratios and 95% confidence intervals were calculated using Statcalc Software (EpiInfo version 5, Statcalc, December 1990).

Results

Two different data sets were studied: Dutch RA patients with their parents and English RA patients with their brothers, sisters and parents. The characteristics of both data sets at the time of taking the blood sample for HLA-DRB1 typing are listed in Table 1.

Table 1. Clinical and laboratory characteristics of the Dutch and English patients used for this study.

	Dutch (n=88)	English (n=223*)
Age at onset (years)	30	32
Disease duration (years)	7.8	11.5
female sex (%)	86.5	79.8
Rheumatoid Factor positive (%)	57	84
SE positive (%)	74	86
Erosive disease (%)	87	84

*The age at onset and disease duration show the mean values in years. Disease duration is the duration of rheumatoid arthritis at the time of taking the sample for HLA-DRB1 typing. The positivity of rheumatoid factor was also determined at the time of taking the blood sample for HLA-DRB1 typing. *out of 91 (multi-case) families.*

Both patient populations had a comparable age of onset, sex distribution and a similar percentage of patients with joint erosions. The young age at onset is probably due to the selection of patients with living parents. The English patients were more often rheumatoid factor (RF) and SE positive and had a longer disease duration at the time the blood sample for HLA-DRB1 typing was taken. These differences are most probably the consequence of including multi-case families in the English data set and mainly single case families (only 1 multi-case family) in the Dutch data set. Patients of

multi-case families more often are carriers of predisposing HLA-DRB1 alleles (the SE-alleles), often have more severe disease and therefore have a higher frequency of rheumatoid factor antibodies (15). Since these differences were as expected and were not considered to interfere with our research question, the patients from both data sets were pooled for some analyses.

The frequency of "DERAA"-containing HLA-DRB1 alleles present in the Dutch RA patients ("DERAA"-positive RA patients) (14.6%) was significantly lower than that of the Dutch healthy control population (29.3%; p=0.007). A similar observation was made in the English patients (only the oldest RA child of every family was included) as the frequency of "DERAA"-containing HLA-DRB1 alleles (8.6%) was significantly lower than that of the English control population (23.8%; p=0.002). These data confirm the protective effect associated with "DERAA"-containing HLA-DRB1 alleles. Before studying a possible effect of "DERAA"-containing HLA-DRB1 alleles as NIMA, we studied whether there was no difference in inheritance of "DERAA"-containing HLA-DRB1 alleles from fathers or mothers to their children. Therefore, we analyzed the frequency of fathers and mothers that have passed on a "DERAA"-containing HLA-DRB1 allele to "DERAA"-positive RA patients. As expected, "DERAA"-containing HLA-DRB1 alleles were equally inherited from fathers or mothers in both the Dutch and English families (data not shown). These data indicate that there is no gender difference in inheritance of "DERAA"-containing HLA-DRB1 alleles.

If non-inherited "DERAA"-containing HLA-DRB1 alleles of the mother protect the child to RA development, it is expected that the frequency of mothers of RA patients bearing a "DERAA"-containing HLA-DRB1 allele is lower compared to the general population. Therefore, we determined whether the frequency of "DERAA"-containing HLA-DRB1 alleles of mothers and fathers of RA patients was different as compared to controls. The frequencies of "DERAA"-containing HLA-DRB1 alleles of the mothers and fathers of the 88 Dutch RA families were therefore compared with the frequency of "DERAA"-containing HLA-DRB1 alleles of a Dutch healthy control population (Table 2). Twenty-two Dutch fathers (26.2%) carried a "DERAA"-containing HLA-DRB1 allele whereas in only 14 mothers (16.1%) a "DERAA"-containing HLA-allele was present. When these frequencies were compared to the frequency of "DERAA"-containing HLA-DRB1 alleles in a Dutch healthy control population (29.3 %), the mothers showed a significantly lower frequency (p=0.02) compared to the control

population. In contrast, the frequencies of the fathers of the RA patients and the individuals of the healthy control group were comparable.

Table 2 "DERAA" frequency of mothers and fathers of Dutch and English RA patients compared with healthy Dutch and English controls.

	"DERAA"+ n =	"DERAA"- n =	frequency (%)	OR	(95% CI)	p-value
Dutch						
Mothers of RA patients	14	73	16.1	0.46	(0.24-0.88)	**0.02***
Fathers of RA patients	22	62	26.2	0.86	(0.49-1.50)	0.66
Contr. Fam. Mothers	67	141	32.2	1.15	(0.79-1.67)	0.51
Healthy controls	124	299	29.3			
English						
Mothers of RA patients	9	82	9.9	0.35	(0.15-0.80)	**0.01***
Fathers of RA patients	14	75	15.7	0.60	(0.29-1.22)	0.18
Healthy controls	42	135	23.8			

"DERAA"+: carriership of one or two "DERAA" containing HLA-DRB1 alleles. "DERAA"-: no "DERAA"- containing HLA-DRB1 allele present. Contr. Fam. Mothers: Mothers of the control population from the Department of Obstetrics of the Leiden University Medical Centre. OR= odds ratio compared to healthy controls. 95% CI= 95% confidence interval.

These findings were replicated in the English multi-case families from Manchester. In these English RA families 9 mothers out of a total of 91 (9.9%) carried a "DERAA"-containing HLA-DRB1 allele, compared to 14 fathers (15.7%). When these frequencies were compared to the frequency of "DERAA"-containing HLA-DRB1 alleles in the population of English Caucasians (23.8%), the frequency of "DERAA"-containing HLA-DRB1 alleles of the mothers was significantly reduced (p=0.01) in contrast to the frequency of "DERAA"-containing HLA-DRB1 alleles of the fathers (p=0.18). The fact that the "DERAA" frequency of the fathers was also (non-significantly) lower that that of the controls is probably due to the fact that the English families were multi-case families which are expected to have a lower frequency of the protective "DERAA"-containing DRB1 alleles.

To exclude the possibility that the difference in “DERAA”-containing HLA-DRB1 allele frequency between the mothers and fathers is due to a general difference in “DERAA”-containing HLA-DRB1 allele frequency between males and females, the frequencies of “DERAA”-containing HLA-DRB1 alleles in males and females of the Dutch healthy control cohort were analyzed. Fifty out of 186 women carried one or two “DERAA”-containing HLA-DRB1 alleles (26.8%) compared to 67 out of 232 men (29.5%). These frequencies were not significantly different (OR= 0.91; 95%CI 0.58-1.42; p=0.73), indicating that the lower frequency of “DERAA”-containing HLA-DRB1 alleles in the mothers as compared to the fathers of RA patients points to a mother-specific effect of “DERAA”-containing HLA-DRB1 alleles on the child.

To further ascertain that the observed difference in frequency of “DERAA”-containing HLA-DRB1 alleles between mothers and fathers of RA patients could indeed be attributed to an effect of non-inherited HLA-antigens, the “DERAA”-positive families with a “DERAA”-negative child (the RA patient) were selected for further analysis. The patient characteristics of this group were comparable to the data shown in Table 1 except for a borderline significant difference in sex in the English patient group (p=0.04). Since the patient characteristics between the Dutch and English patients (as shown in Table 1) only differed for the expected characteristics (RF, SE and disease duration), the patients were pooled for further analysis. From the 45 families fulfilling the selection criterion, 17 “DERAA”-positive mothers and 32 “DERAA”-positive fathers were identified (Table 3).

Table 3 Mothers of “DERAA”-negative RA patients carry less often a “DERAA”-containing HLA-DRB1 allele than fathers.

	DERAA+ n =	DERAA- n =	frequency (%)	OR	(95% CI)	p-value
Mothers	17	28	37.8	0.25	(0.09-0.65)	0.003*
Fathers	32	13	71.1			

The data of the English and Dutch families are combined.
“DERAA”+: carriership of at least one “DERAA”-containing HLA-DRB1 allele.
“DERAA”-: no “DERAA”- containing HLA-DRB1 allele present. The frequency is the percentage DERAA-positive individuals. OR= odds ratio of mothers compared to fathers.
95% CI= 95% confidence interval.

The odds ratio (OR) for "DERAA"-negative RA patients of having a "DERAA"-positive mother compared to a "DERAA"-positive father was 0.25 (95% CI 0.09-0.65; p=0.003). The observed frequency of "DERAA"-positive mothers (37.8%) was also significantly decreased compared to the expected frequency (53.6%; p=0.02). When the data of the 45 families were stratified for SE status of the patient (i.e. either no SE alleles or heterozygous or homozygous for SE) no significant differences were observed between the OR of the DERAA-NIMA versus -NIPA between the different subgroups (data not shown), indicating that the observed NIMA effect of DERAA-containing HLA-DRB1 alleles is probably independent of SE status. However the numbers in the different subgroups were small, particularly for the SE negative patients.

Finally to exclude that also in non-RA families there is a NIMA effect of "DERAA"-containing HLA-DRB1 alleles, a Dutch control population (mother-child pairs from the LUMC Department of Obstetrics) was analyzed. The frequency of "DERAA"-containing HLA-DRB1 alleles in both the mothers (32.2%, Table 2) and children (30.3%) were comparable with that of the Dutch healthy control population (29.3%), showing that there is no (NIMA) effect of "DERAA"- containing HLA-DRB1 alleles in healthy control families. These results together show that there is a protective effect of "DERAA"-containing HLA-DRB1 alleles as NIMA on development of RA of the child.

Discussion

It has been proposed that not only inherited but also non-inherited HLA-antigens from the mother (NIMA) as opposed to those from the father (NIPA) can influence the immune reactivity of an individual. A beneficial NIMA effect has been demonstrated in organ and bone marrow transplantations (6, 9, 16) and a susceptibility-effect of HLA class II molecules as NIMA were shown to be associated with susceptibility to rheumatoid arthritis and diabetes (7, 10, 11). Although it has been shown that diabetes is transmitted less frequently to the offspring of diabetic women than those of diabetic men, no relationship with HLA alleles or other genetic variations was described (17, 18) and therefore, direct evidence for a protective effect of HLA antigens as NIMA in autoimmune diseases is thus far lacking. In this study we show that there is a protective effect of HLA-DRB1 molecules that contain the amino acid sequence "DERAA" as NIMA on the development of RA. The odds ratio (OR) for "DERAA"-negative RA patients of having a "DERAA"-positive mother compared to a

"DERAA"-positive father was 0.25. These data show a protective NIMA-effect in a human autoimmune disease and indicate that a "DERAA"-positive mother can transfer protection against RA to her "DERAA"-negative child.

HLA-DRB1 molecules play a large role in the genetic risk of developing RA. At position 70-74 of the HLA-DRB1 molecules either the amino acids of the SE (R(Q)K(R)RAA) can be present or the amino acids "DERAA". The odds ratio of individuals carrying HLA-DRB1 alleles that express the "DERAA"-sequence (HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304) compared to individuals with "neutral" (SE- and "DERAA"-negative) HLA-DRB1 alleles to develop RA is 0.5-0.7, indicating that "DERAA"-positive individuals have a lower susceptibility to develop RA (5, 19-21). Since the odds ratio of "DERAA" was corrected for SE-alleles, it can be concluded that the "DERAA"-containing HLA-DRB1 alleles are independently associated with a reduced risk to develop RA. The mechanism of protection is unknown, but it has been proposed that it is mediated by T cells recognizing peptides containing the "DERAA"-sequence presented by HLA-DQ molecules (22). Whether these T cells have a regulatory phenotype or are deleted in the thymus by negative selection is still a subject of research. Our observation of a protective effect of "DERAA"-containing HLA-DRB1 alleles as NIMA on RA development gives a new dimension to the direction of this research.

During pregnancy, cells of the mother migrate to the fetus and may induce lifelong microchimerism in the child (23-25). Maternal microchimerism has been shown in mice to induce neonatal B cell (26) and probably also T cell (27) tolerance and is therefore one of the possible mechanisms for NIMA effects (28). Although speculative, we postulate therefore that the protective effect of the DERAA-containing HLA-DRB1 alleles as NIMA on the development of RA is most probably mediated by maternal cells entering the bloodstream and tissues of the child which exert their effect through a change in the immune repertoire and most likely the T cell repertoire of the child. These maternal cells might influence thymic selection or act in the peripheral lymphoid organs, for example as a consequence of the sustained presence of cells from the mother in the child. It has been shown that maternal microchimeric cells can be present in many different cell subsets (29) in both healthy and diseased individuals (30, 31) in which they may exert different effects (32, 33). Likewise, immune regulatory mechanisms might directly be induced in the fetus as it has recently been described that the fetus can already develop cytotoxic T cells directed at a maternal minor H antigen

in utero (34) or becomes sensitized against foreign antigens to which the mother is exposed during pregnancy (35).
Further studies on the intriguing interplay between the developing immune system of the child and cells from the mother are needed both to increase our understanding on how NIMA can influence the immune system of the child and to learn whether and if so how this might be used to combat autoimmune diseases.

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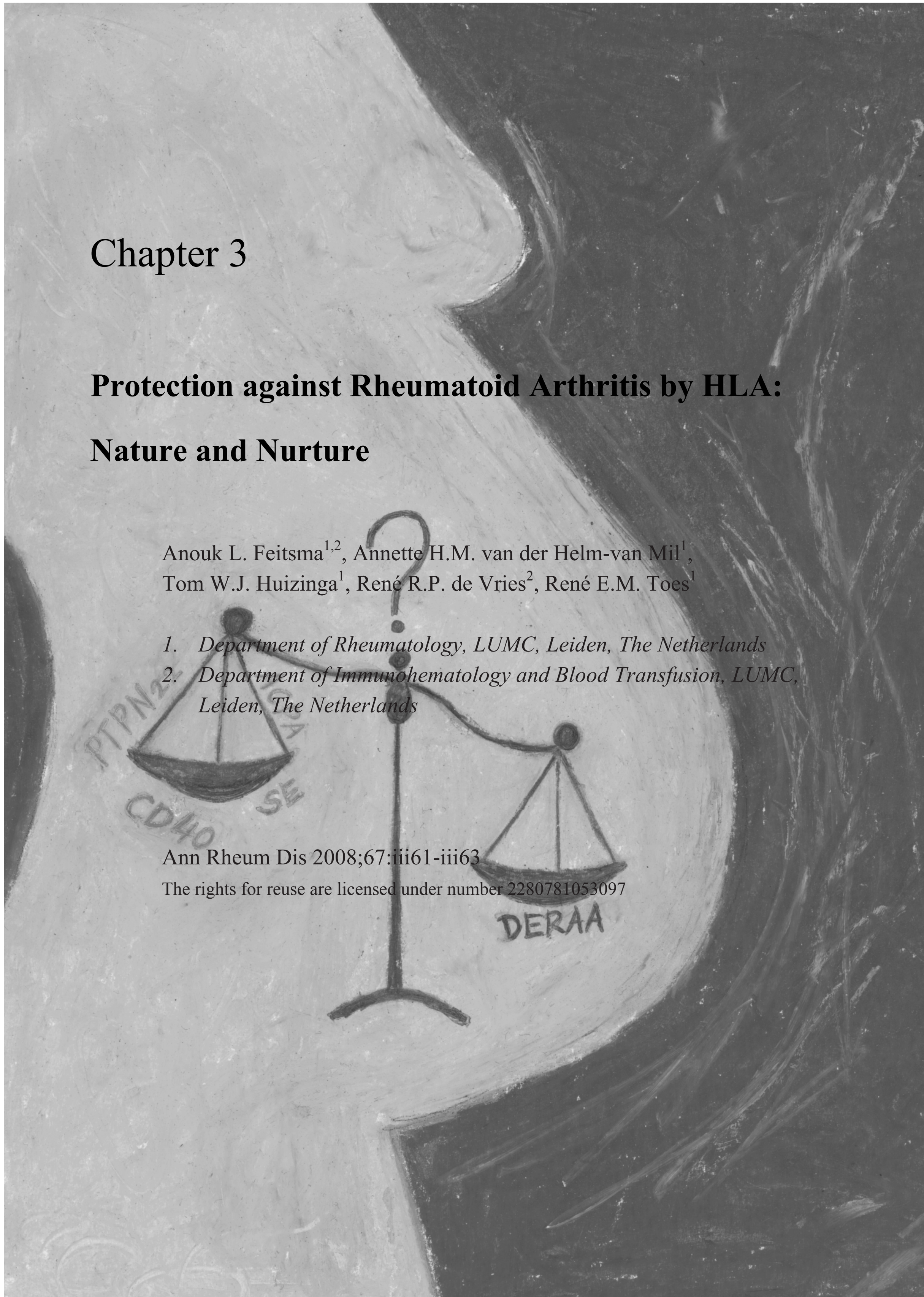

Chapter 3

Protection against Rheumatoid Arthritis by HLA: Nature and Nurture

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Abstract

The human HLA-region contributes most to the genetic risk for Rheumatoid arthritis (RA), either in a predisposing- or a protective fashion. HLA-DRB1-molecules containing the amino-acid sequence “DERAA” at position 70-74 of the DRbeta chain are less often present in RA-patients compared to controls. It has been proposed that antigens transmitted from the mother, but not genetically inherited (called NIMA), can influence RA-susceptibility. Confrontation of the fetal/newborn immune system with the NIMA is supposed to have a lifelong modulating impact on the immune response of the child and thus on the chance to develop RA. Up to now, no protective NIMAs were described in autoimmunity. Recently, we studied whether “DERAA”-containing HLA-DRB1-alleles as NIMA are associated with a protective effect. We showed a protective NIMA-effect in RA as “DERAA”-positive mothers -in contrast to “DERAA”-positive fathers- could transfer protection to their “DERAA”-negative child. The implications of this finding as well as possible explanations are discussed.

Summary

Rheumatoid arthritis (RA) is a complex genetic disorder in which the HLA-region contributes most to the genetic risk. HLA-DRB1-molecules containing the amino-acid sequence QKRAA/QRRAA/RRRAA (i.e. HLA-DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001 and *1402) at position 70 to 74 in the third hypervariable region of the DRB1 chain are associated with susceptibility to RA. HLA-DRB1 molecules containing the amino acids “DERAA” (i.e. HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304) at the same position are associated with protection from RA.

Interestingly, not only inherited but also non-inherited HLA-antigens from the mother can influence RA-susceptibility. We have recently described a protective effect of “DERAA”-containing HLA-DRB1 alleles as non-inherited maternal antigen (NIMA).

The underlying mechanism of this protective effect is currently unknown, although a possible explanation is covered below. In this review, an overview of the current knowledge on protection against RA is given and the inherited and NIMA effect of “DERAA”-containing HLA-DRB1 alleles are compared.

HLA-DRB1 "DERAA"-positive alleles protect against RA

Rheumatoid arthritis (RA) is a complex genetic disorder in which the HLA-region contributes most to the genetic risk. Especially HLA-DRB1 molecules sharing a common epitope, R(Q)K(R)RAA, (i.e. the amino acids Arginine, (Glutamine), Lysine, (Arginine), Arginine, Alanine, Alanine) at position 70-74 in the third hypervariable region of the DRB1 chain, the so-called shared epitope (SE), are associated with both susceptibility to and severity of RA (1-4). The shared epitope is present in the HLA-DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001 and *1402 molecules. At the same position as the SE, the amino acids “DERAA” (i.e. the amino acids Aspartic acid, Glutamic acid, Arginine, Alanine, Alanine) can be present in other HLA-DRB1 molecules (i.e. HLA-DRB1*0103, *0402, *1102, *1103, *1301, *1302 and *1304). Individuals carrying HLA-DRB1 alleles that express this “DERAA”-sequence display a lower susceptibility to develop RA and suffer from less severe disease as compared to individuals with ‘neutral’ (SE- and “DERAA”-negative) HLA-DRB1 alleles. The odds ratio of individuals carrying HLA-DRB1 alleles that express the “DERAA”-sequence compared to individuals with “neutral” (SE- and “DERAA”-negative) HLA-DRB1 alleles to develop RA is 0.5-0.7, indicating that “DERAA”-

positive individuals have a lower susceptibility to develop RA (5-8). The protective effect associated with “DERAA” is also found after stratification for the presence or absence of HLA-SE alleles. This indicates that the protective effect associated with “DERAA”-expression cannot be explained by an overrepresenation of SE alleles in patients, resulting automatically in a lower frequency of other HLA alleles in RA patients. Thus, the “DERAA”-containing HLA-DRB1 alleles are independently associated with a reduced risk to develop RA (5).
It is unclear whether the entire “DERAA” motif is essential for the protection or that only certain amino acids of this motif confer the same effect. In contrast to several reports showing the protective effects by “DERAA”-containing HLA-DRB1 alleles to the development and severity of RA (5,9,10), other reports hypothesize that the amino acids “RAA” at position 72-74 in the third hypervariable region influence the susceptibility to RA development whereas the amino acids at position 70 and 71 modulate this effect (11,12). In these articles it is indicated that HLA alleles expressing the ^{70}ERAA^{74} sequence or the Aspartic acid (D) at position 70 both have a lower frequency in RA patients as compared to healthy controls. Further, it has also been described that protection is mainly associated with the Aspartic acid (D) at position 70 (8,13).
Thus, despite these differences in nomenclature and stratification, it is getting increasingly clear that some HLA alleles confer susceptibility, whereas others are associated with protection.
The mechanism of protection is unknown, but it has been proposed that it is mediated by T cells recognizing peptides containing the “DERAA”-sequence presented by HLA-DQ molecules (14). Whether these T cells have a regulatory phenotype or are deleted in the thymus by negative selection is still a subject of research.

Non-inherited "DERAA" from the mother also gives protection to the child for RA development

In 1954 Owen *et al.* described that Rhesus D (RhD) negative children were tolerant to the RhD antigen when they had a RhD positive mother, probably due to exposure to the RhD antigens during pregnancy (15). This was the first time that a biological effect of non-inherited maternal antigen (NIMA) was described. This terminology is exemplified in Figure 1. Confrontation of the fetal/newborn immune system with the NIMA may have a lifelong influence on the immune response of the child. This phenomenon has considerable implications for transplantation and most studies on

NIMA are coming from the transplantation field. Claas *et al.* described that renal transplant patients often do not generate antibodies against the mismatched HLA antigens of their mother in comparison to those of their father and are therefore tolerant for this HLA mismatch when they are transplanted. This is associated with a longer transplant survival (16-18). This is exemplified best in a study by Burlingham showing that haplo-identical NIMA-mismatched sibling transplants have a graft survival similar to that of HLA-identical siblings, whereas NIPA-mismatched sibling transplants did as poorly as did recipients of maternal and paternal grafts (19).

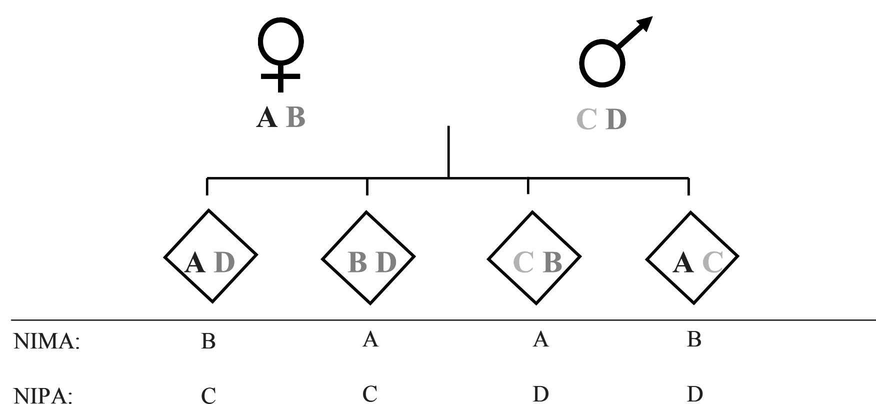

Figure 1. Terminology of non-inherited maternal antigen (NIMA) and non-inherited paternal antigen (NIPA). The terminology is orientated from the point of view of the child. ◊ gender can be male or female.

We have recently shown that there is also a protective effect on the development of RA of HLA-DRB1 molecules that contain the amino acid sequence “DERAA” when presented as NIMA on the development of RA (20). We anticipated that if non-inherited “DERAA”-containing HLA-DRB1 alleles of the mother protect the child to RA development, it is expected that the frequency of mothers of RA patients bearing a “DERAA”-containing HLA-DRB1 allele is lower compared to the general population. Indeed, using a cohort of Dutch RA patients together with their parents, we were able to show that the mothers of RA patients showed a significantly lower frequency (16.1%) of “DERAA”-containing HLA-DRB1 alleles compared to the Dutch control population (29.3%; $p = 0.02$). In contrast, the frequencies of “DERAA”-containing HLA-DRB1 alleles in the fathers of the RA patients (26.2%) and the individuals of the healthy control group were comparable. These findings were replicated in the English

multi-case families from Manchester. To further ascertain that the observed difference in frequency of “DERAA”-containing HLA-DRB1 alleles between mothers and fathers of RA patients could indeed be attributed to an effect of non-inherited HLA-antigens, the “DERAA”-positive families with a “DERAA”-negative child (the RA patient) were selected for further analysis. For this analysis, the patients from the UK and the Netherlands were pooled. The odds ratio (OR) for “DERAA”-negative RA patients of having a “DERAA”-positive mother compared to a “DERAA”-positive father was 0.25 (95% CI 0.09-0.65; p=0.003). These results together show that there is a protective effect of “DERAA”-containing HLA-DRB1 alleles as NIMA on development of RA of the child.

Table 1. Comparison of the inherited and NIMA effect of “DERAA”

	Mother	Child	RA patients (n = 89)	Controls (n = 206)
A	pos	pos	7	39
B	neg	pos	6	23
C	pos	neg	8	26
D	neg	neg	68	118

The Dutch families were used for this analysis [20]. Group B vs D is the inherited effect. OR = 0.45 (0.16-1.25); Group C vs D reflects the NIMA effect. OR = 0.53 (0.21-1.31). Pos = positive (hetero-zygote) for “DERAA”-containing HLA-DRB1 alleles. Neg = negative for “DERAA”-containing HLA-DRB1 alleles.

Thus, together these data indicate that both “DERAA”-containing HLA-DRB1 alleles inherited from one of the parents and the presence of “DERAA” containing HLA-DRB1 alleles as a NIMA protect against the development of rheumatoid arthritis. The question that arises from these observations is how the strengths of both effects compare to each other. To answer this question, both effects were compared in the same set of patient and control families. Only Dutch families (20) were included in this analysis for a proper comparison to the control families. The data depicted in table 1 indicate that, indeed, the effect of “DERAA”-containing HLA-DRB1 alleles as NIMA is as strong as the effect observed in case the “DERAA”-alleles are inherited directly from one of the parents. Although not significant (over 7000 families would be required to discriminate whether the inherited and non-inherited protection differ significantly or not), these data indicate that both effects are of the same magnitude.

The comparable effect size described here is similar as the observations made in the transplantation setting (19).

This result would be in line with the assumption that only very few cells can exert the protective effect. One of the few cell populations that can give rise to many different cell types and has a life long existence are the stem cells. During pregnancy the immune systems of mother and child are in close contact and trafficking of cells, antibodies and/or antigens can occur. Therefore the most plausible explanation for the observed NIMA effect of "DERAA"-containing HLA-DRB1 molecules is maternal microchimerism.

Moreover, because the NIMA effect is not taken into account in most studies analyzing the contribution of the HLA system to RA susceptibility, these data also indicate that the association would be even more prominent in case an effect of "DERAA" as NIMA would have been considered.

Microchimerism as a possible mechanism of the NIMA-effect

During pregnancy there is a bidirectional maternal-fetal lymphocytic transfer (21). Occurrence of these cells starts after about three months of gestation and persists till delivery (22). It is shown that the levels of fetal DNA in the circulation of the mother increase during these six months and disappear for the largest part after delivery (23). During pregnancy also cells of the mother migrate to the fetus and may induce lifelong microchimerism in the child (21,24,25). Maternal microchimerism has been shown in mice to induce neonatal B cell (26) and probably also T cell (27) tolerance and is therefore one of the possible mechanisms for NIMA effects (28) Although speculative, we postulate therefore that the protective effect of the DERAA-containing HLA-DRB1 alleles as NIMA on the development of RA is most probably mediated by maternal cells entering the bloodstream and tissues of the child which exert their effect through a change in the immune repertoire and most likely the T cell repertoire of the child. These maternal cells might influence thymic selection or act in the peripheral lymphoid organs, for example as a consequence of the sustained presence of cells from the mother in the child. It has been shown that maternal microchimeric cells can be present in many different cell subsets (29) in both healthy and diseased individuals (30,31) in which they may exert different effects (32,33). Likewise, immune regulatory mechanisms might directly be induced in the fetus as it has recently been described that

the fetus can already develop cytotoxic T cells directed at a maternal minor H antigen *in utero* (34) or becomes sensitized against foreign antigens to which the mother is exposed during pregnancy (35). Although the presence of maternal microchimerism is not rare, there are several reports that the amount of microchimerism influences the sensitivity of an individual to certain diseases (30,31,36,37).
The observation that the inherited and NIMA effect of "DERAA" have approximately similar effect size strengthens the idea that the NIMA effect is caused by lifelong circulating microchimeric cells that play a role in the thymic selection and therefore influence the T cell repertoire. Only when the inherited and the NIMA acquired "DERAA" have the same mechanism of induction of protection, the similar strength can be explained.

Overall, we can conclude from the data presented in this review that the presence of "DERAA"-containing HLA-DRB1 molecules can protect an individual against the development of rheumatoid arthritis. The "DERAA"-containing HLA-DRB1 molecules can either be present since the individual has inherited them directly or because the individual had a "DERAA"-positive mother and acquired some of the "DERAA"-containing HLA-DRB1 molecules during fetal and/or neonatal life. The protective effect that is acquired in either way is of similar strength, which suggests that already a low amount of cells can initiate this protective effect. Further research is required to elucidate the mechanism of protection of both the inherited as the NIMA effect of the "DERAA"-containing HLA-DRB1 molecules. Such research might be very rewarding as it could guide the way to the development of novel therapies initiating protection in a similar manner as provided by "DERAA"-positive mothers to their "DERAA"-negative children.

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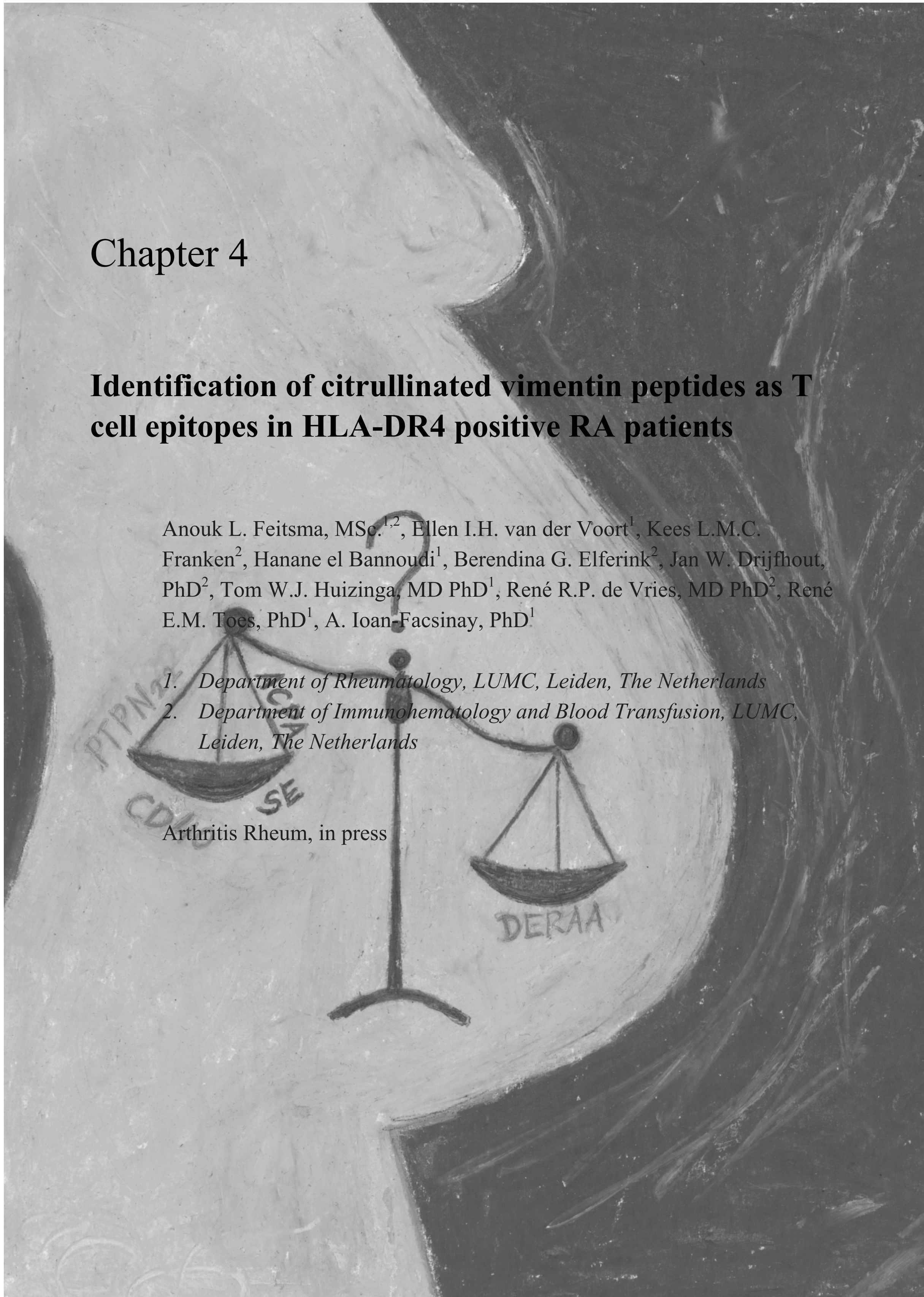

Chapter 4

Identification of citrullinated vimentin peptides as T cell epitopes in HLA-DR4 positive RA patients

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Abstract

Objective. Antibodies directed against citrullinated proteins (ACPA) are highly specific for rheumatoid arthritis (RA). The production of ACPA is most likely dependent on the presence of T cells as ACPA have undergone isotype-switching and associate with the shared epitope-containing HLA-DRB1 alleles (SE). Vimentin is a likely candidate-protein for T cell recognition since over 90% of patients harbouring ACPA reactive with (peptides derived from) citrullinated vimentin carry SE-containing HLA-DRB1 alleles.

The aim of this study was to identify citrullinated vimentin-peptides presented to HLA-DRB1*0401 restricted T cells.

Methods. HLA-DR4-transgenic mice were immunized with all possible citrulline-containing peptides derived from vimentin and T cell reactivity was analyzed. Peptides recognized in a “citrulline”-specific manner by T cells were selected and analyzed for their ability to be processed from the entire vimentin protein. A first inventory for recognition of selected epitopes by T cells from HLA-DR4^{+} ACPA^{+} RA patients was performed.

Results. A “citrulline”-specific response was observed for two of the peptides analyzed. These peptides are naturally processed from the vimentin protein as citrullinated vimentin was recognized by peptide-specific T cells. T cell reactivity against these peptides was also observed in cell cultures from RA patients.

Conclusion. We have identified for the first time two naturally processed peptides from vimentin that are recognized by HLA-DRB1*0401 restricted T cells in a “citrulline”-specific fashion. These peptides can be recognized by T cells from HLA-DR4^{+} ACPA^{+} RA patients as shown in a first inventory.

Introduction

Rheumatoid Arthritis (RA) is a chronic, systemic, inflammatory autoimmune disease, characterized by the presence of autoantibodies. Among the autoantibodies described in RA, antibodies directed against citrullinated proteins (ACPA) are highly specific and predictive for RA (1-3) and can be detected in approximately 70-80% of long-standing RA patients. Antigens recognized by ACPA are present in the inflamed joint (4;5). The production of ACPA is most likely dependent on the presence of T cells as ACPA have undergone isotype-switching.
HLA class II alleles are the most important genetic risk factor for RA. Notably HLA-DRB1 molecules sharing a common epitope, R(Q)K(R)RAA, at position 70-74 in the third hypervariable region of the DRB1 chain, the so-called shared epitope (SE), are associated with both susceptibility to and severity of RA (6-9). It has been shown that the SE-containing HLA-DRB1 alleles predispose to ACPA$^+$ disease, but not to ACPA$^-$ RA (10), and that they are associated with the production of ACPA (11). The latter provides an additional indication for the existence of T cell responses underlying the production of ACPA.

Several human proteins were found to be citrullinated *in vivo*, one of which is vimentin, also known as the Sa-antigen (12-15). Citrullinated vimentin has been shown to be present in the synovial fluid of RA patients and to be recognized by ACPA in approximately 40% of RA patients (13;16-18). Furthermore, we have shown that over 90% of ACPA$^+$ RA patients recognizing a citrullinated peptide derived from human vimentin (19) and 80% of ACPA$^+$ RA patients recognizing the Sa-antigen (unpublished data) carry at least one SE-containing HLA-DRB1 allele. These observations not only show that the SE-containing HLA-DRB1 alleles influence the specificity of the ACPA-response, but also suggest that vimentin could be a protein involved in the recruitment of T cell help for ACPA-producing B cells.
Here, we examined whether we could identify CD4$^+$ T cells that are specific for citrullinated peptides from the human vimentin protein presented in the context of the most frequent SE-containing HLA-DRB1 allele, HLA-DRB1*0401. To this end, we used an unbiased approach by testing the capacity of all possible citrullinated peptides derived from human vimentin to induce T cell responses in DR4-transgenic mice. Epitope mapping in HLA-transgenic mice, both for class I and II, has been shown to be a reliable method to identify T cell epitopes that are also recognized by human T cells (20-24), and therefore we anticipate that the epitopes identified in HLA-transgenic mice are prime candidates for recognition by human T cells. We identified two

naturally-processed peptides of citrullinated vimentin that were recognized by HLA-DRB1*0401 restricted T cells in a "citrulline"-specific manner. Reactivity against these two peptides was also observed with peripheral blood mononuclear cells (PBMC) of HLA-DRB1*04$^+$ ACPA$^+$ RA patients.
These studies identified, for the first time, human "citrulline"-specific T cell responses against naturally processed epitopes from an autoantigen present in the inflamed joint. Therefore, they provide a rationale for more comprehensive analyses in RA patients.

Material and Methods

Mice

DR4-Transgenic mice (HLA-DRB1*0401, -DRA1*0101, hCD4 transgenic mice) lacking endogenous MHC class II were kindly provided by L. Fugger (25) and bred in the in-house mice facility.

Patients and Controls

Blood was obtained from HLA-DRB1*04$^+$ healthy donors after informed consent and PBMC were isolated by ficoll-paque. Patients carrying at least one HLA-DRB1*04 allele who were positive for ACPA, were recruited from the Leiden Early Arthritis Clinic (EAC) (26). Patient characteristics are shown in Table 1.

Peptides/protein

The vimentin gene was amplified by PCR and cloned by Gateway Technology (Invitrogen, San Diego, CA) in a bacterial expression vector containing an N-terminal histidine tag. The protein was overexpressed in Escherichia coli BL21(DE3) and purified by immobilized metal chelate affinity chromatography on Ni-NTA beads, as described before (27). The protein was citrullinated in a 0.1 M Tris pH7.6 solution by adding PAD type II (20 U/ml) (from rabbit skeletal muscle, Sigma Aldrich) and 10 mM $CaCl_2$ for 3 hours at 55 ºC.
The peptides were chemically synthesized at the peptide facility of the Leiden University Medical Centre (LUMC) and dissolved in PBS/0.05% DMSO. Every peptide was designed with a citrulline in the middle of the peptide with a total length of 19 amino acids. In total, the vimentin protein contains 43 arginine residues, but 33 peptides were synthesized (vim1-33, Table 2) in citrullinated form since some peptides contain two citrulline-residues in close proximity. Peptides able to induce a T cell

response in DR4-transgenic mice were also synthesized in non-citrullinated form. The citrullinated peptides were grouped in six pools of five peptides and one pool of three peptides.

Table 1. Patient characteristics

Patient	Age	gender	HLA-DRB1	
1	62	female	0404	1101
2	53	female	0301	0401
3	75	female	0401	0408
4	49	female	0101	0401
5	49	male	0401	0101
6	59	female	0401	0901
7	68	male	0401	14
8	38	male	0401	1404
9	62	female	0401	10
10	54	female	0404	1301

PBMC from 10 ACPA+ RA patients were isolated and tested for the presence of T cell responses against the identified citrullinated vimentin epitopes. The age (in years), gender and HLA-DRB1 alleles of each patient are depicted. From the patients tested for anti-Sa antibodies, 66% was positive.

Chapter 4

Immunization protocol and epitope mapping

DR4-transgenic mice were injected with 100 μl of the peptide pools or individual peptides (100 μg/peptide) emulsified in complete Freunds adjuvant (CFA, Difco) subcutaneously in the base of the tail. On day 21, the mice were boosted with the same peptide pool or peptide (100 μg/peptide) emulsified in incomplete Freunds adjuvant (IFA, Difco) subcutaneously in the flank. On day 42-49 after the first immunization, spleen cells were isolated and restimulated once with the immunizing antigen (10 μg/ml peptide) at a density of 4x10E6 cells per well in 24-wells plates in culture medium (IMDM/8%FCS/penicillin/streptomycin/0.02 mM β-mercapto ethanol). Four days later, cells were harvested with 2mM EDTA and centrifuged on ficoll-paque gradient. Next, cells were rested for another 3 days at a density of 10E6 cells per well in the presence of 3 cU/ml rIL2. On day 7, cells were harvested and tested at the

Table 2. Peptides synthesized from the vimentin protein

nr	AA sequence citrullinated peptide	AA sequence non-citrullinated peptide
1	MST**X**SVSSSSY**XX**MFGGPG	
2	**X**SVSSSSY**XX**MFGGPGTAS	
3	MFGGPGTAS**X**PSSS**X**SYVT	
4	GTAS**X**PSSS**X**SYVTTST**X**T	
5	SS**X**SYVTTST**X**TYSLGSAL	SS**R**SYVTTST**R**TYSLGSAL
6	**X**TYSLGSAL**X**PSTS**X**SLYA	
7	GSAL**X**PSTS**X**SLYASSPGG	
8	SSPGGVYAT**X**SSAV**X**L**X**SS	
9	YAT**X**SSAV**X**L**X**SSVPGV**X**L	
10	**X**L**X**SSVPGV**X**LLQDSVDFS	
11	AINTEFKNT**X**TNEKVELQE	
12	EKVELQELND**X**FANYIDKV	
13	**X**FANYIDKV**X**FLEQQNKIL	
14	EQLKGQGKS**X**LGDLYEEEM	
15	DLYEEEM**X**EL**XX**QVDQLTN	
16	VDQLTNDKA**X**VEVE**X**DNLA	
17	NDKA**X**VEVE**X**DNLAEDIM**X**	
18	DNLAEDIM**X**L**X**EKLQEEML	
19	EKLQEEMLQ**X**EEAENTLQS	
20	EAENTLQSF**X**QDVDNASLA	
21	DNASLA**X**LDLE**X**KVESLQE	
22	KPDLTAAL**X**DV**X**QQYESVA	
23	FADLSEAAN**X**NNDAL**X**QAK	
24	AAN**X**NNDAL**X**QAKQESTEY	
25	QAKQESTEY**XX**QVQSLTCE	
26	KGTNESLE**X**QM**X**EMEENFA	
27	AANYQDTIG**X**LQDEIQNMK	
28	QNMKEEMA**X**HL**X**EYQDLLN	
29	ALDIEIATY**X**KLLEGEES**X**	
30	**X**KLLEGEES**X**ISLPLPNFS	
31	LPNFSSLNL**X**ETNLDSLPL	LPNFSSLNL**R**ETNLDSLPL
32	LPLVDTHSK**X**TLLIKTVET	
33	TLLIKTVET**X**DGQVINETS	

Every peptide number (nr) is followed by the amino acid (AA) sequence of the synthesized peptide. X = Citrulline, R = Arginine

indicated cell concentrations in round-bottom 96-wells plates. 100,000 irradiated spleen cells from a naive mouse and the different peptide pools/peptides (10 μg/ml/peptide) or recombinant (citrullinated) vimentin (20 μg/ml) were added to each well. As a positive control, either 20 cU/ml rIL2 or PHA (1 μg/ml) was used. Every condition was tested in triplo. Four days later, ^{3}[H]-Thymidine was added to the wells for 16 hours. The plates were harvested in the Tom-Tec Mach3 harvester (Perkin Elmer, The Netherlands) and counts were measured using the 1450 Microbeta counter (Perkin Elmer, Groningen, The Netherlands). To determine the restriction of the T cell response, blocking antibodies against HLA-DR (B8.11.2) (28) were used.

ELISA

Supernatants from the stimulated spleen cells were removed before addition of ^{3}[H]-Thymidine and IFNγ was measured using a standard sandwich ELISA. The rat-anti-mouse coating- and detection antibodies were purchased from BD Pharmingen. Streptavidin-HRP (Sanquin) and ABTS (Sigma-Aldrich) were used as enzyme and substrate, respectively. Stimulation indices (SI) were calculated by dividing the amount of IFNγ produced upon antigenic stimulation by the amount produced by non-stimulated cells. The error bars represent the standard error of the mean.

Intracellular cytokine staining

Peripheral blood mononuclear cells (PBMC) from healthy individuals or patients from the EAC cohort were isolated and 3x10E6 cells/well were cultured for 2 hours in a 24-wells plate with the different citrullinated peptides (10 μg/ml) or Memory Mix (mix of Candida Albicans (0.005%), Tetanus Toxoid (0.75 Lf/ml) and tuberculin purified protein derivative (PPD) (5 μg/ml)) as a positive control. After removal of the antigen, the cells were cultured for 4 days in IMDM/5% pooled human serum/penicilline/ streptomycin. On day 4, 2x10E6 autologous PBMC were plated in 24-wells plates and non-adherent cells were removed after two hours of incubation. 1-2x10E6 Cultured cells were added to the adherent antigen presenting cells and restimulated with antigen overnight. For the last 14 hours of stimulation, 3 μg/ml Brefeldin A (Sigma-Aldrich) was added to the wells. Next, cells were stained for the cell surface markers CD3, CD4 and CD45RA (20 min, on ice) and intracellular IFNγ was stained using the BD cytofix/cytoperm^{TM} fixation/ permeabilization solution kit (BD Biosciences) according to the manufacturers instructions. Antibodies both for surface and intracellular markers were purchased from BD Biosciences. Data acquisition and analysis were performed on a LSRII with FACS DIVA Software (BD Biosciences).

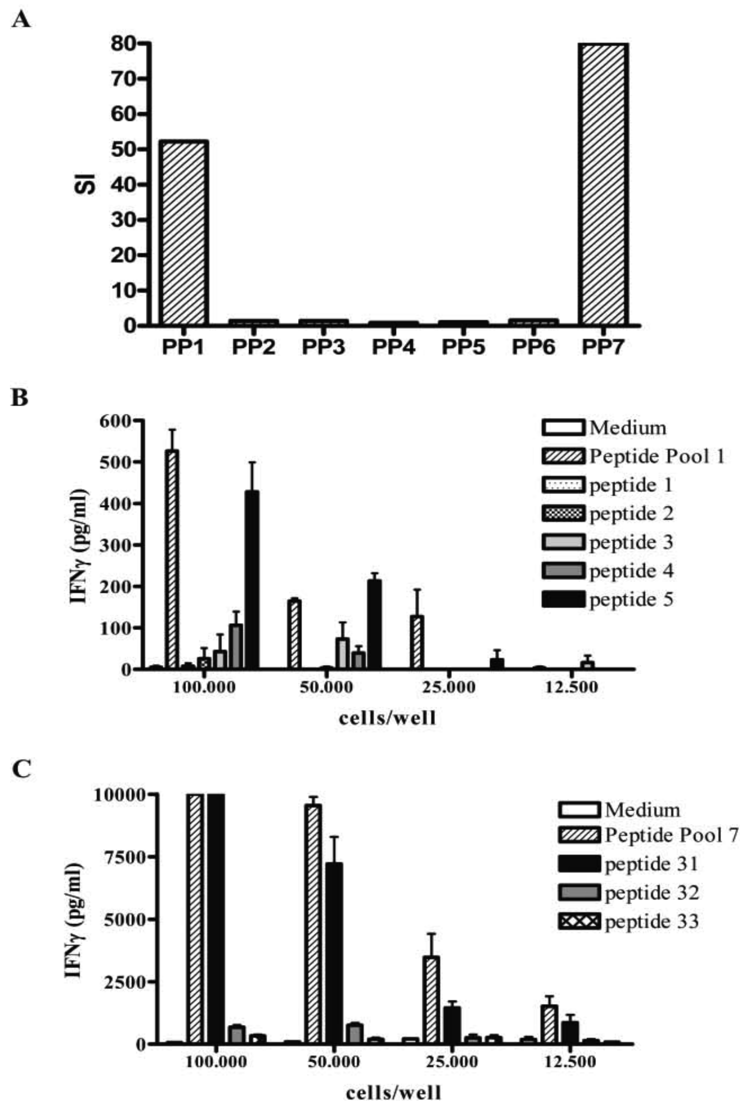

Figure 1. IFNγ production of bulk spleen cell cultures from DR4-transgenic mice immunized with the indicated peptide pools.
A. Reactivity against the different peptide pools (PP; hatched bars) with 100,000 c/w. The bars represent the mean stimulation indices (SI) against each peptide pool of two independent experiments.
B. IFNγ production against peptide pool 1 and the individual peptides (p1-p5) from this pool after immunization with the peptide pool at the indicated cell concentrations. C. Idem for peptide pool 7. Bars in B and C represent the mean of triplicates and the standard error of the mean (SEM).

Statistics

For the statistical analysis of the peptide and protein responses, paired T-tests were performed in Graphpad Prism version 4.0. p-values lower than 0.05 taking into account the 95% confidence interval were considered significant.

Results

Based upon several lines of evidence (12-17;19), we hypothesize that vimentin represents a relevant candidate autoantigen recognized by HLA-SE-restricted T cells. To identify vimentin epitopes recognized by T cells, we have chosen an unbiased approach in which all possible citrullinated peptides of human vimentin (Table 2) were analyzed for their ability to induce a T cell response in DR4-transgenic mice.

Peptide Pools inducing antigen-specific T cell responses

To enable efficient analyses of the large number of peptides generated, we made a first selection of potential epitopes by immunizing DR4-transgenic mice with seven peptide pools. "Peptide-pool"-specific T cell responses were repeatedly observed in bulk cultures obtained from mice immunized with peptide pool 1 and 7 (Figure 1A). No response was observed against these peptide pools when spleen cells from naive mice were tested (data not shown). These results indicate that the immunogenic citrullinated T cell epitopes are among the peptides contained in peptide pools 1 and 7.

Characterization of immunogenic peptides

Next, we wished to identify the individual peptides responsible for the induction of T cell responses by the respective peptide pool. Therefore, DR4-transgenic mice were immunized with peptide pool 1 or 7 and the T cell reactivity to the peptide pool as well as to the individual peptides from the pool was analyzed. A dose-dependent T cell response of spleen cells from mice immunized with peptide pool 1 was observed after stimulation with peptide 5 (vim26-44) (Figure 1B). Likewise, peptide 31 (vim415-433) was consistently recognized by spleen cells from mice immunized with peptide pool 7 (Figure 1C). Together, these results indicate that the citrullinated peptides 5 and 31 are immunogenic in HLA-DR4-transgenic mice.

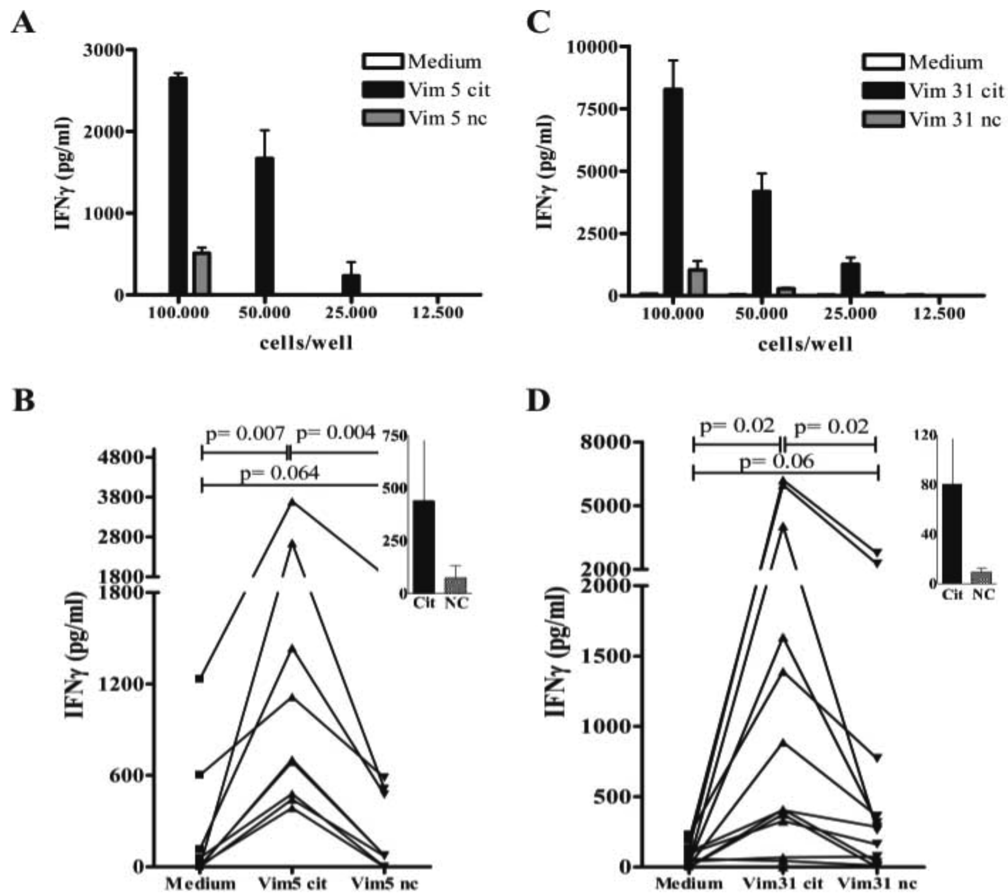

Figure 2. IFNγ response of bulk spleen cell cultures from DR4-transgenic mice immunized with the indicated citrullinated peptides and tested against the citullinated (cit; black bars) and non-citrullinated (nc; grey bars) peptide.
A. Cell titration showing the response to vim5 from one representative experiment.
B. Overview of the responses against vim5 with 100,000 c/w.
C. Cell titration for the response against vim31 from one representative experiment.
D. Overview of the responses against vim31 with 50,000 c/w.
The bars in A and C represent the mean response measured in triplicate +/- the SEM. In B and D each symbol represents the mean of a triplicate and one set of symbols connected by a line represents one independent experiment. The inlays show the stimulation indices (SI) against the citrullinated (black bar) and non-citrullinated (grey bar) peptides +/- the SEM.

To determine whether the response observed against these two citrullinated vimentin peptides is "citrulline"-specific, mice were immunized with these peptides and their spleen cells were restimulated *in vitro* with the same peptides. Subsequent proliferation of spleen cells against either the citrullinated or non-citrullinated form of the peptides was assessed. A dose-dependent T cell response was observed against citrullinated vim5 while no responses could be observed in response to the non-citrullinated counterpart (Figure 2A&B). On average, the stimulation index (SI) of the cultures restimulated with citrullinated vim5 was approximately six times higher compared to cultures stimulated with the non-citrullinated control peptide (Figure 2B, inlay). Similar results were obtained for vim31 (Figure 2C&D). In this case the T cell response was on average nine times higher upon stimulation with the citrullinated peptide as compared to the non-citrullinated peptide (Figure 2D, inlay). No IFNγ was detected when spleen cells from sham-immunized mice (i.e. CFA/IFA without peptide) restimulated with either citrullinated peptide were used (data not shown). Moreover, we have confirmed by intracellular cytokine staining the presence of CD4+ T cells producing IFNγ in the spleen cells of mice immunized and challenged with the citrullinated peptides and not with their non-citrullinated counterparts (data not shown). As expected, peptide-specific responses were impaired in the presence of anti-HLA-DR antibodies, confirming that the responses against citrullinated vim5 and 31 are HLA-DR restricted (Figure 3A&B). Together, these data indicate that the two peptides identified induce a "citrulline"-specific T cell response in a HLA-DRB1*0401 restricted manner.

Natural processing of the immunogenetic peptides from citrullinated vimentin protein

To analyze whether the citrullinated peptides recognized by T cells can be naturally processed and presented from the entire vimentin protein, we next tested the reactivity of spleen cells from peptide-immunized mice against the recombinant human vimentin either in citrullinated or non-citrullinated form. In three independent experiments, a significant response against the citrullinated vimentin protein compared to the medium or non-citrullinated protein (Figure 3C) was observed with spleen cells from mice immunized with citrullinated vim5. Similar results were obtained with spleen cells from mice immunized with vim31 (Figure 3D). No reactivity was observed when spleen cells of naive mice were tested against the citrullinated vimentin protein (data

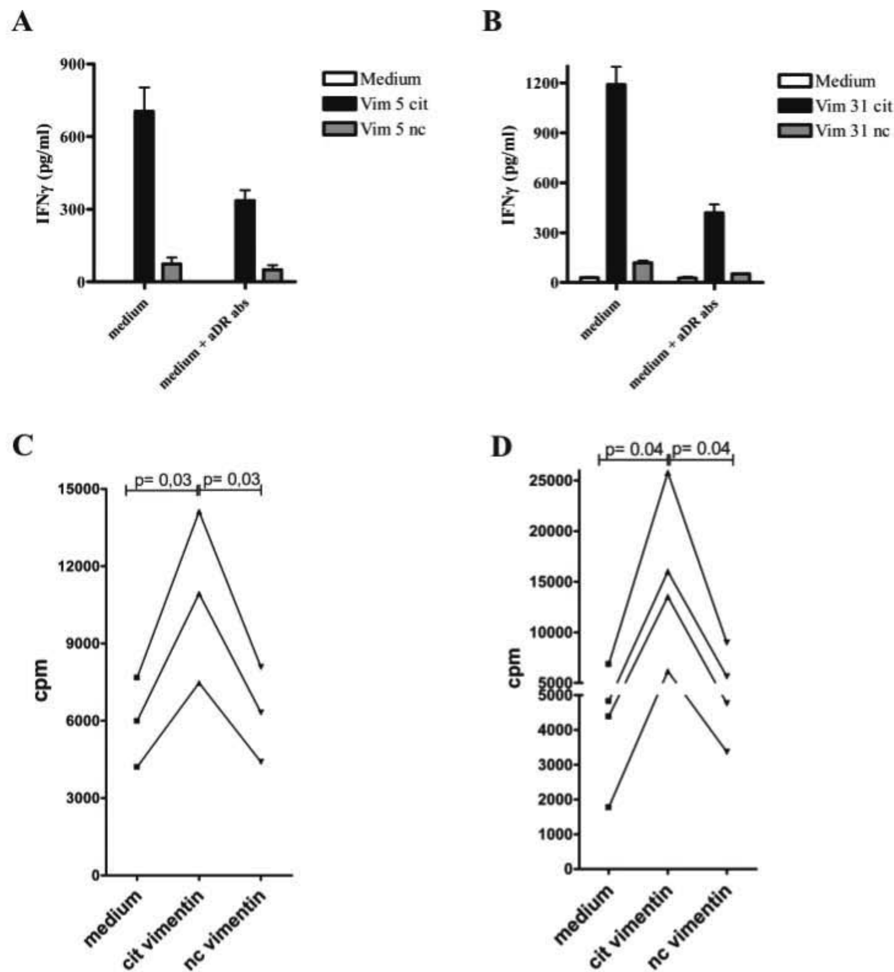

Figure 3. A&B. HLA-restriction of the T cell response. Spleen cells from mice immunized with vim5 Cit (A) or vim31 Cit (B) were restimulated with the indicated peptides in the presence or absence of anti-HLA-DR antibodies. Bars represent the mean of a triplicate measurement in supernatants of cells without stimulation (white bars), stimulated with citrullinated (black bars) or non-citrullinated (grey bars) peptide. The error bars represent the SEM. B&D show an overview of the responses of bulk spleen cell cultures from DR4-transgenic mice immunized with vim5 Cit (C) or vim31 Cit (D) against the citrullinated and non-citrullinated vimentin protein. Every line represents one experiment where the mean response (measured in triplicate) against the indicated antigens and the background are connected.

not shown). These results indicate that the epitopes identified can be naturally processed from citrullinated vimentin.

Recognition of both vim5 and vim31 by T cells in RA peripheral blood

Next, we wished to analyze whether the two citrullinated vimentin peptides, vim5 and 31, identified as DRB1*0401-restricted T cell epitopes in DR4-transgenic mice can be recognized by T cells from RA patients. Because the presence of IgG ACPA in patients implies the existence of memory T-helper cell responses that have provided help to ACPA-producing B cells, we have investigated, as a first inventory, the presence of T cells with a memory phenotype specific for vim5 or vim31 in 10 $ACPA^{+}$ HLA-$DRB1*04^{+}$ RA patients from the Leiden Early Arthritis Cohort (EAC) (Patient characteristics are shown in Table 1). PBMC from these patients were tested for IFNγ production by intracellular cytokine staining after stimulation with (citrullinated) vim5 or 31. After gating of the $CD3^{+}CD4^{+}CD45RA^{-}$ lymphocytes, cells from three of the patients responded to citrullinated but not against non-citrullinated vim5 (Figure 4A&B). Next to the patients, also five healthy controls were tested. The percentages of $CD3^{+}CD4^{+}CD45RA^{-}$ cells producing cytokines were much lower compared to the patients and no significant differences between the different culture conditions were observed (Figure 4B, right panel). From the nine RA patients tested for (citrullinated) vim31 reactivity, a marginal, but detectable, response was observed for three patients (Figure 4C and 4D, left panel). From these three patients, one also responded to the non-citrullinated peptide, although to a less extent. In contrast, no responses were observed against both citrullinated and non-citrullinated vim31 in the healthy individuals (Figure 4D, right panel). All patients and controls showed T cell reactivity against a control antigen (Memory Mix, data not shown). These data suggest that both identified vimentin epitopes can be recognized by T cells from $ACPA^{+}$ HLA-$DRB1*04^{+}$ RA patients.

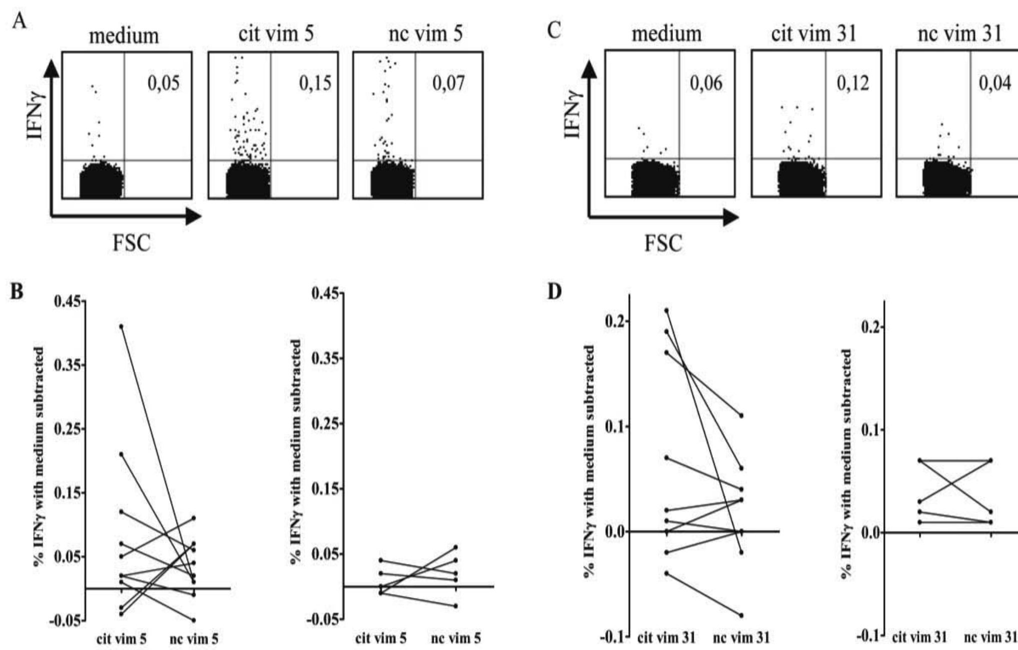

Figure 4. T cell response of PBMC cultures from human individuals. PBMC from ACPA+ HLA-DRB1*04+ RA patients and healthy individuals were cultured and tested against citrullinated and non-citrullinated vim5 (A&B) or 31 (C&D). One representative patient is depicted in the dot plots for reactivity against vim5 (A) or vim31 (C) in which the percentages represent the IFNγ-producing cells in the CD3+CD4+CD45RA- lymphocyte population. Quadrants are based on the isotype control. The overview of all patients (left panels) and controls (right panels) is depicted in B and D where each line represents one individual and each dot represents the percentage of IFNγ-producing cells stimulated with the (non)-citrullinated peptide minus the background (medium).

Discussion

At present, only limited data are available on potential T cell epitopes that can be recognized in a "citrulline'-dependent manner by RA patients. Therefore, an unbiased inventory, focusing on relevant autoantigens recognized by ACPA, such as performed in the experiments described in this manuscript is highly relevant.

We examined in this study whether we could identify $CD4^{+}$ T cells specific for citrullinated peptides derived from human vimentin. In total 33 peptides were synthesized in their citrullinated form and tested for T cell reactivity in DR4-transgenic mice. A "citrulline"-specific response was observed against two of the peptides, vim5 (vim26-44) and vim31 (vim415-433). We have shown that these peptides are naturally processed epitopes of human vimentin and provided data indicating that they can be recognized by T cells with a memory phenotype from RA patients.

The two T cell epitopes identified in this study have not been described before to be either involved in B cell or T cell responses. Previous studies identified T cell reactivity against a vimentin-derived peptide in HLA-DR4-positive mice (29). Although the peptide sequence used by Hill *et al.* was similar, it was not homologous to the sequence present in vimentin (30). A (large) Leucine present in vimentin (position 69), was replaced by the small Alanine, thereby possibly influencing the binding capacity to the HLA molecule (31). Our study identifies two vimentin epitopes that can be recognized by HLA-DRB1*0401 restricted T cells without the apparent requirement for additional amino acid changes, such as described by Hill *et al*. Furthermore, the peptide of the study by Hill *et al.* was selected on the basis of a prediction program focusing on the ability of Arginine/Citrulline to bind the anchor region shared by the SE-containing HLA-DRB1 alleles. We, however, used an unbiased approach in which all possible peptides from the vimentin protein were tested by positioning every Arginine present in the middle of a peptide. Although the Arginine is centred, it can bind to every anchor position of the binding pocket since the peptide is 19 amino acids long.

The observed T cell responses in HLA-DR4 transgenic mice are HLA-restricted, as shown using an HLA-DR-blocking antibody. However, these IFNγ-responses are only partially blocked in the presence of the anti-HLA-DR antibody. This is probably due to the high abundance of HLA-DR molecules on the cell surface of antigen-presenting cells or to the relatively low affinity of the anti-DR antibody to HLA-DRB1*0401, as

the capacity of this antibody to inhibit T cells responses was previously shown to vary depending on the DR molecule involved (32).
Because binding of the peptides to the HLA-DR4 molecule is indispensable for induction of a T cell response, we tested for both citrullinated and non-citrullinated vim5 and 31 their binding to HLA-DRB1*0401 in a competitive binding assay using the biotinylated HA-peptide (HA309-320) as a competitor. Only weak inhibition of the biotinylated peptide could be observed at high peptide concentrations (data not shown). Therefore, no conclusions could be drawn about the differences in binding capacity between the identified citrullinated and non-citrullinated version of the vimentin peptides to the HLA-DR4 molecule. This observation is in line with other observations showing that low affinity peptides can also efficiently induce T cell responses, as is described for an insulin peptide in NOD mice (33) and recently for a dominant gluten peptide in DQ8-transgenic mice (34). A low net binding value to MHC class II can be a consequence of both the association and dissociation rate with the MHC molecule being high, while the T cell response is readily observed (34-36). Although our results might be counterintuitive, they are very intriguing as it has been proposed that low affinity peptides play an important role in the induction of autoimmunity since they escape tolerance induction (37;38).

The IFNγ production observed with PBMC from RA patients was rather low. However, this would be in line with the view that the expected precursor frequency of T cells reacting with citrullinated vimentin peptides is low. Even the T cell fraction reactive to recall antigens (i.e. a mix of Tetanus Toxoid, Candida Albicans and tuberculin purified protein derivative) is only 3% on average after restimulation. Furthermore, it has been shown that PBMC from RA patients produce less IFNγ compared to healthy individuals in response to recall antigens, probably due to immuno-suppressive drugs (39).
In this study, we have performed an inventory of T cell responses against the identified epitopes in 10 ACPA$^+$ HLA-DRB1*04$^+$ RA patients and 5 HLA-DRB1*04$^+$ healthy controls. To obtain a comprehensive view of the pattern of reactivity of citrullinated vimentin-specific T cells, several different aspects remain to be elucidated in a larger cohort of patients and controls. Future studies include assessing recognition of these T cell epitopes in ACPA$^+$, as well as ACPA$^-$ patients, and the requirement for SE-containing HLA-DRB1 alleles for the recognition. Likewise, a more extensive characterization of the cytokine profile of these T cells would be informative, as it is conceivable that the cytokine profile changed from a regulatory type in healthy controls

to pro-inflammatory in RA patients as it was previously shown for another RA candidate autoantigen (39). Furthermore, since our approach focused specifically on the identification of “citrulline”-specific T cells, it cannot be excluded that also T cells reacting against non-citrullinated peptides from the vimentin protein or another protein that is internalized and presented together with citrullinated vimentin exist. These studies would involve immunization with non-citrullinated peptides from vimentin or other proteins that could be associated with vimentin *in vivo*. All these aspects imply the necessity of further extensive studies. Nonetheless, our study is the first to identify “citrulline”-specific T cell responses in humans, recognizing epitopes from an autoantigen present in the inflamed joint of RA patients. As such, these results provide a valuable basis for future, more extensive studies.

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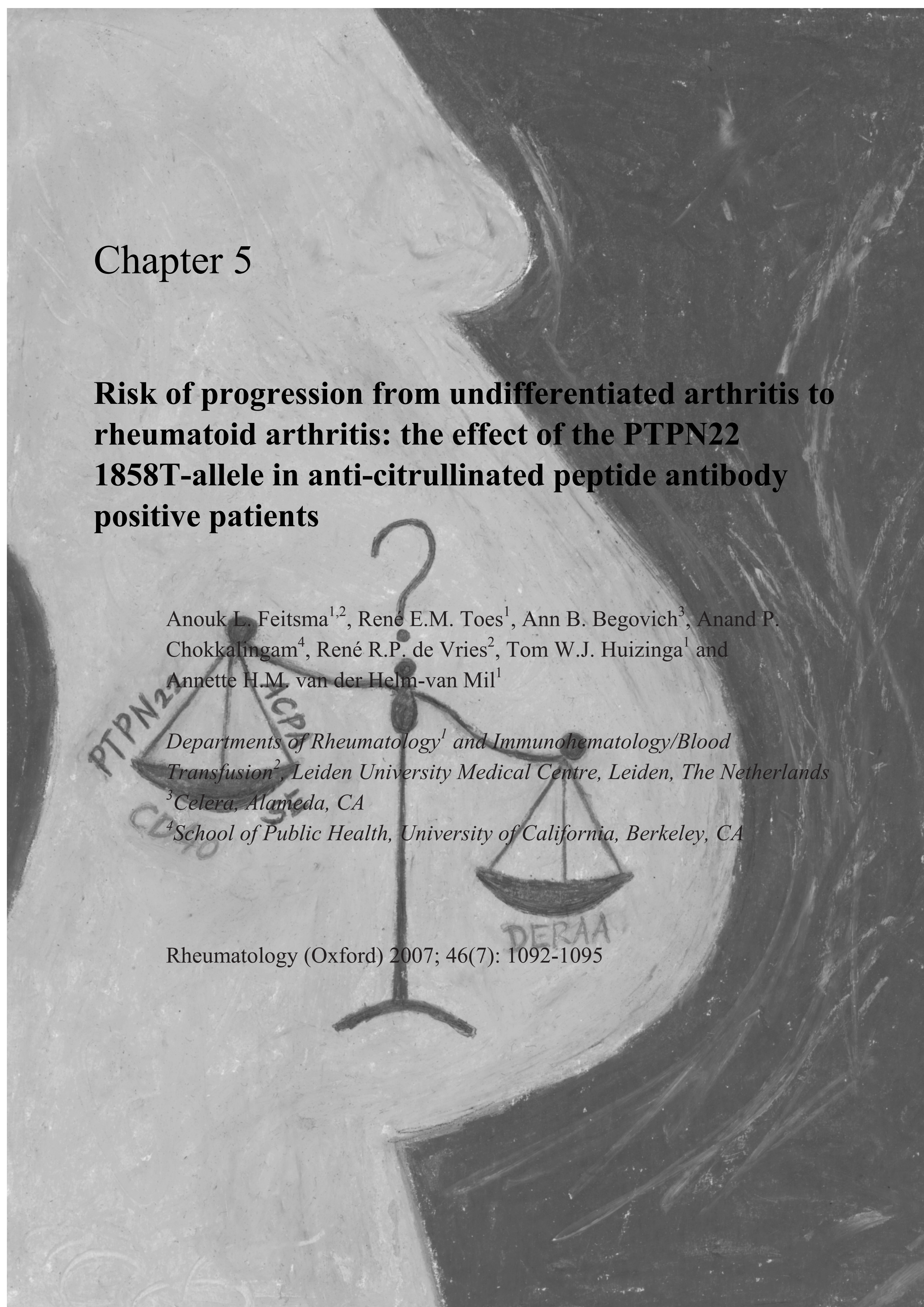

Chapter 5

Risk of progression from undifferentiated arthritis to rheumatoid arthritis: the effect of the PTPN22 1858T-allele in anti-citrullinated peptide antibody positive patients

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Abstract

Objectives. Anti-citrullinated peptide antibodies (ACPA) and the C1858T missense single-nucleotide polymorphism (SNP) in the *PTPN22* gene are both associated with the development of rheumatoid arthritis (RA). We investigated whether the combination of these two biomarkers yielded better test characteristics to predict progression from undifferentiated arthritis (UA) to RA compared to ACPA alone.
Methods. Three-hundred ninety-four individuals with UA from a Dutch population-based inception cohort were included in this study. At baseline ACPA were measured and the *PTPN22* C1858T and HLA-DRB1 genotypes determined. Progression to RA was monitored at one year after entry into the cohort.
Results. A priori, UA patients had a 35% (95% CI 30-40%) risk of developing RA, which increased to 66% (95% CI 57-75%) in patients who were ACPA-positive. There was an additional although non-significant (p = 0.34) increase in RA risk to 76% (95%CI 57-90%) when patients were positive for both ACPA and the *PTPN22* 1858T-allele. The area under the receiver operator characteristic curve increased from 0.68 for ACPA-status alone to 0.70 for the combination of ACPA-status and the *PTPN22* C1858T polymorphism. In logistic regression analysis, ACPA predicted RA-development independent of *PTPN22*, while the *PTPN22* polymorphism had no independent effect. In HLA-DRB1 shared epitope positive, ACPA-positive UA patients, ACPA-levels were significantly increased in *PTPN22* 1858T allele carriers compared to non-1858T carriers.
Conclusions. In this Dutch cohort of UA-patients, the *PTPN22* 1858T allele does not markedly improve individual decision-making to predict RA-development over ACPA alone, but it is associated with higher ACPA-levels.

Introduction

Anti-citrullinated peptide antibodies (ACPA), which can be detected years before disease onset, are highly specific for rheumatoid arthritis (RA) (1-3). Approximately 72% of patients presenting with an early form of arthritis that cannot be properly classified (undifferentiated arthritis, UA) and who are positive for ACPA develop RA (4). The C1858T missense single-nucleotide polymorphism in the *PTPN22* gene is also associated with RA, UA (5-11) and several other autoimmune diseases (for a review see 12). The relevance for ACPA-determination is increasingly accepted in clinical practice (3); however, the role of the *PTPN22* 1858T-allele in RA prediction has not been studied. Consequently, we investigated whether the combination of these two biomarkers improved prediction of progression from UA to RA compared to ACPA alone. Since the *PTPN22* 1858T-allele confers risk to ACPA-positive arthritis (10) we hypothesized that the *PTPN22* C1858T polymorphism may also influence auto-antibody levels. Therefore we examined whether ACPA-levels differed between ACPA-positive UA-patients with and without the *PTPN22* T-allele.

Material and Methods

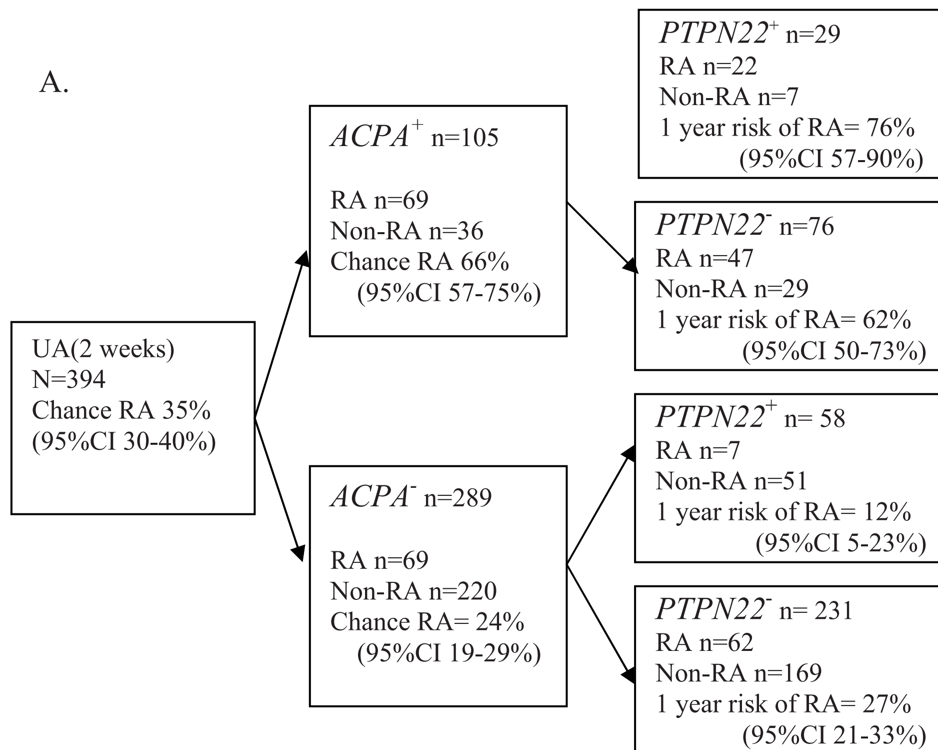
Study population.

The Leiden Early Arthritis Clinic (EAC) is a population-based inception cohort of white Dutch patients with recent-onset arthritis (median symptom duration 112 days, IQR 55-239 days) which, at present, includes 1944 individuals (for a detailed description of this cohort see ref 13). A subset of these patients (n=394), who could not be classified according to the 1987 ACR criteria at baseline and were categorized as having undifferentiated arthritis (UA), were included in this study. At one year of follow-up, subjects were re-evaluated as having RA or not according to the ACR 1987 criteria (14). Patients who during the one year of follow-up cumulatively fulfilled the ACR criteria were diagnosed as RA and all other patients as non-RA.

Laboratory methods.

Blood specimens, obtained at baseline, were typed for anti-CCP2 antibodies (ACPA) using an ELISA (Immunoscan RA Mark 2; Euro-diagnostica, Arnhem, The Netherlands). The cut-off level for ACPA positivity was set at 25 arbitrary units, according to the manufacturer’s instructions. Rheumatoid factor levels were also

A.



B.

	Sensitivity		**Specificity**		**PPV**		**NPV**	
	%	*95% CI*	*%*	*95% CI*	*%*	*95% CI*	*%*	*95% CI*
ACPA	50	42-58	86	82-90	66	57-75	76	71-81
PTPN22	21	14-28	77	72-83	33	23-43	64	59-70
PTPN22 given ACPA$^+$	32	21-44	81	64-92	76	57-90	38	27-50
PTPN22 given ACPA$^-$	10	4-20	77	71-82	12	5-23	73	67-79

Figure 1. Characteristics of the study population. A. Description of the patient cohort. The RA diagnosis is based on the diagnosis after 1 year of inclusion in the cohort. UA=undifferentiated arthritis, n=number of patients in this group. B. Proportional values for RA-development taking into account ACPA status (positive or negative), the PTPN22 C1858T-polymorphism (T-allele positive or negative) or a combination of these two markers. PPV = positive predictive value, NPV = negative predictive value.

measured at baseline with an ELISA. The *PTPN22* C1858T polymorphism and HLA-DRB1-alleles were genotyped as previously described (5,15).

Analysis.

Statistical analyses were performed using SPSS_12.0 Software (Chicago, IL, USA). 95% confidence intervals (95%CI) of the proportional data were calculated with CIA software (16). Predictive values were compared by logistic regression analysis and by determination of the area under the receiver operator characteristic curve (AUC). The Mann-Whitney test and linear regression analysis were used to compare ACPA levels.

Results

Of the 394 UA patients, 138 patients (35%) progressed to RA within one year (Figure 1A). Of these 138, 69 (50%) were positive for ACPA at baseline, compared to 36 of the 256 individuals (14%) in the non-RA group. The *PTPN22* 1858T-allele was present in 21% (n=29) of UA-patients who progressed to RA and in 23% (n=58) of the non-RA group. The unadjusted relative risk (RR) for RA-development was 2.75 (95%CI 2.15-3.53) for ACPA-positivity and 0.94 (95%CI 0.67-1.31) for carriage of the *PTPN22* 1858T allele.

Of the 105 ACPA-positive patients, 66% (95%CI 57-75%) developed RA in 1 year, compared to 24% (95%CI 19-29%) of the 289 ACPA-negative patients. When the ACPA-positive and ACPA-negative patient groups were further stratified for *PTPN22* 1858T allele carriage, 76% (22 of 29, 95%CI 57-90%) of the ACPA-positive, *PTPN22* 1858T-allele positive patients developed RA, compared to 62% (47 of 76, 95%CI 50-73%) of the ACPA-positive *PTPN22* 1858T-allele negative patients. Although these results show an increased predictive value if the *PTPN22* C1858T polymorphism is combined with ACPA status, this difference was not statistically significant (p=0.34). While ACPA-negative patients had a 24% risk of progressing to RA, information on the presence or absence of *PTPN22* 1858T allele did not significantly alter this probability (Figure 1A). A similar analysis examining the effect of the *PTPN22* 1858T allele conditional on baseline levels of rheumatoid factor showed no significant differences in the probabilities for developing RA (data not shown).

To further understand the role of ACPA and the *PTPN22* 1858T allele in RA risk prediction, we used logistic regression analysis to examine how these factors influenced risk for RA-development, mutually adjusting for both the presence of ACPA and the *PTPN22* 1858T allele. Only ACPA-positivity was identified as an

independent predictive variable in this model (OR 6.3, 95%CI 3.9-10.3, p<0.0001), whereas the *PTPN22* 1858T-allele was not (OR=0.7, 95% CI 0.4-1.3, p=0.282). Addition of the *PTPN22* C1858T polymorphism to ACPA status in the regression model increased the AUC marginally (AUC 0.68 for ACPA alone and 0.70 for both).

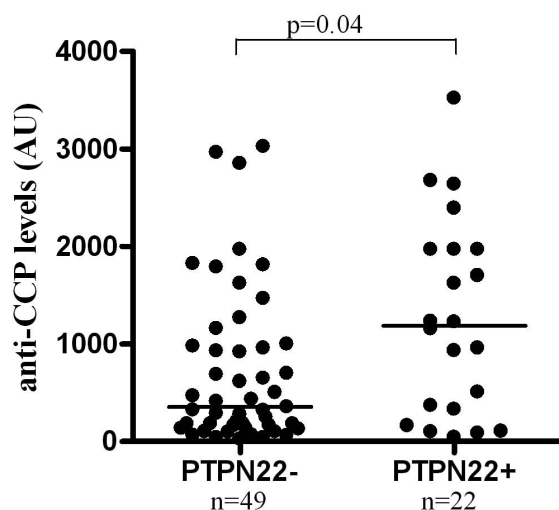


Figure 2. Influence of PTPN22-polymorphism on anti-CCP (ACPA) levels.
Ninety-six of the 105 UA patients who had ACPA were successfully genotyped for the DRB1 Shared Epitope (SE) alleles (defined as DRB1*0101, 0102, 0401, 0404, 0405, 0408, 0410, 1001 and 1402) and both SE+ and SE- patients were divided in two groups based on the presence or absence of the PTPN22 1858T allele. Only the SE+ patients are shown in the graph.
n = number of patients in each group, AU=arbitrary units. The lines represent the median of the ACPA antibody levels in each group.

Because we previously observed a stronger association of the *PTPN22* C1858T polymorphism with ACPA-positive RA than ACPA-negative RA (10), we stratified and compared the ACPA levels of all 96 ACPA-positive subjects according to *PTPN22* 1858T carriage status. As HLA-DRB1 alleles that encode for a common amino acid sequence, the shared epitope (SE), dominantly influence the level of ACPA (4), this analysis was performed after stratification for the presence or absence of SE-alleles (defined as DRB1*0101, 0102, 0401, 0404, 0405, 0408, 0410, 1001 or 1402). Within the 71 SE-positive ACPA-positive patients, the 22 carrying the *PTPN22* 1858T allele had significantly higher levels of ACPA (median 1190 units, IQR 286-1970 units) compared to the 49 who did not carry this allele (median 357 units, IQR 152-988 units)

(p=0.04) (Figure 2). No difference by *PTPN22* 1858T allele was observed among the SE-negative patients (SE^-*PTPN22*$^-$ n= 21, median 346 units, IQR 80-1712 units; SE^- *PTPN22*$^+$, n=4 median 584 units, IQR 43-1848 units); however, the sample sizes were too small to draw a statistically sound conclusion. Linear regression analysis adjusting for HLA-SE confirmed the independent association of the *PTPN22* C1858T polymorphism with ACPA levels in ACPA positive patients (B= 452 units, 95%CI 58-847 units, p=0.03).

Discussion

Previous studies showed that the *PTPN22* C1858T polymorphism and ACPA-status are both associated with development of RA in the general population (1,2,5-11). Our study demonstrates an independent association of ACPA but not the *PTPN22* 1858T allele with progression to RA among patients presenting with UA.

Johansson *et al.* observed that the specificity for future onset of RA in a population of healthy blood donors increased from 98.6%, when only ACPA-status was taken into account, to 100% when both ACPA and *PTPN22* C1858T polymorphism were analyzed together (17). They did not determine the sensitivity and specificity of subsequently APCA alone and the combination of ACPA and the *PTPN22* 1858T allele, but compared the patients positive for both ACPA and the *PTPN22* 1858T allele with all other patients (including patients positive for either ACPA or the *PTPN22* 1858T allele or negative for both). This resulted in different values for specificity and sensitivity compared to this study. Since the value of a positive test result as for an individual patient in clinical practice is not measured by the sensitivity or specificity, but is reflected by the a priori and post priori probabilities, the current study evaluated the risk of a cohort of patients presenting with UA to progress to RA given their ACPA and *PTPN22* 1858T status. The risk to develop RA increased significantly in the case of ACPA-positivity. This risk increased marginally for those ACPA-positive patients who also harbored the *PTPN22* 1858T allele; however, this increase was not significant. We cannot exclude a Type-II error although, given the effect size, approximately 3000 UA-patients, from which approximately 800 are ACPA positive, would be needed to detect a significant difference. In the ACPA-negative group, knowledge of the *PTPN22* C1858T polymorphism did not increase the predictive value of RA development. Moreover, only ACPA was identified as a significant independent predictor for RA-development. These data indicate that in clinical practice, testing for

the *PTPN22* polymorphism in addition to ACPA-status will be of limited relevance for patients with UA.

The notion that for clinical practice testing of the *PTPN22* C1858T polymorphism has no additive value to ACPA-determination is not in contrast with the findings that the *PTPN22* C1858T polymorphism is involved in the pathogenesis of RA. The *PTPN22* C1858T polymorphism is only associated with ACPA-positive RA, indicating that the *PTPN22* genotype confers risk to a serologically defined subset of RA. Thus, pathophysiologically both factors are on the same pathway, and by measuring ACPA the effect of the *PTPN22* 1858T-allele when present, is already included.

It should also be noted that, given there is a gradient in the frequency of the *PTPN22* 1858T allele that increases from the south (2-3% in Italy) to the north (15.5% in Finland) (for a review see 12) of Europe, the utility of the SNP may vary in different populations.

We also showed that the presence of the *PTPN22* 1858T-allele in shared epitope positive, ACPA positive UA-patients was associated with higher levels of ACPA. This is of interest as a recent study revealed that ACPA-levels were a predictor for RA-development in ACPA-positive UA-patients (18). The observation that ACPA-positive patients who carry the *PTPN22* disease-associated 1858T-allele have higher ACPA-levels than those who are homozygous for 1858C is interesting and may shed some light on how the *PTPN22* 1858T polymorphism, which appears to be a "gain of function" allele, mediates susceptibility to multiple autoimmune diseases (5,19,20).

Given the findings of this study we conclude that (i) testing for the *PTPN22* C1858T-polymorphism in ACPA-positive UA-patients does not significantly improve prediction of RA development and (ii) the *PTPN22* T-allele is associated with higher ACPA levels.

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Chapter 6

A single nucleotide polymorphism in *CD40* associates with the rate of joint destruction in Rheumatoid Arthritis

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Abstract

Objective: The severity of joint destruction in Rheumatoid Arthritis (RA) is highly variable between patients and influenced by genetic factors. Genome-wide association studies (GWAs) have boosted the field of the genetics of susceptibility to RA enormously, but risk loci for severity of RA remain poorly defined. A recent meta-analysis of GWAs identified 6 genetic regions for susceptibility to autoantibody-positive RA, i.e. *CD40, KIF5A-PIP4K2C, CDK6, CCL21, PRKCQ* and *MMEL1-TNFRSF14*. We have investigated whether these newly described genetic regions associate with the rate of joint destruction.

Methods: RA-patients enrolled in the Leiden Early Arthritis Clinic were studied (n=563). Yearly radiographs were scored using the Sharp-van der Heijde method (median follow-up 5 years, maximal follow-up 9 years). The rate of joint destruction between genotype groups was compared using a linear mixed model correcting for age, gender and treatment-strategies. 393 ACPA-positive-RA-patients included in the NARAC with radiographic data were used for replication.

Results: The TT and CC/CG genotypes of two SNPs, rs4810485 *(CD40)* and rs42041 *(CDK6)* respectively, were associated with a higher rate of joint destruction in ACPA-positive RA (p=0.003 and 0.012), of which rs4810485 was significant after Bonferroni correction for multiple testing. The association of the *CD40* minor allele with radiographic progression rate was replicated in the NARAC cohort (p=0.021).

Conclusion: A polymorphism in the *CD40* locus is associated with the rate of joint destruction in ACPA-positive RA and provides one of the first non-HLA-related genetic severity-factors that is replicated.

Introduction

Rheumatoid arthritis (RA) is characterized by inflammatory arthritis and localized destruction of bone and cartilage. The severity of joint destruction is highly variable between patients and, according to twin studies, substantially influenced by genetic factors (1). Nevertheless, the precise contribution of genetic factors still has to be determined. To date only a small number of genetic risk-factors has been identified, and apart from HLA, none of these factors have been convincingly replicated.

In contrast, the genetics of susceptibility to RA has been boosted considerably, largely due to genome-wide association studies. In addition to the HLA-DRB1 shared epitope alleles, several new susceptibility-factors, *PTPN22*, *TRAF1-C5*, *OLIG3-TNFAIP3* and *STAT4*, have been identified and were independently replicated. Intriguingly, for many of these genetic risk-factors the associations are confined to anti-citrullinated protein antibodies (ACPA)-positive RA-patients. Whether genetic factors also differently affect the severity of joint destruction in ACPA-positive and ACPA-negative RA remains unknown. Nonetheless, compelling evidence demonstrates that ACPA-positive RA-patients have a more destructive disease course compared to ACPA-negative patients.

A recent meta-analysis on two genome-wide association studies identified six new risk loci (rs4810485 (*CD40*), rs1678542 (*KIF5A-PIP4K2C*), rs42041 (*CDK6*), rs2812378 (*CCL21*), rs4750316 (*PRKCQ*) and rs3890745 (*MMEL1-TNFRSF14*)) as susceptibility factors for autoantibody-positive RA (2). The present study aimed to investigate the association between these single-nucleotide-polymorphisms (SNPs) and the rate of radiological joint destruction in RA, and ACPA+ RA in particular, using a large longitudinal cohort. A cohort of ACPA-positive RA-patients was used for replication. This study shows that a genetic variant in the *CD40* gene associates with the rate of joint destruction in ACPA-positive RA.

Patients and methods

Patients

Five hundred sixty three RA-patients, consecutively included in the Leiden Early Arthritis Cohort (EAC) between 1993 and 2006 with both DNA and radiographs available were studied. The RA-patients fulfilled the 1987 ACR-criteria. Follow-up visits were performed yearly. Treatment strategies changed in time and differed for different inclusion periods (before 1996, 1996-1998, 1999-2001, after 2001) (see ref (3) for detailed description of the EAC). Anti-CCP2 antibodies were measured using stored baseline serum samples (Immunoscan RA Mark 2; Euro-Diagnostica, The Netherlands).

Replication cohort

393 ACPA-positive RA-patients that were included in the North American Rheumatoid Arthritis Consortium (NARAC) that had hand radiographs available were studied. As the radiographs were taken at different disease durations, the estimated radiological progression per year was determined by dividing the total Sharp-van der Heijde score of the hands by the disease duration at the time of the radiograph.

SNP genotyping

The six recently identified risk loci (2) were genotyped in the 563 RA-patients from the Leiden EAC using allele-specific kinetic PCR as previously described (4). The data were hand-curated without knowledge of clinical characteristics before statistical analysis with a 98% genotyping success rate; previous analyses suggest a genotyping accuracy of >99%. For the *MMEL1-TNFRSF14* locus, a perfect proxy of rs3890745 (reported in (2)) was used (rs6684865, r^2=1).

In the NARAC genotyping was performed using the Illumina Hapmap500 BeadChip, as described (5). Rs4810485 was not typed in the whole genome study, but a perfect proxy for this variant was genotyped (rs1569723, r^2=1). For *CDK6*, neither rs42041 nor a perfect proxy were genotyped and therefore the data on rs42041 was imputed as described (2).

Radiographs

In the EAC, radiographs of hands and feet, taken on consecutive years, were scored according to the Sharp-van der Heijde method (6). To encompass a reliable sample

size, radiographic follow-up data were restricted to a maximum of 9 years with a median of 5 years. All radiographs were scored by one experienced scorer who was blinded with respect to clinical and genetic data. 499 radiographs were rescored (149 baseline radiographs and 350 radiographs during follow-up from 60 randomly selected RA-patients). Intraclass-observer correlation coefficients (ICC) were 0.91 for all radiographs, 0.84 for baseline radiographs and 0.97 for the radiographic progression rate. In the NARAC the radiographs were scored by one reader blinded to clinical or genetic data. 25% of the radiographs were re-scored, the ICC was 0.99.

Statistical Analysis

Analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL). As radiographic data were not normally distributed, the raw data on the Sharp-van der Heijde scores are presented using medians (Figure 1-2) and were log-transformed in preparation for analysis. In the EAC, a linear model for longitudinal data was used to compare progression rates between groups. Age, gender, inclusion period (a proxy for treatment strategy) and their interactions with time were entered in the model to correct for possible confounding effects (see also the supplementary methods). As six SNPs were evaluated, a Bonferroni correction for multiple testing was applied; the p-value for significance was set at $p<0.008$. Only the SNPs that were clearly related to the progression rate in the EAC were analyzed in the replication cohort. In the NARAC, the estimated radiological progression per year was compared with the Kruskal Wallis test. No corrections were made for age, gender or treatment in this cohort.

Results

Baseline characteristics of the RA-patients are shown in Table 1. In the EAC, the minor allele frequencies were 0.242, 0.340, 0.267, 0.366, 0.204 and 0.307 for rs4810485, rs1678542, rs42041, rs2812378, rs4750316 and rs6684865 respectively and in agreement with previous results (2). The raw data on the Sharp-van der Heijde scores for the three genotypes at each SNP are depicted in Figure 1. To study the influence of the SNPs on the rate of joint destruction, a linear mixed model analysis was performed for each SNP. For rs4810485 (*CD40*) the GG and GT genotypes showed comparable radiographic scores, therefore the genotype data were combined and carriership-analysis was performed. Similarly, the CC and CG genotypes of rs42041 (*CDK6*) were pooled. In the total group of RA-patients an association was observed for rs42041 (*CDK6*) ($p=0.033$). For the other SNPs no significant association

Table 1. Patient characteristics at baseline

Patient Characteristics EAC	**N=563**
Age at inclusion (yrs), mean (SD)	56.0 (± 15.6)
Female, N (%)	394 (70.0)
Symptom duration at inclusion (months), mean (SD)	6.7 (± 10.5)
Swollen Joint Count, mean (SD)	5.72 (± 3.3)
Ritchie score, mean (SD)	10.3 (± 7.8)
ACPA-positive, N (%)†	250 (55.9)
IgM-RF-positive, N (%)†	322 (58.4)
HLA-DRB1 Shared Epitope +, N (%)†	339 (67.1)
CRP (mg/l), mean (SD)†	29.4 (± 34.2)
ESR (mm/h), mean (SD)†	39.5 (± 27.5)
HAQ, mean (SD)	1.1 (± 0.7)
Total Sharp-score, median (IQR)	5 (2-11)
Patient Characteristics NARAC	**N=393**
Age at disease onset	40.8 (± 11.9)
Female, N (%)	286 (72.8)
ACPA-positive, (%)§	100%
HLA-DRB1 Shared Epitope +, N (%)§	100%

† *Data on ACPA-, RF- and HLA DRB1 SE-status and CRP and ESR-levels were available in the EAC in 447, 551, 441, 520 and 544 out of 563 genotyped patients respectively.*

§ *Data on ACPA- and HLA DRB1 SE-status was available for all of the 393 genotyped patients*.

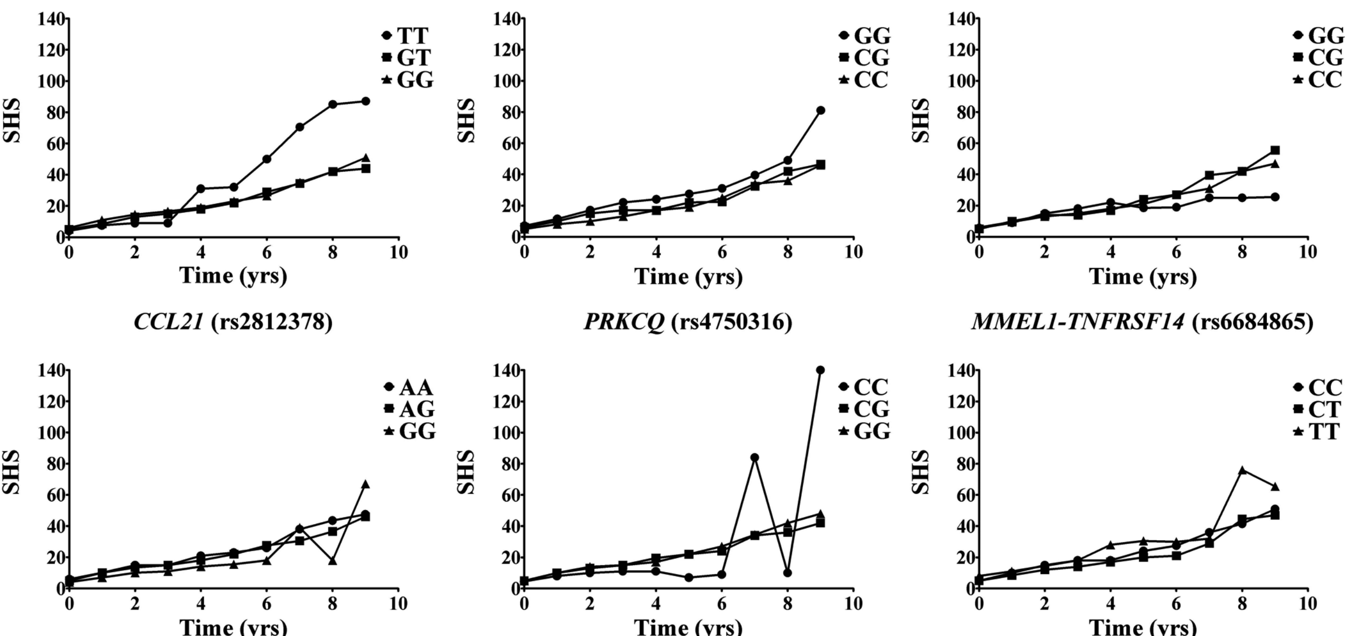
with the radiological progression over time was detected (p=0.268, 0.369, 0.679, 0.583 and 0.451 for rs4810485, rs1678542, rs2812378, rs4750316 and rs6684865 respectively). Because the genetic regions studied are thus far observed to be susceptibility-factors only for autoantibody-positive RA-patients, analyses were repeated in the ACPA-positive subgroup. Here, two polymorphisms, rs4810485 (*CD40*) and rs42041 (*CDK6*), affected the rate of joint destruction (Figure 2). For rs4810485, the G-allele was associated with a lower progression rate (GG/GT vs. TT, p=0.003). Back transforming the regression coefficient of the genotype in the model to the original scale yielded a 1.12 (95% CI 1.04-1.21) times larger increase in Sharp-score per year for carrying the risk genotype. For rs42041, the C-allele was associated with a higher rate of joint destruction (CC/CG vs. GG, p=0.012). For carriership of the C-allele a 1.09 (95% CI 1.02-1.16) larger yearly increase in Sharp-score was observed. Only rs4810485 was statistically significant after correction for multiple testing. The interaction between inclusion period and time was significant in all six analyses ($p<0.001$), demonstrating the effect of inclusion period on the radiological progression rate. Gender and age were not independently associated with progression.

To find replication, the effect of *CD40* and *CDK6* on radiological progression was analysed in 393 ACPA-positive RA-patients from the NARAC. Using a perfect proxy for rs4810485, the genotype associated with severity in the EAC also revealed a higher estimated radiological progression per year in the NARAC: 3.40 Sharp-units/year (n=23) vs. 2.83 and 1.83 Sharp-units/year (n=122 and 248, p=0.021). Using imputed data for rs42041 no significant differences between the three genotypes were observed (2.76, 2.38 and 2.07 Sharp-units/year, n=32, 163 and 188 respectively, p=0.327). The total number of patients available for analysis of rs42041 was 383; genotyping data were missing in 10 cases.

Discussion

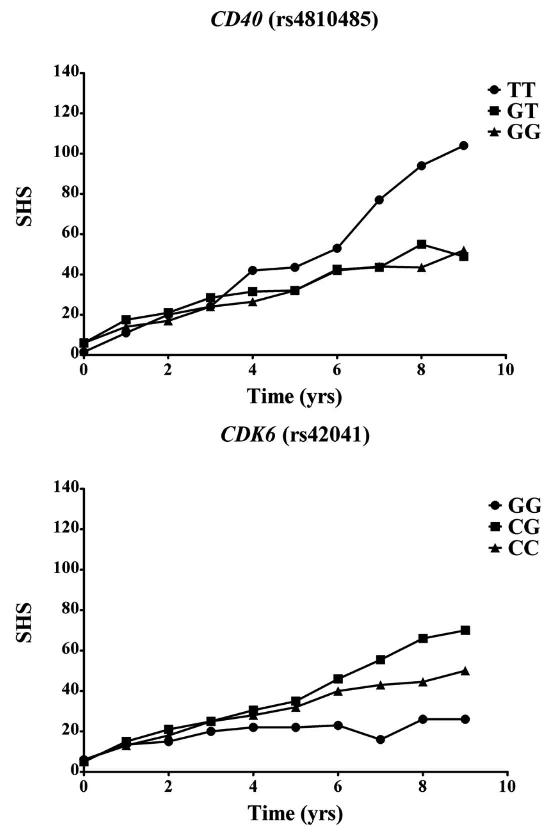
Although several clinical and serological risk factors for RA-severity are known, thus far the inter-individual variance in joint destruction is insufficiently explained and genetic factors are scarcely investigated. A better comprehension of the factors that mediate joint damage in RA may lead to the development targeted therapies or may contribute to prediction of the disease outcome in individual RA-patients. Most recently, six new loci were described to predispose to autoantibody-positive RA (2). Although susceptibility-factors do not necessarily affect disease progression, this study

Figure 1 Median Sharp-van der Heijde scores for the different SNPs per genotype in all RA-patients



Overview of the raw Sharp-van der Heijde scores, expressed as medians, of all 6 SNPs per genotype for the total patient population (n=563). The risk-alleles predisposing to RA in the study of Raychaudhuri et al (2) were the G-, C-, G-, G-, G- and T-allele for the rs4810485, rs1678542, rs42041, rs2812378, rs4750316 and rs6684865 SNPs respectively. The number of available radiographs varied per time-point and declined to 466 after 1 year of follow-up, 426, 357, 299 and 269 after 2 till 5 years of follow-up and 206, 154, 116 and 84 radiographs after 6, 7, 8 and 9 years of follow-up respectively. The number of patients in the different genotype groups were respectively: GG:280, GT:198, TT:22 (rs4810485); CC:247, CG:248, GG:67 (rs1678542); CC:305, CG:215, GG:43 (rs42041); AA:217, AG:279, GG:66 (rs2812378); CC:23, CG:183, GG:355 (rs4750316); CC:166, CT:170, TT:26 (rs6684865)*. *Due to technical difficulties genotyping was not successful in 63 and 201 of cases for rs4810485 and rs6684865 respectively. SHS: Sharp-van der Heijde score.*

Figure 2 Median Sharp-van der Heijde scores for rs4810485 and rs42041 in ACPA-positive RA



Overview of the raw Sharp-van der Heijde scores, expressed as medians, in ACPA-positive RA (n=250). The G-allele was the risk-allele predisposing to RA in the study of Raychaudhuri et al (2) for both the rs4810485 and rs42041 SNPs. The number of patients in the different genotype groups were for rs4810485*: GG: 128, GT: 88, TT: 11 and for rs42041: CC: 131, CG: 101, GG: 18. *genotype data for rs4810485 was not available in 23 cases. SHS: Sharp-van der Heijde score.

investigated whether these six SNPs are also risk-factors for a severe course of RA, measured by the rate of joint damage. The present data suggest that two SNPs, rs4810485 (*CD40*) and rs42041 (*CDK6*), influence the rate of joint destruction in ACPA-positive RA. Of these, only rs4810485 was significantly associated after correction for multiple testing and was replicated in an independent cohort of ACPA-positive RA-patients. As such, *CD40* is the first non-HLA-related genetic risk-factor for RA-severity that is independently replicated.

A recent study (2) reported a common variant at the *CD40* locus (the minor T-allele) to be protective for the development of RA. Surprisingly, here the minor T-allele associates with a higher rate of joint destruction in two cohorts. This finding is counter-intuitive, if one assumes that genetic variants associating with susceptibility also associate with severity. Although our findings were observed in two independent cohorts, and thus replicated, a type I error cannot be ruled out. The disease associated (common) allele marks a haplotype of *CD40* that contains a polymorphism in the upstream Kozak sequence that results in increased surface expression on B cells (7). To our knowledge, the effect of this haplotype on CD40 surface expression in synovial fibroblasts has not been directly studied. However, CD40-expression is increased on synoviocytes in RA and triggering of CD40 in synovial fibroblasts is associated with production of proinflammatory cytokines and osteoclastogenesis (8;9). It is likely that the biological pathways underlying susceptibility and severity are distinct with respect to CD40 triggering. This would provide an explanation for the finding that the minor T-allele has a protective effect in susceptibility studies but associates with a more severe disease course. Clearly it is essential to perform further studies on the mechanisms by which *CD40* polymorphisms associate with erosive outcome in RA.

A second SNP tended to associate with the rate of joint damage in RA in the EAC, rs42041. Absence of replication in the NARAC indicates that the observed association with the progression rate in the EAC cannot be interpreted. Nonetheless, it will be interesting to see the results on other studies analyzing *CDK6* and RA-severity. Thus, at present, of the two SNPs that tended to show an association with the rate of joint destruction, only the genetic variant in *CD40* is statistically significant after correction for multiple testing and is replicated and is therefore identified as a severity-factor for RA.

The other four studied SNPs in the loci encoding for *KIF5A-PIP4K2C, CCL21, PRKCQ* and *MMEL1-TNFRSF14* were not observed to associate with the severity of joint destruction. Therefore, these polymorphisms appear to be genetic risk-factors that are primarily associated with RA-susceptibility. Indeed, all of these SNPs were recently replicated as true susceptible loci in RA-patients of European ancestry (10).

The prospective nature of the data of the EAC strengthens the impact of the findings because higher radiological scores for risk-genotypes were present at subsequent time points; as such the present data set is advantageous in comparison to studies that

assessed cross-sectional radiological data. The fact that a large number of patients with a long follow-up of up to 9 years were included for analysis is clearly an advantage, but also has a limitation. Inherent to the design of an inception cohort, not all patients had achieved maximum follow-up, so the number of missing data that the mixed-model had to take into account increased with longer follow-up. Small numbers of radiographs available at the latest time points are also the most likely explanation for the observed "bump" at the 8 year time point for the genotypes GG, CC and TT of the SNPs rs2812378, rs4750316 and rs6684865 respectively (Figure 1).

Evaluation of the effect of genetic factors on the rate of joint destruction during the disease course inevitably implies that other factors that affect the disease course should be taken into consideration as well. Analyses for all six SNPs revealed that inclusion period, a proxy for treatment strategy, was significantly associated with the rate of joint damage, which is in line with previous results from the EAC (11). The analyses on *CD40* and *CDK6* showed that these SNPs were associated with joint damage, independent from treatment strategy. Nevertheless, corrections for treatment strategy were made on group-level and thus were an approximation for the real effect of treatment on the rate of joint destruction for individual RA-patients.

In conclusion, a polymorphism in the *CD40* locus shows a significant association with the rate of joint destruction in ACPA-positive RA, a finding that is replicated in an independent cohort. Although further studies are needed to identify the causal variant, the data presented provide a foundation for further investigations of the role of CD40 in joint destruction in RA.

Supplementary Methods

Statistical Analysis

To take advantage of the prospective character of the data of the EAC, consisting of repeated measurements, and to avoid multiple testing by performing statistical tests for each time point, a linear model for longitudinal data was used, with the log transformed Sharp-score as response variable, to compare the radiological progression rates between genotype groups. Different correlation structures between the repeated measurements were explored, and based on the Akaike's information criterion, an autoregressive correlation structure with heterogeneous variances was chosen. Due to

the study design (an inception cohort) not all patients achieved a similar duration of follow-up. The model takes missing observations into account, assuming that the missing is at random. Differences in progression rates between the different genotypes were tested by considering the significance of the interaction between genotype and time with time as linear covariate. Age, gender and inclusion period (before 1996, 1996-1998, 1999-2001, after 2001) and their interactions with time were entered in the model to correct for possible confounding effects. In order to prevent overfitting of the data no corrections were applied for other variables. Inclusion period is a proxy for treatment modalities, because treatment strategies improved over time and an influence of the treatment strategy on the progression of radiographic joint damage was observed previously, as well as in the present study. The following treatment strategies were applied in the subsequent inclusion periods. Patients included between 1993 and 1995 were treated initially with analgesics and subsequently with chloroquine or sulfasalazin if they had persistent active disease (delayed treatment). From 1996 to 1998 RA-patients were promptly treated with either chloroquine or sulfasalazin (early treatment) (3). From 1998 to 2002 patients were promptly treated with either sulfasalazin or methotrexate (early treatment) and patients included in 2002 or later were promptly treated with either sulfasalazin or methotrexate combined with treatment adjustments based on the disease activity (early and disease activity based treatment).

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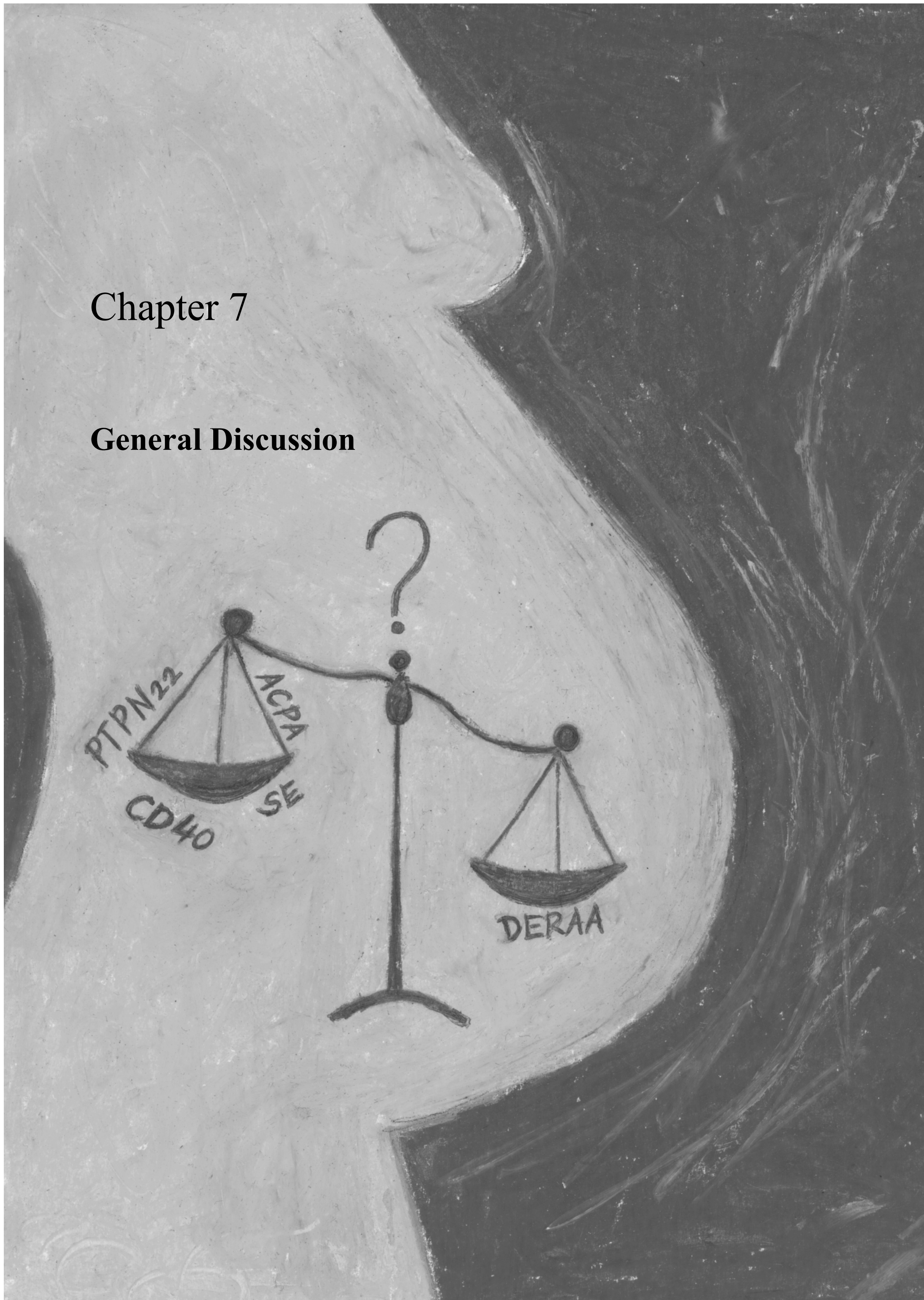
Chapter 7

General Discussion



Many cell types take part in the immunological processes contributing to the synovial and systemic inflammation present in patients with rheumatoid arthritis (RA), including T cells and B cells. They exert their own functions and interact with each other, resulting in the chronic inflammation observed in RA. The research presented in this thesis focused on the identification of several risk factors and specific T cell responses that are thought to play a role in the pathogenesis of RA.

Four major topics were studied. Firstly, we showed that "DERAA"-containing HLA-DRB1 alleles are less frequently present in RA patients as compared to controls both when these alleles are inherited directly as well as when they are acquired as non-inherited maternal antigen (NIMA) (**Chapter 2 and 3**). Secondly, two naturally processed epitopes derived from the human citrullinated vimentin protein were identified in mice transgenic for the most frequent SE-containing HLA-DRB1 allele in Caucasians, HLA-DRB1*0401. IFNγ-production by $CD4^+$ T cells against these peptides could be observed in RA patients (**Chapter 4**). These T cells could be involved in providing help to ACPA-producing B cells. Furthermore, we showed in **Chapter 5** that the C1858T polymorphism of the PTPN22 gene is not informative for the prediction of RA development in UA patients in addition to ACPA status, but does seem to affect ACPA-levels of RA patients. In **Chapter 6**, we observed that a newly identified risk factor for RA, CD40, also influences the severity of the disease as measured by radiological damage.

Regarding these findings, the following topics will be discussed in further detail in the different sections of this chapter:

1. "DERAA"-containing HLA-DRB1 alleles
    a. The possible mechanism of the observed protection
    b. Maternal michrochimerism as mechanism of the observed NIMA effect
    c. Associations of these HLA-DRB1 alleles with other diseases
2. PTPN22 and ACPA
3. The role of CD40 on ACPA production and RA development
4. Directions for further research

1a. Possible mechanism of DERAA protection

It has been shown by several groups that the frequency of "DERAA"-containing HLA-DRB1 alleles is reduced in RA patients as compared to healthy controls (1-4). We have described in **Chapter 2 and 3** of this thesis that this protective effect is present both when these HLA-DRB1 alleles are inherited, as well as when the gene products are

acquired as a non-inherited maternal antigen (NIMA). Although it is becoming increasingly clear that some HLA-DRB1 alleles confer protection to RA (5;6), it is unclear whether the entire "DERAA"-motif is essential for protection or whether only certain amino acids of this motif may confer the same effect. In contrast to several reports showing the protective effects by "DERAA"-containing HLA-DRB1 alleles on the development and severity of RA (1-4), other reports conclude that the amino acids "RAA" at position 72-74 in the third hypervariable region influence the susceptibility to RA development whereas the amino acids at position 70 and 71 modulate this effect (7;8). In these articles it is indicated that not only HLA-DRB1 alleles expressing the ^{71}ERAA^{74} sequence but also alleles that only contain the Aspartic acid (D) at position 70 have a lower frequency in RA patients as compared to healthy controls. The hypothesis that protection is mainly associated with the Aspartic acid (D) at position 70 is supported by Ruiz-Morales *et al*. (9) and Mattey *et al* (10). A meta-analysis including large group sizes and different study populations has to be performed to elucidate which of the amino acids are essential for the observed protective effect. In the section below it is assumed that the "DERAA"-motif is responsible for the observed protection.

Table 1. Human proteins containing the amino acid sequence DERAA. The sequences are depicted as peptides with "DERAA" in the centre and 7 flanking residues on each side.

Protein name	Sequence
Vinculin	PNREEVF**DERAA**NFENHSG
MHC	QKDI(L/F)LE**DERAA**VDTYCRH
Parkin	TTQAYRV**DERAA**EQARWEA
PER1 (period circadian protein)	SCLFQDV**DERAA**PLLGYLP

The mechanism by which the "DERAA"-containing HLA-DRB1 alleles influence the susceptibility to and the severity of RA is unknown, but it has been proposed that it is mediated by T cells recognizing peptides containing the "DERAA"-sequence presented by HLA-DQ molecules (11). As it is well-known that many peptides presented by HLA-class II molecules are derived from other HLA-molecules (12-18), it is hypothesized that the T cell repertoire of individuals carrying the "DERAA"-containing HLA-DRB1 alleles, in contrast to "DERAA"-negative individuals, is tolerized for "DERAA"-containing antigens. The "DERAA"-sequence has been

described to be only present in four human proteins *(19)*: i.e. parkin, PER1, the "DERAA"-containing HLA-DRB1 molecules and vinculin (*Table 1*). Parkin and PER1 are both brain-specific proteins and are therefore probably not involved in the protection against RA. It has been shown that vinculin, a cytoskeletal protein, can be found under certain conditions (e.g. during apoptosis) on the cell surface, and that cross-priming to vinculin-specific cytotoxic T lymphocytes can occur (20;21). These findings indicate that vinculin-specific T cells can exist and thus are not tolerized in the thymus, despite the fact that vinculin is an abundantly expressed self-protein present in the cytoskeleton of every cell in the body. We have observed that PBMC from "DERAA"-negative individuals can respond with significantly more IFNγ to the "DERAA"-containing peptide derived from vinculin than those of "DERAA"-positive individuals (data not shown). It is hypothesized that the vinculin peptide is recognized as self in "DERAA"-positive individuals since the "DERAA"-containing HLA-DRB1 peptide is presented by another HLA molecule to the immune system, therefore resulting either in deletion of vinculin-reactive T cells or skewing of the cytokine profile to a suppressive profile without IFNγ.
Since it is not likely that IFNγ-producing T cells reactive against self-proteins are induced by such self-proteins, giving rise to autoimmunity, these vinculin-reactive T cells possibly result from e.g. an infection. Indeed, the phenomenon that molecular mimicry from a pathogen to a self-protein can lead to autoimmunity has been reviewed and observed several times (22-28). Many pathogens, such as Influenza, Measles, Bordetella Pertussis and Salmonella, can express proteins containing the "DERAA"-sequence. We therefore hypothesize that "DERAA"-directed T cells stimulated by a pathogen-derived "DERAA"-containing peptide accidentally can cross-react with human self-proteins, e.g. vinculin. Therefore, they aggravate an ongoing inflammation in the joints and play a role in the development of RA (Figure 1). These "DERAA"-(cross)reactive T cells only exist in "DERAA"-negative individuals (Figure 1A) in contrast to "DERAA"-positive individuals (Figure 1B) who are tolerant to the "DERAA"-sequence.

Since it was shown that the "DERAA"-containing peptide derived from the HLA-DRB1*0402 molecule can be presented by HLA-DQ8 (11), evidence supporting the hypothesis formulated above was obtained by analyzing T cell reactivity against the "DERAA"-containing peptide derived from Influenza A in DQ8-transgenic mice. Our preliminary data indicate that T cells specific for the "DERAA"-containing peptide derived from vinculin can be observed after immunization with this peptide, but not

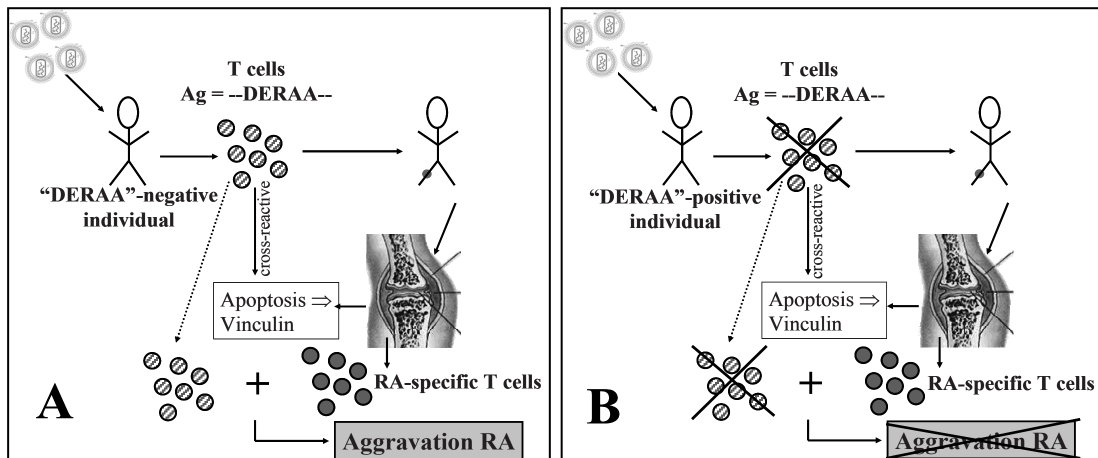


Figure 1. Proposed mechanism for the effect of "DERAA"-containing HLA-DRB1 molecules on RA. An individual is infected with a "DERAA"-containing pathogen. Depending on whether it is a "DERAA"-negative (A) or a "DERAA"-positive (B) individual T cells reactive with the "DERAA"-containing peptide processed from the pathogen are triggered or not. These T cells, which are only present in "DERAA"-negative individuals (A) can lead together with RA-specific T cells to the aggravation of the inflammation leading to the diagnosis of RA.

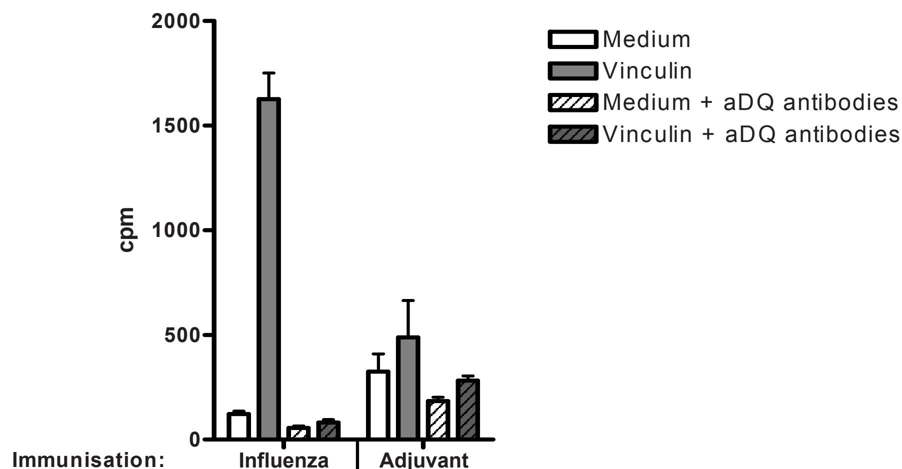


Figure 2. Crossreactivity to vinculin in DQ8-transgenic mice. Mice were immunized with the "DERAA"-containing peptide derived from Influenza (Influenza) or PBS in adjuvant (Adjuvant). Spleen cells were cultured for 4 weeks with the immunizing flu peptide and tested afterwards in a proliferation assay with 10,000 cells/well. No (open bars) or anti-HLA-DQ antibodies (hatched bars) were added to the wells without stimulation (white bars) or stimulated with the "DERAA"-containing peptide derived from vinculin (grey bars). Bars represent the mean amount of counts after addition of 3[H]-Thymidine overnight measured in triplicate with the SEM.

in mice immunized with a control peptide (data not shown). T cells triggered against the "DERAA"-peptide derived from Influenza A and cross-reactive with the vinculin-derived "DERAA"-peptide were HLA-DQ restricted (Figure 2, left part). These T cells are absent in mice immunized with adjuvant only (Figure 2, right). Together, these data suggest that T cells can show reactivity to the vinculin peptide after initial triggering against the "DERAA"-peptide derived from a pathogen, e.g. Influenza A.

When the decreased frequency of "DERAA"-containing HLA-DRB1 alleles in RA patients is studied in more detail, several articles showed that "DERAA"-containing HLA-DRB1 alleles protect against the development of $ACPA^{+}$ RA (1;6). Since it has been shown that SE-containing HLA-DRB1 alleles associate with the production of ACPA and the so-called SE is located at the same position in the HLA-DRB1 molecule as the amino acids "DERAA", this association has to be corrected for the presence of SE-containing HLA-DRB1 alleles. Lundstrom *et al.* showed that also after stratification for SE-containing HLA-DRB1 alleles, the frequency of HLA-DRB1*13 alleles (most frequent "DERAA"-containing HLA-DRB1 alleles) is significantly decreased in $ACPA^{+}$ compared to $ACPA^{-}$ RA patients (29), indicating that the "DERAA"-containing HLA-DRB1*13 alleles can protect against the development of ACPA. In a meta-analysis with RA patients and controls from four different countries (including our own EAC and BEST cohort) it was shown that HLA-DRB1*1301 alleles protect against the development of $ACPA^{+}$ RA in contrast to $ACPA^{-}$ RA after stratification for SE-containing HLA-DRB1 alleles. Since the HLA-DRB1*13 alleles account for 78-93% of the "DERAA"-containing HLA-DRB1 alleles present in the studied patients, the meta-analysis was still underpowered to prove or exclude HLA-DRB1*0103 and *0402 for the protective effect on $ACPA^{+}$ RA (30). Therefore, future studies have to clarify which HLA-DRB1 alleles confer protection to ACPA.

1b. Maternal microchimerism as mechanism of the observed NIMA effect

When the protection induced by both inherited and non-inherited "DERAA"-containing HLA-DRB1 alleles on RA is working via molecular mimicry, it also could explain the mechanism of the NIMA effect.

In **Chapter 2 and 3** of this thesis we hypothesize that the observed NIMA effect of "DERAA"-containing HLA-DRB1 alleles is caused by maternal microchimerism. It has been shown in humans that there is long-term persistence of microchimerism (31) and that these microchimeric cells can differentiate into different tissue-specific cell types (32;33). It can be hypothesized that due to the presence of "DERAA"-containing

HLA-molecules on the surface of microchimeric maternal cells the child will recognize "DERAA"-containing antigens as self and therefore the observed T cell responses will resemble the reactivity of a "DERAA"-positive individual. This will result either in regulatory T cells or deletion of "DERAA"-reactive T cells, as occurs with the inherited effect of "DERAA"-containing HLA-DRB1 molecules.
It has been shown that *in utero* there is a much higher percentage of regulatory T cells in the fetus compared to after birth. T cell tolerance to alloantigens (e.g. NIMA) present *in utero* may, in some cases, be maintained after birth through the establishment of long-lived regulatory T cells (34). Following this scenario, the acquirement of "DERAA"-containing antigens *in utero* might result in the lifelong absence of pro-inflammatory "DERAA"-reactive T cells.
Another possibility is that the maternal microchimeric cells end up in the thymus serving as antigen presenting cell (APC), thereby inducing regulatory T cells or deletion of T cells, e.g. against "DERAA"-containing antigens. It is also hypothesized by Dutta and Burlingham that it is not the microchimeric APC themselves, but maternal antigen acquisition by host APC from these few microchimeric cells that drives the balance of T effector and regulatory T cells in favor of the latter (35).

1c. Effects of "DERAA"-containing HLA-DRB1 alleles in other diseases

Thus far we have focused on the association of "DERAA"-containing HLA-DRB1 alleles with RA. We wondered however whether the "DERAA"-containing HLA-DRB1 alleles might also be associated with other (immunological) disorders. Therefore a preliminary literature search was performed to analyze this. The most common "DERAA"-containing HLA-DRB1 alleles, HLA-DRB1*13 (from which approximately 97% will be "DERAA"-containing HLA-DRB1*13 alleles in the Caucasian population (36;37)), are associated with Hepatitis B (38-44) and –C infections (45-48), cervical cancer (49-54), HIV(55-57) and systemic lupus erythematosus (58-61). In all these different diseases, carriership of HLA-DRB1*13 protects either for the development of the disease or chronicity/ complications of the disease. Therefore, "DERAA"-associated protection does not seem to be specific for RA. It is unknown whether the "DERAA"-sequence of the HLA-DR13 molecules is directly involved in the observed protection. When future research indicates that these effects can be attributed to the presence of "DERAA" in the HLA-molecule, these observations can help to elucidate the mechanism by which these "DERAA"-containing HLA-DRB1 alleles can influence the immune response.

2. PTPN22 and ACPA

The most studied SNP of the PTPN22 gene is the C1858T polymorphism. This single nucleotide change results in an amino acid substitution from Arginine to Tryptophan in the Lyp protein transcribed from the PTPN22 gene. This substitution is located in a binding domain important for the function of Lyp, which acts as a Tyrosine phosphatase (62). The function of Lyp is the negative regulation of T cell receptor signaling, either direct or indirect, by influencing the phosphorylation status of different molecules involved in the signaling cascade that leads to T cell activation (63-69). The functional consequences of the amino acid change resulting from the C1858T polymorphism are not entirely clear as it has not only been shown that it leads to more T cell activation since there is less inhibition of the activation signal by Lyp (70), but also that cells from carriers of the T variant produce less cytokine (71). In mice it has been shown that a knock-out of Pep, the mouse ortholog of the Lyp protein, displays a hyperreactive T cell response (72). Lyp is expressed in different cell types and probably exerts different functions in e.g. T and B cells.

In **chapter 5** of this thesis, the predictive value of the C1858T polymorphism was studied next to ACPA status. Our study demonstrated an independent association of ACPA but not of the PTPN22 C1858T polymorphism with progression to RA among patients presenting with UA, although the presence of this SNP is associated with an increased level of ACPA in ACPA$^+$ patients. This finding was confirmed by another study (73). In many articles, it has been shown that the odds to be a carrier of the T variant of the C1858T polymorphism is about two times increased in ACPA$^+$ compared to ACPA$^-$ individuals, indicating that carriership of the T variant of the C1858T polymorphism is associated with ACPA production (74-77). The production of ACPA is also associated with the SE-containing HLA-DRB1 alleles. Intriguingly the association of PTPN22 with RA is also present only in SE-positive individuals and not in SE-negative individuals (76), indicating that SE and the PTPN22 allele are in the same biological pathway. As both genetic risk factors associate with ACPA$^+$ disease, the contribution of PTPN22 is probably found in setting the balance for ACPA production. It is therefore conceivable that PTPN22 associates with ACPA production because it has a direct impact on the activity of the B cell receptor as it has been found that the T-variant of the C1858T polymorphism results in less B cell receptor signaling (78;79). More extensive studies have to show how these data relate to each other.

Thus, pathophysiologically both the SE-containing HLA-DRB1 alleles, ACPA and PTPN22 are players in the same pathway (*see Figure 3*), and by measuring ACPA the effect of the PTPN22 1858T-allele when present, is already included.

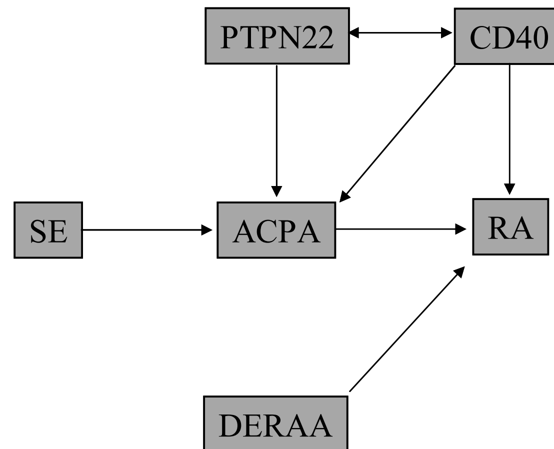


Figure 3. Schematic representation of the risk factors for RA that were studied and discussed in this thesis. The arrows indicate the direction of the putative interactions.

3. The role of CD40 on ACPA production and RA development

Recently, a SNP located in the intron of the gene encoding for CD40 was identified to associate with RA in a genome wide association study (GWAS) (80). The susceptibility allele of this SNP associates with less severity or progression of RA, as described in **chapter 6** of this thesis. CD40 is a well-known molecule expressed on B cells and other antigen presenting cells required for optimal cell activation by T cells. Its ligand, CD40L, is expressed on activated $CD4^{+}$ T cells. Triggering of CD40 is involved in B cell proliferation, antibody production, class-switching and B cell memory formation. It is unknown what functional consequences the SNP has on the expression of CD40 protein, but the effect can be either on the B cell or other professional APC such as dendritic cells. Moreover, CD40 is also reported to be expressed on synovial fibroblasts.

Regarding the contribution of CD40 to the process of disease development of RA, it can either influence the production of ACPA and thereby the susceptibility and severity of RA or play an independent role on the pathogenesis of RA (Figure 3). There is only one article on the influence of CD40 on the production of ACPA. In this article it is

shown that B cells from the peripheral blood (both from RA patients and healthy controls), synovial fluid and bone marrow all start to produce ACPA in response to CD40 triggering. Although B cells from ACPA-positive individuals already produce ACPA without *in vitro* stimulation, the ACPA production is increased after CD40 stimulation (81). For rheumatoid factor (which are autoantibodies specific for the Fc portion of IgG) it has been shown that CD40 signaling plays a major role in the survival of rheumatoid factor producing B cells and therefore in the rheumatoid factor production (82).

CD40 signaling into the antigen presenting cell is mediated by the (de)phosphorylation of the Tyrosine kinases (83) on which Lyp also exerts its function. Therefore the CD40 signaling pathway and the effect of the PTPN22 SNP in B cells can probably influence each other, and it would by intriguing to know whether a similar relationship between CD40 and PTPN22 and/or SE-containing HLA-DRB1 alleles can be formed as described for the PTPN22-HLA-SE interaction.

On the T cell site CD40-CD40L interaction plays an important role in the amplification of the T cell response by a positive feedback loop for the production of co-stimulatory molecules on dendritic cells (84).

The role of CD40-CD40L interaction in arthritis has also been studied in different mouse models. It has been shown that blocking of the CD40L molecule results in prevention of collagen-induced arthritis (CIA), both measured in clinical scoring and in the absence of anti-collagen antibodies (85). The inhibition or prevention of arthritis in both the K/BxN model and DBA1 mice by prevention of the CD40-CD40L interaction has also been shown, but this does not affect established arthritis indicating that the CD40-CD40L interaction plays a role in the initiation rather than in the exacerbation phase of the arthritis (86-88).

CD40 is not only expressed on B cells but also on other antigen presenting cells. Therefore the influence of CD40 on RA may also be attributable to induction of fibroblast proliferation (89), the induction of TNFα production by synovial cells (90-92), chondrocytes (93) or osteoclasts (94).

A sheme showing the relationship of the risk factors studied in this thesis to RA is shown in figure 3.

4. Directions for further research

As already indicated in several parts of the discussion, future research has to be performed to elucidate the phenomena observed and studied in this thesis. Here I will indicate future directions that could be followed and that are not mentioned in the previous sections.

"DERAA"-containing HLA-DRB1 alleles protect against the development and severity of RA, both when they are inherited and when they are acquired as a NIMA. We observed that the effect of the "DERAA"-containing HLA-DRB1 alleles is of a similar strength when they are inherited compared to acquired as a NIMA. The underlying mechanism of the effect of "DERAA"-containing HLA-DRB1 alleles has been discussed in the previous sections but certainly is not proven up to now. The T cell reactivity observed against the peptide derived from the human cytoskeletal protein vinculin needs to be shown also for naturally processed peptides from the whole protein. The possibility of cross-reactivity of T cells triggered against a pathogen-derived peptide containing the "DERAA"-sequence, i.e. from Influenza A, with the "DERAA"-containing peptide derived from vinculin is demonstrated in DQ8-transgenic mice. It is important to know whether "DERAA"-specific T cells are triggered when a mouse or individual is infected with a "DERAA"-containing strain of the Influenza A virus. Furthermore, extensive studies for cross-reactivity of other pathogen-derived "DERAA"-containing peptides with the vinculin-peptide have to be performed both in mice and in humans. An extensive meta-analysis for the effect of "DERAA"-containing HLA-DRB1 alleles in other diseases and infections can probably help to elucidate the underlying mechanism.

An extensive family study in which individuals from three generations can be studied possibly will give more insight in the mechanism underlying the observed effect of "DERAA"-containing HLA-DRB1 alleles as a NIMA. It is important to know in which immunological status the acquirement of "DERAA"-containing antigens can still lead to a protective effect; is it necessary to acquire this NIMA in a fetal stage or is induction of the effect still possible when acquired in adulthood? The latter would have implications to treat "DERAA"-negative individuals with "DERAA"-containing molecules (e.g. by means of transfusing cells expressing the "DERAA"-containing HLA-DRB1 molecules) to induce protection against the development of RA in individuals who are at risk or ameliorate existing disease in "DERAA"-negative patients.

When more is known about the fine-specificity of ACPA, the interplay between T cells and their help to ACPA-producing B cells can be studied in more detail. This both accounts for “citrulline”-specific T cells and for T cells specific for a connected protein (e.g. vinculin) helping an ACPA-producing B cell. Since these studies rely on a delicate choice of at least HLA-type, ACPA-status and ACPA-specificity, large cohorts are necessary to perform these studies.
Both for the C1858T polymorphism of the PTPN22 gene and the CD40 SNP, functional studies are necessary to study what the precise effects are of the nucleotide change and whether the already studied SNPs are the most informative or whether they are in linkage disequilibrium with another SNP that is the causative SNP for the functional effect. After this, the cell type important for the observed effect has to be defined.

In conclusion, several different aspects playing a role in the pathogenesis of RA were studied in this thesis. Answers were found, opening new perspectives for further research, but also raising many new questions, waiting to be answered.

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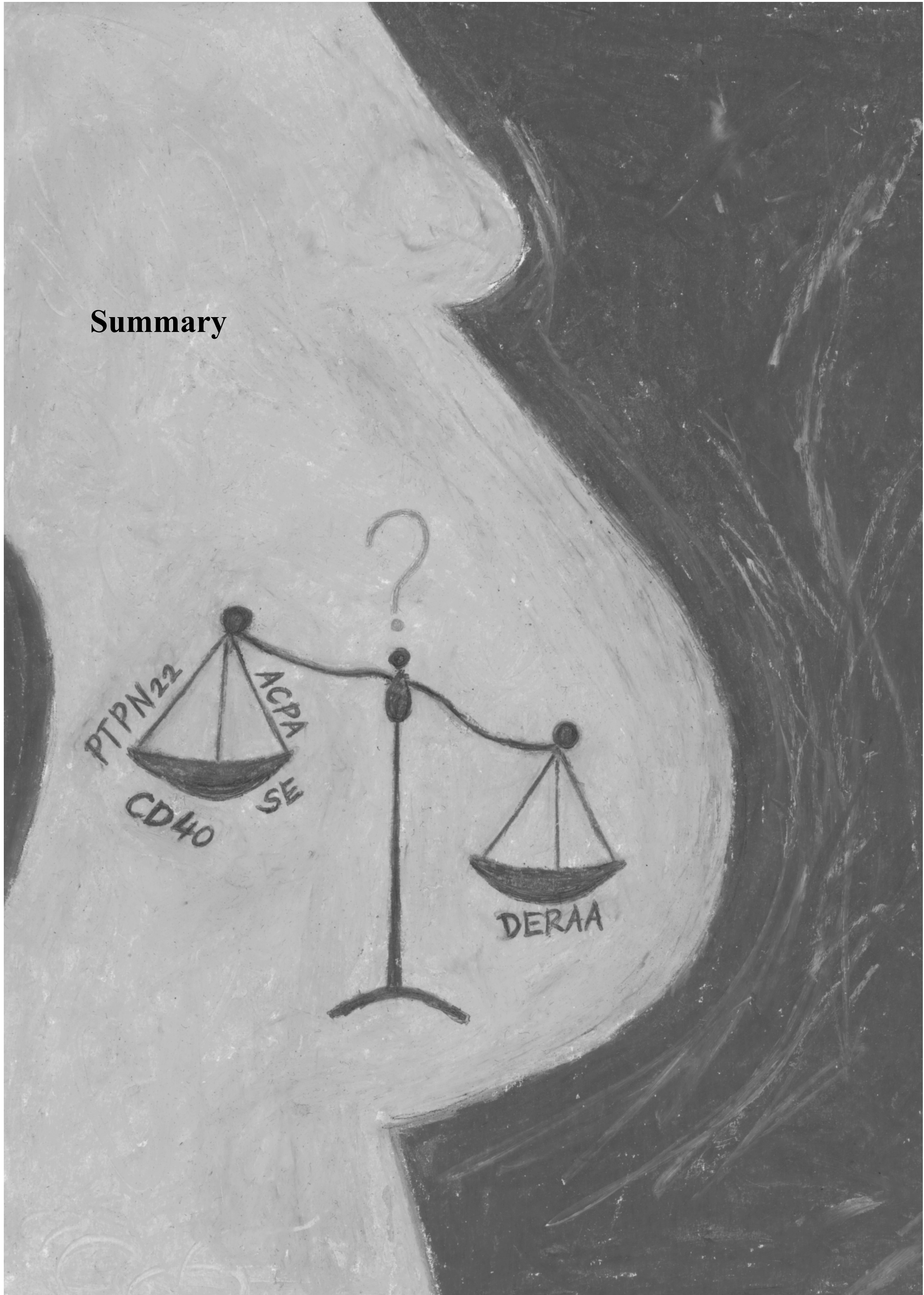

Summary
PTPN22
ACPA
CD40
SE
DERAA

Rheumatoid arthritis

Arthritis is a group of conditions characterized by joint inflammation. This inflammation can be caused by different mechanisms (e.g. autoimmunity, fractures, wearing, or infection) and can lead to breakdown of the cartilage in the joints. Rheumatoid arthritis (RA) is a chronic inflammation of several joints caused by autoimmunity. The diagnosis of RA is based on a list of seven criteria: morning stiffness, arthritis of 3 or more joint areas, arthritis of hand joints, symmetric arthritis, rheumatoid nodules, serum rheumatoid factor and radiographic changes. Since only four of these seven ACR criteria have to be fulfilled for the diagnosis of RA, the RA patient population is clinically heterogeneous. The occurrence of RA varies among countries and areas over the world; in Europe it has a prevalence of approximately 1% within the Caucasian population. In the Dutch population, women are affected by RA approximately two times more frequently than men. The progression of the disease is often measured by making x-rays of the joints (e.g. each year) in addition to assessments of physical function and questionnaires. The mechanism of RA development is complex and largely unknown, but it is generally accepted that both genetic and environmental factors play a role. In this thesis, on the one hand, factors involved in the protection against RA are studied. On the other hand, processes and genetic factors involved in the severity of RA and an increased risk of RA development have been investigated. In this summary several basic immunological aspects will be discussed and all chapters will be discussed briefly.

The immune system

The immune system is comprised of cells involved in adaptive and innate immune responses, each cell exerting his own function. Adaptive immune responses are characterized by the fact that they are antigen-specific immune responses of cells having a receptor specific for the antigen it responds to, which are the T cell receptor and B cell receptor in this case. When a B cell recognizes the antigen with its B cell receptor, this can result in the production of antibodies. An antibody is a protein that binds specifically to its corresponding antigen and thereby pathogens are neutralized or prepared for uptake and destruction by phagocytes. Adaptive immune responses occur during lifetime and involve specific antigen recognition and expansion of B and T cells. This distinguishes such responses from innate immunity, which includes the engulfment and digestion of pathogens by phagocytic cells that are immediately available. The innate immune response is directed against common components of

pathogens and not against an individual pathogen. Innate and adaptive immune responses are interdependent, and are exerted mainly by white blood cells (leukocytes). The different cells from the immune system all exert their own function. Antigen presenting cells (APC), e.g. dendritic and B cells, scan the whole body for its content, both self-proteins and infectious agents. They can take up these proteins, cut them in pieces (called peptides) and present these peptides via what is called a HLA molecule to a T cell (**Figure 1**). These HLA molecules are the most important genetic risk factor involved in RA development. Regarding the risk of RA development, three variants can be discriminated; either by a variant that increases the risk of RA development (called shared epitope (SE)), or by a variant that gives a lower chance of RA development (called DERAA), or by a neutral variant that does not have an influence on the development of RA.

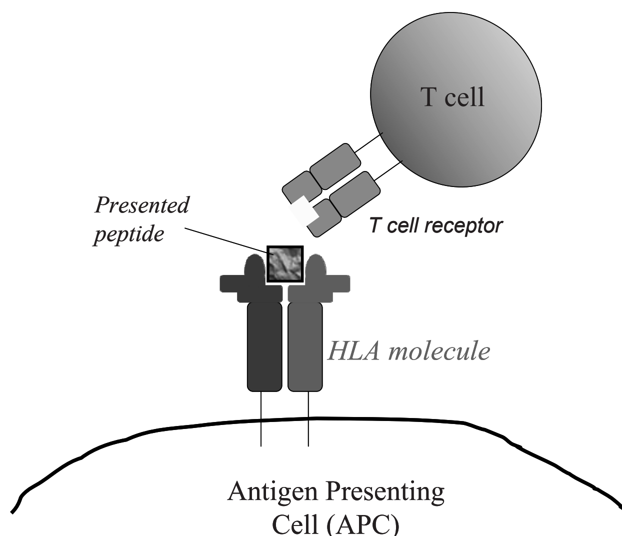


Figure 1. Presentation of a peptide to a T cell by an antigen presenting cell (APC). The peptide is presented bound in a HLA molecule to the T cell via its specific receptor.

Protection by "DERAA"

Different individuals can have different HLA molecules. Each variant has a slightly different composition of the building blocks, called amino acids, it consists of. There are 20 different amino acids which can be indicated with a one letter code. The variants containing at a specific position the amino acids that are coded by the letters "DERAA", give an individual a lower chance to develop RA. This effect had been described already, but we describe in **Chapter 2** and **Chapter 3** of this thesis that a mother with a HLA molecule containing this DERAA-motif confers a life-long

protection to her child against the development of RA both with and without passing the gene responsible for this HLA molecule to the child. This in contrast to a father that has a DERAA-containing HLA molecule. The father can only confer protection to the child when his DERAA-containing HLA molecule is inherited by the child.

Vimentin-specific T cells

RA patients can develop different kinds of antibodies, from which some can react against proteins from the body which have a little difference compared to the regular protein. This change is called citrullination and therefore these antibodies are called anti-citrullinated protein antibodies (ACPA). Citrullination is a common natural process present in all individuals, but only RA patients can develop these antibodies. One of the proteins these antibodies can react to is vimentin. This is a self-protein that is present in every cell of an individual. B cells can receive signals from activated T cells to produce antibodies. In **Chapter 4** of this thesis it was studied whether we could identify the peptides from the vimentin protein which an APC presents to a T cell to activate this T cell. We were especially interested in Citrulline-specific T cell responses. This identification was performed with the help of HLA-transgenic mice expressing a human HLA-molecule on their cells. Two peptides inducing a Citrulline-specific T cell response were identified. The cells from 10 RA patients and 5 healthy controls were tested for reactivity against these two peptides. Several RA patients showed a Citrulline-specific response, which was absent in the 5 healthy controls. Since the amount of tested individuals was small, more extensive studies have to be performed to identify the presence/absence of T cell responses in different groups of individuals.

PTPN22 and CD40

Next to HLA molecules there are several other genetic factors involved in the risk of RA development. In this thesis, two of these genes are studied. They are called PTPN22 and CD40.

The sequence of building blocks of a gene, called nucleotides, can differ between different individuals in a population. When the frequency of a single nucleotide change is equal or higher than 1%, this is called a single nucleotide polymorphism (SNP). The most studied SNP of the PTPN22 gene is associated with RA and several other auto-immune diseases. There were indications that there is also a relation between this SNP of the PTPN22 gene and the development of ACPA antibodies. We investigated in **Chapter 5** whether this SNP can give additive value to the prediction of the

development of RA when information about the PTPN22 gene is combined with presence/absence of ACPA antibodies. The analyses showed that both information on the PTPN22 SNP and the presence/absence of ACPA can help to predict the development of RA, but both factors combined do not result in additive value for prediction.

Because nowadays, RA patients are seen in an earlier phase of the disease, before the appearance of well established indicators of poor prognosis such as erosions and nodules, markers which have a good predictive value on radiographic damage in an early phase of the disease will become more important. Therefore, we investigated in **Chapter 6** whether different SNPs, including one in the CD40 gene, are involved in the progression and severity of RA once patients are diagnosed. It was shown that the SNP of the CD40 gene was indeed associated with the severity and progression of RA. The CD40 gene encodes a molecule, CD40, that is involved in the interactive signaling between B and T cells to activate them. Further studies have to show the functional effects of the SNP in the CD40 protein.

The studies included in this thesis are quite diverse but all deal with different aspects playing a role in RA development or progression of the disease. Several questions have been answered, but all result in a range of new questions that need to be answered in future studies. I hope this summary will explain a little bit the struggles I have been trying to answer in the last 5 years. Of course everybody is invited to read the detailed versions of each article in the indicated chapters!

Nederlandse samenvatting

Reumatoïde artritis

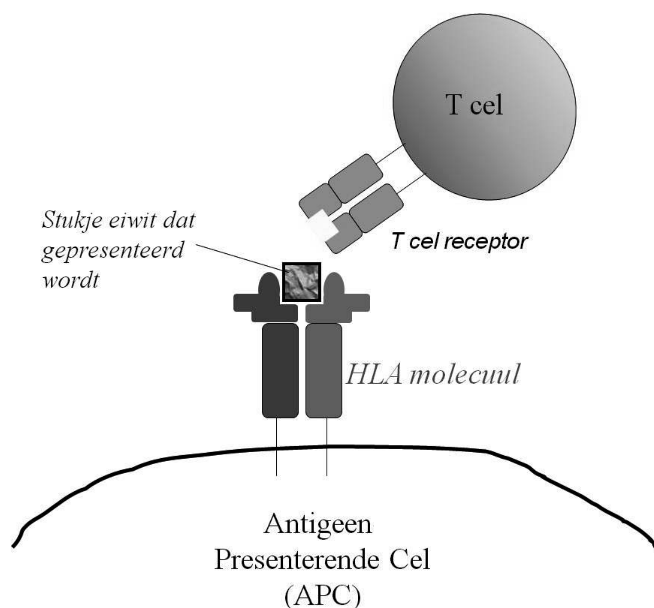
Artritis is een groep van verschillende aandoeningen die worden gekarakteriseerd door gewrichtsontsteking. Deze ontsteking kan worden veroorzaakt door verschillende mechanismen, bijv. autoimmuniteit (de ontsteking is gericht tegen eiwitten/cellen van het eigen lichaam), breuken, slijtage of infectie en kan leiden tot de afbraak van het kraakbeen dat zich in de gewrichten bevindt. De diagnoses van de verschillende vormen van artritis worden gesteld aan de hand van ziektecriteria. Reumatoïde artritis (RA) is een chronische ontsteking van verschillende gewrichten veroorzaakt door een autoimmuun-reactie. De diagnose ervan is gebaseerd op een lijst van zeven symptomen: ochtendstijfheid, artritis aan 3 of meer gewrichten, artritis van de hand-gewrichten, symmetrie van de artritis, reumatoïde nodules, positief voor reumafactor (bepaald antilichaam) en veranderingen in de gewrichten die te zien zijn op röntgenfoto's. Als er aan minstens vier van deze symptomen wordt voldaan, wordt de diagnose RA gesteld. Dit betekent dat twee verschillende personen met RA maar één symptoom overeenkomstig hoeven te hebben en verder hele verschillende klachten laten zien. De ernst van het verloop van RA wordt vaak gemeten door elk jaar röntgenfoto's te maken naast het lichamelijk onderzoek en vragenlijsten. In verschillende landen is de mate waarin RA voorkomt verschillend, maar in de landen binnen Europa heeft ongeveer 1% van de Caucasische populatie RA. Van de patiënten met RA in Nederland is het percentage vrouwen twee keer zo groot als het percentage mannen. De ontwikkeling van de ziekte is complex en het verloop ervan nog onduidelijk, maar onderzoek heeft aangetoond dat erfelijke en omgevingsfactoren een rol spelen. In dit proefschrift wordt enerzijds naar factoren gekeken die bescherming geven tegen de ontwikkeling van RA en anderzijds juist naar processen en factoren die een rol spelen in de (versnelde) ontwikkeling of verergering van RA. In deze samenvatting zullen eerst een aantal aspecten van het immuunsysteem worden besproken en vervolgens zal er kort worden ingegaan op de onderwerpen en bevindingen van de individuele hoofdstukken.

Het afweersysteem

Ons afweersysteem bestaat uit verschillende soorten cellen die elk hun eigen rol spelen in het ontwikkelen van een ontsteking of afweerreactie. Globaal kan het afweersysteem worden onderverdeeld in een specifiek en aangeboren afweersysteem. De reacties van het specifieke afweersysteem worden getypeerd door het feit dat de reactie specifiek gericht is tegen een bepaald stukje eiwit, een antigeen genoemd. Deze antigenen worden door zogenoemde B-cellen herkend met de B-cel receptor en door T-cellen met de T-cel receptor. In het geval van een B-cel leidt de herkenning van het antigeen tot de productie van antilichamen door de B-cel. Een antilichaam is een beschermingsstof die in een organisme wordt gevormd als specifieke reactie op een antigeen. Het antilichaam bindt aan het antigeen dat hij herkent, wat vervolgens resulteert in het onschadelijk maken van de cel of het eiwit waarvan dit antigeen van afkomstig is. Als eenmaal een reactie is opgetreden tegen een bepaald antigeen, dan blijven er levenslang B of T-cellen aanwezig in het lichaam die specifiek tegen dit antigeen kunnen reageren als ze het opnieuw tegenkomen in het lichaam. Dit onderscheidt de cellen die betrokken zijn bij specifieke afweerreacties van cellen van het aangeboren immuunsysteem. Bij reacties van het aangeboren immuunsysteem worden ziekteverwekkers door cellen van het afweersysteem die altijd beschikbaar zijn 'opgegeten' (fagocytose genoemd). Deze cellen kunnen dan vervolgens deze ziekteverwekkers afbreken in de cel en daarmee de ziekteverwekker voor het lichaam opruimen. De reacties van het aangeboren afweersysteem zijn gericht tegen moleculen en eiwitten die in veel verschillende ziekteverwekkers voorkomen en niet tegen een molecuul dat specifiek voor één virus of bacterie is. De cellen van het aangeboren en specifieke immuunsysteem behoren vrijwel allemaal tot de 'witte' bloedcellen (leukocyten en lymfocyten) en werken samen om een optimale afweerrespons te krijgen. In dit proefschrift is onderzoek gedaan aan de zogenoemde T en B-cellen die onderdeel uitmaken van het specifieke afweersysteem.

Zoals hierboven al is beschreven, heeft elke cel van het afweersysteem zijn eigen functie. Zo zijn er de antigeen-presenterende cellen (bijvoorbeeld B-cellen en dendritische cellen), die het hele lichaam doorreizen en daarbij aftasten wat er allemaal in het lichaam aanwezig is, zowel alles wat er hoort te zitten als eventuele ziekteverwekkers. Zij kunnen deze cellen of eiwitten dan opnemen, in stukjes knippen en vervolgens als een soort ober, de gevonden stukjes eiwit (peptides), op een dienblad (een HLA-molecuul in de mens) aan T-cellen presenteren (**Figuur 1**). Deze HLA-moleculen zijn de belangrijkste genetische risico factor voor de ontwikkeling van RA.

Met betrekking tot het risico voor de ontwikkeling van RA, zijn er drie varianten van de HLA-moleculen; een variant die het risico op de ontwikkeling van RA verhoogt (die wordt de 'Shared Epitope' (SE) genoemd), een variant die de kans op de ontwikkeling van RA verlaagt ("DERAA" genoemd) en een variant die geen (beschreven) invloed heeft op de ontwikkeling van RA.



Figuur 1. Presentatie van een stukje eiwit (peptide) door een antigeen presenterende cel (APC) aan een T-cel. Het peptide wordt gepresenteerd door een HLA-molecuul op de APC aan de bijpassende T-cel receptor die aanwezig is op de T-cel.

Bescherming door "DERAA"

Verschillende individuen hebben verschillende HLA moleculen. De bouwstenen waaruit een HLA-molecuul bestaat heten aminozuren. Er zijn 20 verschillende aminozuren en elk type HLA-molecuul heeft een net andere samenstelling van deze aminozuren. Deze aminozuren kunnen met een éénletterige code worden aangegeven. De varianten van de HLA-moleculen die de aminozuren gecodeerd door "DERAA" bevatten, geven een bescherming tegen de ontwikkeling van RA. Dit effect was reeds beschreven bij de start van mijn promotie, maar daarnaast is gebleken dat als een moeder een "DERAA"-bevattend HLA-molecuul heeft, zij haar kind bescherming meegeeft voor de ontwikkeling van RA ongeacht of zij het "DERAA"-molecuul genetisch aan haar kind doorgeeft of niet. Dit geldt alleen voor de moeder en niet voor de vader van het kind. Een vader kan zijn kind alleen bescherming meegeven als het

kind zijn “DERAA”-bevattende HLA-molecuul erft. Dit onderzoek staat beschreven in **hoofdstuk 2** en **hoofdstuk 3** van dit proefschrift.

Vimentine-specifieke T-cellen

RA patiënten kunnen verschillende soorten antilichamen vormen, waaronder antilichamen die gericht zijn tegen eiwitten die een kleine verandering hebben ondergaan ten opzichte van het ‘gewone’ lichaamseigen eiwit. Deze verandering wordt aangeduid met citrullinatie en de antilichamen worden daarom “anti-Citrullinated protein antibodies” (ACPA) genoemd. Citrullinatie is een proces dat in ieder individu optreedt, maar alleen RA patiënten maken antilichamen tegen deze eiwitten. Eén van de eiwitten waar deze antilichamen tegen kunnen reageren is vimentine. Vimentine is een lichaamseigen eiwit dat aanwezig is in elke cel van het lichaam. Antilichamen worden geproduceerd door B-cellen als deze B-cellen signalen krijgen van geactiveerde T-cellen om antilichamen te gaan produceren. In **hoofdstuk 4** van dit proefschrift is onderzocht of we konden ontdekken tegen welk stukje van het veranderde vimentine-eiwit T-cellen kunnen reageren en of ze dan ook specifiek zijn voor het veranderde eiwit of ook tegen het ‘normale’ vimentine kunnen reageren. We hebben hiervoor gebruik gemaakt van een bepaald soort muizen die menselijke HLA-moleculen op hun antigeen presenterende cellen tot expressie brengen zodat we de bijpassende peptides voor de humane situatie konden onderzoeken. In deze studie zijn twee peptides geïdentificeerd waarbij de T-cellen van de gebruikte muizen specifiek reageerden tegen de veranderde (gecitrullineerde) vorm van het peptide en niet tegen het onveranderde peptide. Vervolgens hebben we met cellen van RA patiënten en gezonde controles gekeken of deze ook tegen de geïdentificeerde peptides konden reageren. De T-cellen van een aantal RA patiënten reageerden specifiek tegen het gecitrullineerde peptide en de cellen van gezonde proefpersonen lieten geen reactie tegen dit peptide zien. Omdat er slechts 10 RA patiënten en 5 gezonde controle personen zijn getest, moet er een veel uitgebreider onderzoek worden gedaan naar het voorkomen van dit soort T-cellen in verschillende groepen personen.

PTPN22 en CD40

Naast HLA-moleculen zijn er een aantal andere genetische factoren die de kans om RA te ontwikkelen beïnvloeden. In dit proefschrift zijn twee van deze genetische factoren nog wat verder bestudeerd. Dit zijn CD40 en PTPN22.

De volgorde van de bouwstenen van een gen, die nucleotiden worden genoemd, kan verschillend zijn bij verschillende individuen in een populatie. Als de mate waarin een bepaalde variant voorkomt gelijk of hoger is dan 1% wordt zo'n variant een 'single nucleotide polymorphism' (SNP) genoemd. De meest bestudeerde SNP van het PTPN22 gen is geassocieerd met RA maar ook met een aantal andere auto-immuunziekten. Er lijkt een relatie te zijn tussen deze SNP en de ontwikkeling van de hiervoor beschreven ACPA antilichamen. In **hoofdstuk 5** van dit proefschrift is onderzocht wat de voorspellende waarde op de ontwikkeling van RA is van informatie over de SNP van het PTPN22 gen, het wel/niet hebben van ACPA, of informatie over beide. Hieruit blijkt dat afzonderlijke informatie over of de SNP of het wel/niet hebben van ACPA kan helpen bij de voorspelbaarheid van de ontwikkeling van RA, maar informatie over beide heeft geen aanvullende waarde. Dit betekent dat deze twee genetische factoren betrokken zijn bij dezelfde route voor de ontwikkeling van RA.

Tegenwoordig komen toekomstige RA patiënten al voordat zij genoeg symptomen hebben om een goede diagnose te kunnen stellen bij de dokter. Naast een vroege diagnose zou het heel waardevol zijn om te kunnen voorspellen of iemand met RA een ernstige vorm van RA gaat krijgen of dat het een vorm is met een veel langzamer verloop. Om op dit moment toch een goede diagnose en een daarmee samenhangend behandelplan op te kunnen stellen is het belangrijk om hiervoor nieuwe voorspellende factoren te identificeren. Daarom hebben we in **hoofdstuk 6** bestudeerd of een aantal SNPs waarvan is beschreven dat ze een invloed hebben op de ontwikkeling van RA ook invloed hebben op het verloop van de ziekte wanneer deze al gediagnosticeerd is. We vonden dat één van deze SNPs, een variant in het gen voor CD40, inderdaad van invloed is op de progressie van de ziekte. CD40 is een molecuul dat op het oppervlak van een B-cel voorkomt en een belangrijke rol speelt in de communicatie tussen een B-cel en een T-cel wat kan resulteren in de activatie van de B-cel.

Zoals waarschijnlijk al is opgevallen, zijn er een aantal verschillende aspecten die een rol spelen in de ontwikkeling van RA en de progressie daarvan onderzocht in dit proefschrift. De resultaten hebben een aantal facetten verduidelijkt, maar roepen zeker ook nog vele onopgeloste vragen op die door vervolgstudies opgelost moeten worden. Ik hoop dat deze samenvatting velen van jullie een beetje een idee geeft waar ik me de afgelopen 5 jaar mee bezig heb gehouden. En natuurlijk zijn jullie van harte uitgenodigd om de gedetailleerde versie te lezen in de aangegeven hoofdstukken!

List of abbreviations used in this thesis

ACPA	anti-citrullinated protein antibody
ACR	American College of Rheumatology
DERAA	five amino acids present in certain HLA-DRB1 molecules
EAC	early arthritis cohort
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent activated cell sorter
GWAS	genome wide association study
HLA	human leukocyte antigen
IFNγ	interferon-γ
Ig	Immunoglobulin
NIMA	non-inherited maternal antigen
NIPA	non-inherited paternal antigen
PBMC	Peripheral blood mononuclear cells
PTPN22	protein tyrosine phosphatase N22
RA	rheumatoid arthritis
SE	shared epitope
SNP	single nucleotide polymorphism
UA	undifferentiated arthritis

Curriculum Vitae

Anouk Leonie Feitsma werd geboren op 9 april 1981 te Sneek. Na de basisschool ging zij naar het Titus Brandsma College (later Marne College) in Bolsward waar zij in 1999 haar VWO diploma behaalde. Vervolgens is zij aan de studie Biomedische Wetenschappen in Leiden begonnen, waar zij in 2002 haar Bachelor diploma en in November 2004 haar Master cum laude behaalde.
Tijdens de Masterfase van haar studie heeft zij drie verschillende onderzoeksstages uitgevoerd.
Allereerst bij de afdeling Endocrinologie in het LUMC onder begeleiding van drs. Geertje van der Horst en dr. Marcel Karperien. Zij heeft hier onderzoek gedaan naar de Wnt signalling en stabiele transfectie van osteoblast cellijnen.
In 2003/2004 heeft zij haar afstudeerstage uitgevoerd op de afdeling Reumatologie waarbij zij onder begeleiding van dr. Andreea Ioan en dr. René Toes op zoek is gegaan naar Citrulline-specifieke T-cellen die een rol spelen in reumatoïde artritis. De aanzet die in deze stage is gegeven, heeft zij uiteindelijk in het laatste jaar van haar promotie voortgezet, rekening houdend met de voortschrijdende inzichten. Dit heeft geresulteerd in de identificatie van twee peptiden van het vimentine eiwit waartegen Citrulline-specifieke responsen worden geïnduceerd in HLA-DR4 positieve individuen.
Vervolgens heeft zij nog 3 maanden stage gelopen in Duitsland bij het "Interfakultäres Institut für Zellbiologie" in Tübingen. Hier heeft zij de NKG2D expressie en de lymfocyten die aanwezig zijn in de darm in MICA transgene muizen gekarakteriseerd. Dit onderzoek werd uitgevoerd onder begeleiding van Katrin Wiesner, dr. Alexander Steinle en prof. dr. Hans-Georg Rammenssee.
In November 2004 is Anouk begonnen aan haar promotie-onderzoek op de afeling Reumatologie en Immunohematologie en Bloedtransfusie in het LUMC, waarvan het resultaat dit boekje is. Het promotie-onderzoek werd begeleid door prof. dr. René de Vries, prof. dr. Tom Huizinga en dr. René Toes.
Vanaf mei 2009 is zij werkzaam als Researcher bij de Corporate Research afdeling van Friesland Campina in Deventer. Zij is hier betrokken bij verschillende projecten die tot doel hebben om de producten van Friesland Campina te analyseren en te verbeteren met betrekking tot gezondheidseffecten op het gebied van afweer en de ontwikkeling van zuivelconsumerende baby's.

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