From THE DEPARTMENT OF MEDICINE
Karolinska Institutet, Stockholm, Sweden

SPECIFIC AUTOIMMUNITY IN RHEUMATOID ARTHRITIS - T CELLS, ANTIBODIES AND GENETIC REGULATION

Omri Snir

Karolinska Institutet

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“It a very complicated case,
a lotta ins, lotta outs”

“The Dude”, The Big Lebowski

ABSTRACT

Complex interactions between genes and environmental factors may result in destruction of the body's own cells and tissues by the immune system, i.e. autoimmunity. Rheumatoid arthritis (RA) is a chronic joint inflammation mediated by all arms of the immune system that can lead to tissue destruction and functional disabilities. Many genetic variants and environmental factors that affect the immune system in RA have been revealed in recent years, however it is still not known precisely how they regulate and control autoimmunity. In this work I studied the function and specificity of adaptive immunity in RA, and also addressed influences from known genetic variants that predispose for disease.

First, autoantibody responses to several RA-associated citrullinated autoantigens were studied in a cohort of patients with established RA. Antibody responses and their interrelationships were examined, both in the whole study cohort and following stratification to HLA-DRB1 types, since HLA-DRB1 alleles are the strongest genetic risk factors known for RA. The autoantibodies were found to be highly specific for RA and displayed only limited cross reactivity. HLA-DRB1*04 alleles strongly associated with the presence of these autoantibodies both in sera and synovial fluid.

T cells are believed to be central mediators of RA pathogenesis, however studying T cell specificity has been proven difficult. The stinking HLA-DRB1*04 association with different anti-citrulline antibody responses encouraged us to revisit T cell recognition and responses to citrullinated proteins. We identified an epitope from vimentin that binds HLA-DRB1*0401 in its citrullinated but not in its native form and T cell recognition and function were investigated. CD4 T cells from both HLA-DRB1*0401 RA patients and healthy donors recognized citrullinated vimentin. However, T cells derived from RA patients secreted higher levels of cytokines, suggesting previous activation and/or cytokine dysregulation in RA. This study required a development of an assay sensitive enough to allow detection of rare antigen-specific CD4 T cells. Having such a tool, we further applied the same method to functionally examine type-II collagen (CII)-reactive CD4 T cells from peripheral blood and synovial fluid from HLA-DR*04 RA patients. T cells indeed recognized different variants of the immunodominant T cell epitope of CII and displayed epitope spreading throughout the disease. Synovial fluid derived T cells produced higher levels of inflammatory cytokines as compared to blood suggesting local reactivation.

Many more RA predisposing genetic variants have been identified outside the HLA-DRB1 locus in recent years. We therefore continued to study the association with autoantibody specificities in two independent cohorts of RA patients. Several genetic variants were found to control autoantibodies formation; some associated with several autoantibodies whereas others exclusively linked with a single fine specificity.

In summary, our data suggest that both B and T cells selectively respond to autoantigens in RA and are controlled by HLA and additional RA-predisposing genes. This work emphasizes the importance of multidisciplinary investigation for the understanding of interaction between genes and immunity in order to functionally explain epidemiological findings. We further hope that our findings regarding T and B cell specificities will encourage others to continue in this direction, which may pave the road towards specific therapy in patients following precise gene-immune investigation.

LIST OF PUBLICATIONS

I. Multiple antibody reactivities to citrullinated antigens in sera from patients with rheumatoid arthritis: association with HLA-DRB1 alleles.
Omri Snir*, Mona Widhe*, Caroline von Spee, Johan Lindberg, Leonid Padyukov, Karin Lundberg, Åke Engström, Patrick J Venables, Joakim Lundeberg, Rikard Holmdahl, Lars Klareskog, Vivianne Malmström. *Ann Rheum Dis. 2009; 68:736-743.*

II. Autoantibodies to several citrullinated antigens are enriched in the joints of RA patients.
Omri Snir, Mona Widhe, Monika Hermansson, Caroline von Spee, Johan Lindberg, Sanne Hensen, Karin Lundberg, Åke Engström, Patrick J. W. Venables, René E. M. Toes, Rikard Holmdahl, Lars Klareskog, Vivianne Malmström. *Arthritis & Rheumatism. 2010; 62:44-52.*

III. Identification and functional characterization of T cells reactive to citrullinated-vimentin in HLA-DRB1*0401 humanized mice and RA patients.
Omri Snir*, Mary Rieck*, John A. Gebe, Betty B. Yue, Crystal A. Rawlings, Gerald Nepom, Vivianne Malmström, and Jane H. Buckner. *Arthritis & Rheumatism. 2011. In press.*

IV. Multifunctional T cell reactivity to native and glycosylated type-II collagen in rheumatoid arthritis.
Omri Snir, Johan Bäcklund, Julia Boström, Ida Andersson, Jan Kihlberg, Lars Klareskog, Rikard Holmdahl and Vivianne Malmström. *Submitted*

V. Common variants in non-HLA-DRB1 alleles associate with distinct ACPA fine specificities in RA patients.
Omri Snir, David Gomez-Cabrero, Maria Seddighzadeh, Katharina Ute Klich, Lena Israelsson, Anca I Catrina, Jesper Tegner, Lars Klareskog, Vivianne Malmström and Leonid Padyukov. *Manuscript*

* These authors equally contributed to the work

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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
APC	Antigen presenting cell
CII	Type-II collagen
CCP	Cyclic citrullinated peptides
CD	Cluster of differentiation
CDR3	Complementary-determining region 3
CEP-1	Citrullinated alpha-enolase peptide-1
CI	Confidence interval
CIA	Collagen induced arthritis
DMARD	Disease-modifying anti-rheumatic drugs
EIRA	Epidemiological investigation of rheumatoid arthritis
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
HA	Influenza hemagglutinin antigen
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IL	Interleukin
LD	Linkage disequilibrium
MHC	Major histocompatibility complex
MCV	Mutated citrullinated vimentin
OR	Odds ratio
PAD	Peptidylarginine deiminase
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
R	Arginine
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SE	Shared epitope
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cell
SNP	Single nucleotide polymorphism
Tmr	MHC class-II tetramer
TTx	Tetanus toxoid

1 AIMS OF THIS THESIS

The overall aim of this thesis work was to study B and T cell specificities in RA and to examine the contribution and regulation of the HLA-DRB1 region and additional RA-predisposing genetic variants on autoreactive immune responses in RA.

More specifically the aims of this thesis were:

1. To study ACPA fine specificities in sera and synovial fluid from patients with RA, and to further characterize the division and the interrelation of different ACPAs and evaluate the genetic contribution of the HLA-DRB1 type to immunity against citrullinated proteins (paper 1 & 2).

2. To define a T cell epitope from the citrullinated candidate autoantigen vimentin, and to target and functionally examine citrullinated-vimentin specific T cell in RA (paper 3).

3. To study and compare cytokine responses against different variants of the immunodominant T cell epitope on type-II collagen in HLA-DR*04 positive RA patients' peripheral blood and synovial fluid (paper 4).

4. To examine the contribution of RA-predisposing genetic variants outside the HLA-DRB1 region to antibody responses against citrullinated autoantigens (paper 5).

2 AUTOIMMUNITY IN RHEUMATOID ARTHRITIS

2.1 THE IMMUNE SYSTEM AND AUTOIMMUNITY

The immune system is one of the main defense mechanisms, providing protection against various infections and harmful substances such as toxins. The immune system is comprised of a variety of effector cells and molecules and can be divided into two main arms: innate and adaptive immunity. Having the ability to combat a wide range of pathogens the innate arm rapidly responds and clears most immune insults. If needed the adaptive immunity joins the defense effort and specifically fights the pathogen. The adaptive arm is composed of T and B lymphocytes and develops throughout the lifetime of an individual, building an immunological memory. To efficiently protect an organism against diseases the immune system must fulfill its four main tasks:

i. Recognition, and tolerance (towards self)
ii. Effector function - neutralization and elimination
iii. Self control and regulation
iv. Memorizing, specific for the adaptive immune system

One of the most important features of lymphocytes is the ability to distinguish between self and non-self, not permitting an immune response against the body's own cells and tissues. However, the random gene rearrangements occurring during lymphocyte development in the central lymphoid organs may generate lymphocytes with forbidden affinity to self-antigens. This may lead to a false recognition and a loss of immune tolerance to self, allowing an immune response that may eventually lead to autoimmunity. Autoimmune diseases are mostly characterized by an inappropriately controlled immunity that may result in inflammation and/or functional problems that affect one organ or more, where genes and different environmental factors mutually contribute to the development of autoimmunity. It is estimated that about 3-5% of the population are affected by different autoimmune disease with an overall increasing frequencies in women compared to men[1,2].

2.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chromic inflammatory disease characterized by joint swelling and destruction, leading to severe disabilities and shorter life expectancy due to accelerated atherosclerosis[3-7] and other co-morbidities. RA is considered an autoimmune disease given the presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated proteins antibodies (ACPA) found in patients with rather high specificity[8-11]. Both systemic and local autoimmune inflammatory load drive the progression of the disease. Like many other autoimmune disorders women are more affected than men, with an overall prevalence of 0.5 – 1% in most western populations[12].

To ensure correct diagnosis RA is defined by a combination of several criteria. The American Collage of Rheumatology (ACR) issued a set of criteria used for differentiating RA from other inflammatory arthritidies in 1987[13] (table 1). During 2010 updated criteria were issued by the ACR and the European League Against Rheumatism (EULAR)[14]. The 2010 criteria are a score-based algorithm, by which a

collective score from four categories sets the evaluation (table 2); an overall score of ≥ 6/10 is needed for classification of a patient as having RA[14]. The current recommendations, however, are to use both sets of criteria when classifying the diagnosis of patients that are subject to research.

Table 1 – The ACR Criteria for RA (1987)

	Criterion	Specification
i.	Morning stiffness ≥ 1 hour	Morning stiffness in and around joints lasting ≥ 1 hour
ii.	Arthritis of ≥ 3 joints/joint groups	Three or more joints with soft tissue swelling or fluid in14 possible areas: left and right proximal interphalangeal (PIP), metacarpo-phalangeal (MCP), wrist, elbow, knee, ankle, and metatarsophalangeal (MTP) joints.
iii.	Arthritis of the hand joints	One or more swollen joints in a wrist, MCP or PIP joint.
iv.	Symmetry of arthritis	Simultaneous involvement of the same joint areas.
v.	Subcutaneous nodules	Subcutaneous nodules over bony prominences, extensor surfaces, or juxta- articular regions.
vi.	Rheumatoid factor (RF)	Detected in sera
vii.	Radiographic changes	Typical for rheumatoid arthritis on posteroanterior hand and wrist radiographs. Must include erosions or unequivocal to the involved joints.

***** Criteria 1-4 should be present for at least six weeks. Patients fulfilling ≥ 4 out of 7 criteria are classified as having rheumatoid arthritis. Patients with two clinical diagnoses are not to be excluded.

Table 2 – the 2010 ACR/EULAR scoring criteria for Classification of RA

	Group A – Joint involvement	**Score**
i.	1 large joint* (*refers to shoulder, elbow, hip, knee and ankle)	0
ii.	2-10 large joints	1
iii.	1-3 small joints*, with or without involvement of large joints (*refers to the MTP and PIP joints, second to fifth MTP joints, thumb PIP joints and wrists	2
iv.	4-10 small joints (with or without involvement of large joints)	3
v.	>10 joints (at least one small joint)	5

	Group B – Serology (at least 1 test result is needed for classification)	**Score**
i.	Negative RF and negative ACPA	0
ii.	Low-positive RF or low-positive ACPA	2
iii.	High-positive RF or high-positive ACPA	3

	Group C – Acute-phase reactants (at least one test result is needed for classification)	**Score**
i.	Normal CRP (C-reactive protein) and normal ESR (erythrocyte sedimentation rate) 0	0
ii.	Abnormal CRP *or* normal ESR 1	1

	Group D – Duration of symptoms (self-reported)	**Score**
i.	<6 weeks	0
ii.	≥6 weeks	1

2.3 AUTOIMMUNITY IN RA

2.3.1 Autoantibodies in RA

A wide range of autoantibodies with different specificities is found in RA patients' sera and synovial fluid, which reflects the vigorous activity of the immune system in the disease. In this work we mainly studied ACPAs, however rheumatoid factor (RF) and antibodies against type-II collagen (CII) are also rather specific for RA and have been extensively studied in this context.

2.3.1.1 Rheumatoid factor

Rheumatoid factor is the most studied autoantibody present in RA patients and its discovery back at the late 1930s led to the logical view that RA is an autoimmune disease[15]. It is present in about 2/3 of RA patients and directed towards determinants in the Fc region of IgG. The classical RF is IgM, but also IgA- and IgG-RF are found in sera and synovial fluid of RA patients. However, RF has rather low specificity for RA. It is also found in sera from patients with other rheumatic and chronic inflammatory diseases, viral infections and in up to 5% of healthy individuals with increased frequency in elderly[16,17].

2.3.1.2 Anti-type-II collagen antibodies

Anti-type-II collagen (anti-CII) antibodies are found in sera and synovial fluid of RA patients, and were first characterized in the mid 1970s[18,19]. Anti-CII producing B cells were also found in the rheumatoid synovium and synovial fluid, indicating that CII drives a specific autoimmune response in the rheumatic joint[20-22]. About 3-27% of RA patients develops anti-CII antibodies[23-25] and their presence is correlated with disease severity and radiological changes[24]. These frequencies, however, were measured against the whole CII. Higher frequencies of anti-CII antibodies were reported when antibodies were monitored using triple-helical peptides that represent the immunodominant B cell epitopes on CII, i.e. C1, J1 and U1[26,27]. Moreover, monoclonal antibodies specifically targeting these epitopes can induce arthritis in mice[27,28].

2.3.1.3 Anti-citrullinated protein antibodies

Anti-citrullinated proteins antibodies (ACPAs) have attracted lots of attention in recent years. ACPAs exclusively target modified epitopes on proteins that underwent deimination of arginines and consequently converted to citrulline (this process, i.e. deimination is also called citrullination). Citrullination is mediated by a group of enzymes denoted peptidylarginine deiminases (PADs)[29,30]. Five members of the PAD family are currently known (PAD1-4 and PAD6), having different tissue distribution[29]. PAD2 and PAD 4 are expressed in rheumatoid synovial membrane[31,32], synovial fluid cells[33] and extracellularly in synovial fluid[34]. The activity of these enzymes is dependent on high calcium concentrations and deimination can thus occur intracellularly[35], mainly during apoptosis, or extracellularly in calcium-enriched environments[36]. Of note, the conversion of arginine to citrulline also changes its charge from +1 to neutral, as PAD removes the arginine's positively charged amine group[36] (figure 1). Citrullinated proteins were detected in a number of inflamed tissues, including arthritic joints[37], lungs[38,39], extra-articular inflammatory sites in RA[39], human brain[40] and others. No selectivity of citrullination for certain arginine-containing proteins has been demonstrated to date, and citrullination of many

different proteins in the synovia and in the joint, including fibrinogen[41], vimentin[42,43], CII[44] and α-enolase[45] have been found.

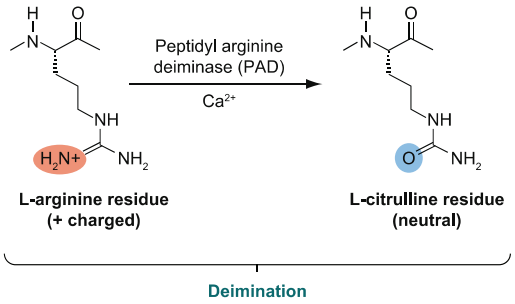


Figure 1 - Conversion of arginine to citrulline by peptidylarginine deiminase (PAD), i.e. citrullination. A posttranslational calcium-dependent enzymatic process results in the loss of one positive charge for every arginine residue converted to a neutral citrulline. *Amended from Klareskog et al. Annu. Rev. Immunol. 2008*

ACPAs are found in approximately 60-70% of RA patients with rather high specificity[46]. About 2% of healthy population are ACPA-positive and relatively few patients with other systemic inflammatory diseases[47]. An overall measurement of ACPA levels is rather simply achieved using the anti-CCP (cyclic citrullinated peptides) ELISA-based assay, which potentially detects all ACPAs regardless of their specificity and currently aids clinical diagnosis of RA[14,47]. ACPA levels are fairly stable during the disease and very few patients shift from being anti-CCP-positive to negative and vice versa[48,49]. Several lines of evidence suggest that ACPAs play a pathological role in RA: *(i)* Anti-CCP antibodies were found in RA sera up to nine years before the clinical onset of RA, and increased concentrations of the antibodies were seen as individuals approached onset of disease [50]. *(ii)* In line with this, the finding that very few patients develop ACPA after disease onset provides an indirect evidence for the contribution of the antibodies to the pathogenesis of ACPA-positive RA *(iii)* Studies of ACPA isotypes show a sustained presence of IgM anti-CCP, indicating a continuous activation of ACPA-reactive B cells throughout the disease course[51]; and *(iv)* it was also demonstrated that a transfer of monoclonal antibodies to citrullinated fibrinogen enhanced a mild arthritis in the mouse although they could not induce arthritis on their own[52].

2.3.2 T cell responses in RA

The precise pathological autoimmune mechanisms that drive RA are still largely unknown. Considerable evidence indicate that CD4 T cells are central in the pathogenesis of RA[53] and possibly other autoimmune diseases. In inflammatory arthritis activated effector CD4 T cells stimulate monocytes/macrophages, synovial fibroblasts and B cells to produce inflammatory cytokines like IL-1, IL-6 and TNFα, and to secret matrix metalloproteinases. This is mediated via cell-surface signaling by means of CD69 and CD11[54] as well as through the release of cytokines such as IFNγ and IL-17 (reviewed in[55]). B cell stimulation by activated T cells occurs via binding of CD154 and CD28 and results in production of antibodies, e.g. rheumatoid factors, anti-CII antibodies and ACPAs. However, the identity of the antigen(s) that are recognized by CD4 T cells in RA is still unknown. Activated and memory T cells are found in large numbers in the synovial tissue and fluid from

patients' inflamed joints. Some express highly homologous T cell receptor (TCR), which represent an oligoclonal expansion[56]. A major genetic contribution to RA comes from several alleles within the HLA-DRB1 loci, dominating by *HLA-DRB1*0401*, *HLA-DRB1*0404* and *HLA-DRB1*0101* in Caucasians[57]. These alleles, as well as other *HLA-DRB1*01* and *HLA-DRB1*04* subtypes, share a motif in the third hypervariable region of the DRβ chain and are therefore expected to present similar antigens to CD4 T cells[58], which also contributed to the thought that CD4 T cells are important in RA. All HLA-DRB1 alleles having this motif are defined as possessing "shared epitope" alleles[58] (the SE theory is further discussed in 2.4.1 HLA). An additional indication for the importance of T cells in RA originates form the presence of autoantibodies; most autoantibodies in RA sera and synovial fluid are of IgG isotype. IgG-producing B cells usually get T cell help for their maturation and differentiation. Thus, in genetically susceptible individuals T cells may encounter an arthritogenic antigen in the context of MHC class-II and initiate autoimmune responses (together with many other cellular and humoral compounds) that eventually result in joint inflammation.

There have been several attempts to find autoreactive T cells and to characterize their specificity and function, where CII, aggrecan and human cartilage gp39 have been commonly studied[59-61]. However, a combination of methodological limitations and uncertainty regarding the true identity of the antigens has provided severe limitations to these studies[59]. Still, there are few reports on T cell reactivity to CII[62,63], heterogeneous nuclear ribonucleoprotein A2 (hnRNP-A2, i.e. RA33)[64,65] and recent reports on T cell reactivity to citrullinated antigens[66,67]. However, no overall consensus has been reached on the potential autoantigens that are involved and the importance of T cells in RA has been challenged[68]. T cell cytokines such as IFNγ and IL-2 are present in relatively low concentrations in the joint, whereas macrophages and fibroblast products are abundant[69]. Paracrine and autocrine cytokines network secreted by macrophages and synovial fibroblasts were proposed in the 1990s as a mechanism that perpetuate synovitis in a T cell-independent manner[70]. This model could also explain the accumulation of T cells to the inflammatory site via chemotaxis. One of the main goals of this work was to revisit the question of T cell specificity in RA using new technology and collaborative efforts with experts in the field. Finding the antigens and the cells that drive the disease may lead to a breakthrough in the understanding of autoimmunity in RA and development of specific therapies.

2.4 GENETIC SUSCEPTIBILITY IN RA

It is long known that the susceptibility to develop RA is heritable. It is supported by studies demonstrating a larger disease concordance in monozygotic twins in comparison with dizygotic[71,72] and estimated to be approximately 60%[71]. The study of genetic risk factors in RA has exploded in the recent years due to advanced genotyping techniques and increased knowledge of the human genome. Today, it is feasible to test more than a million genetic variations in a single experiment, allowing hypothesis-free genetic studies over wide ranges of the human genome. Thus, the list of genes that are associated with the risk of RA has increased. However, all newly discovered genetic risk factors display only a modest risk for developing RA and do not explain the major part of heritability of RA, as they are also rather common in healthy population.

2.4.1 HLA

In a series of studies done in the 1970s Stastny defined the genetic basis for RA susceptibility[73,74]. He reported that mixed lymphocyte reaction from RA patients produced relatively low responses in comparison to healthy controls[73]. Stastny brilliantly interpreted that lymphocytes from RA patients share the same or similar "*lymphocyte-defined determinates*", and further that "*this gene appears to be increased in patients with RA with respect to non-RA controls and may reflect an association of genes within the HLA chromosomal region leading to predisposition for the development of RA*". In a short paper in 1978 Stastny showed that 70 percent of an RA patient cohort were HLA-DRw4 in comparison to 28 percent of healthy controls and therefore concluded that HLA-DRw4 is a genetic risk factor for having RA. Until these days HLA is by far the strongest known genetic susceptibility for the development of RA.

The HLA region is comprised of three different clusters; class I and class II that code for MHC class I and MHC class II, respectively, which are central in antigen presentation to T cells. The class III cluster codes for many other products, mainly immune related molecules, such as cytokines and complement components. The class II cluster in HLA region includes 5 main loci; HLA-*DP*, -*DQ*, -*DR, -DM* and -*DO*. The genetic variants with the most known profound contribution for RA susceptibility are located within the HLA-DR region (i.e. DR*0101, *0102, *0401, *0401, *0405, *0408, *1001, and *1401). As mentioned before, these variants are collectively known as "shared epitope" (SE) alleles because of their sequence similarity within the third hypervariable region (aa70-74: QKRAA, QRRAA, RRRAA). This provides a strong rationale for adaptive immunity having a pathogenic role in RA via MHC class-II-dependent T cell activation.

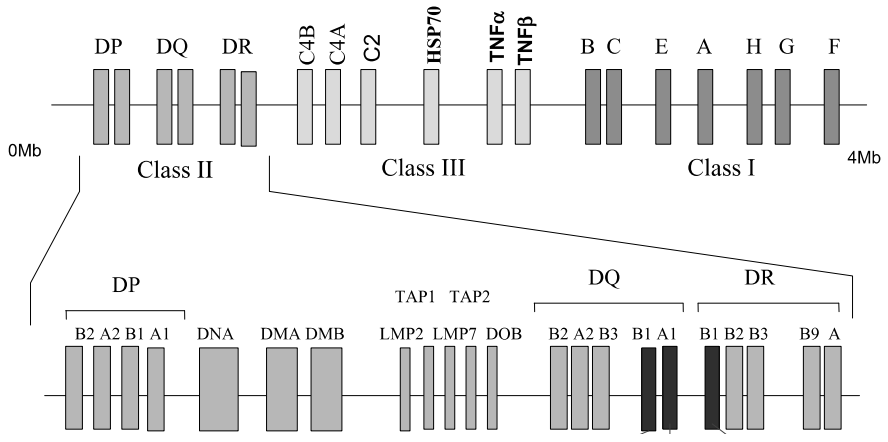


Figure 2 – a simplified map of the human leukocyte antigen (HLA) region. Amended from Simmonds and Gough, *British Medical Bulletin 2005; 71: 93-113*

2.4.2 RA-predisposing genetic variants outside the HLA-DRB1 region

Additional genes outside the HLA-DRB1 region are likely to contribute to disease pathogenesis and heterogeneity, however these have been harder to identify being rather common in the population and having modest effects. A polymorphism in the PTPN22 gene on chromosome 1 is the second most common genetic predisposition for RA and the first known genetic variance outside the HLA region that was found[75,76]. Following this finding additional polymorphisms in non-MHC genes were identified, many that are located in proximity to genes with central immune functions. Several of those genetic variants associate with ACPA-positive rather than with ACPA-negative disease, demonstrating that these are two distinct subsets with similar clinical features[77]. This strengthens the hypothesis for a role of adaptive immunity in RA pathogenesis, in particular for the ACPA-positive subset. A list of genes outside the HLA-DRB1 locus in which a polymorphism was associated with RA is provided in table 3. The contribution of the majority of these genetic variants to the development of anti-citrulline immunity in RA was studied in this work.

Table 3 – non-HLA-DRB1 RA risk alleles

Rs number	Gene(s)	Major allele	Minor allele	Risk allele
rs6314[78]	HTR2A	C	T	C
rs1328674[78]	HTR2A	C	T	T
rs548234[79]	PRDM1	T	C	C
rs4781003[80]	CIITA	C	T	T
rs4535211[81]	PLCL2	G	A	A
rs10431908[80]	CIITA	A	G	G
rs544167	C2	G	T	G
rs12746613[81]	FCGR2A	C	T	T
rs4810485[81,82]	CD40	G	T	G
rs10498441	NID2	A	G	A
rs10499194[81]	TNFAIP3	C	T	C
rs2064476	HLA-DPB2	A	G	A
rs706778[83]	IL2RA	C	T	T
rs2736340[84]	BLK	A	G	G
rs26232	C5orf30	C	T	C
rs540386[81]	RAG1	C	T	C
rs231707[81]	TNIP2	G	A	A
rs10402677	CEACAM1	G	A	A
rs42041[81]	CDK6	C	G	G
rs2024301[85]	DCIR	A	T	T
rs3807306[86]	IRF5	A	C	A
rs10488631[87]	IRF5	T	C	C
rs3761847[88]	TRAF1	A	G	G
rs7026551[89]	C5	A	C	C
rs11586238[81]	CD2,CD58	C	G	G
rs231735[84]	CTLA4	G	T	G
rs13017599[84]	REL	A	G	G
rs394581[81]	TAGAP	T	C	T

Rs number	Gene(s)	Major allele	Minor allele	Risk allele
rs2263484	C21orf74	A	C	C
rs6682654[81]	CD244			
rs6859219	ANKRD55	C	A	C
rs13031237[84]	REL	A	C	C
rs934734	SPRED2	A	G	G
rs11676922	AFF3	A	T	T
rs3087243[79]	CTLA4	G	A	G
rs1678542[79,90]	KIF5A	C	G	C
rs951500	CCL21	A	G	A
rs892188[81]	ICAM5	C	T	T
rs1133104[91]	CLEC4A	G	T	T
rs1980422[81]	CD28	T	C	C
rs1859341	CEACAM8	A	G	G
rs3087456[92]	CIITA	A	G	G
rs2271077	GALNTL2	A	G	A
rs2377422	CLEC4A;POU5F1P3	C	T	T
rs2476601[75,76]	PTPN22	C	T	T
rs2812378[81]	CCL21	A	G	G
rs2240340[93]	PADI4	C	T	T
rs6416647	CIITA	T	C	C
rs3890745[81]	MMEL1	T	C	T
rs4272626[81]	NHLH2	C	T	T
rs10258735	RPA3	A	G	G
rs3093023	CCR6	G	A	A
rs3218253[81]	IL2RB	G	A	A
rs6822844[79,81]	IL2	G	T	G
rs7234029[81]	PTPN2	A	G	G
rs6457620[81]	HLA-DRA	G	C	G
rs6920220[94]	TNFAIP3	G	A	A
rs10413014	CEACAM8	A	G	G
rs7574865[95]	STAT4	G	T	T
rs10468473	MAP2K4	G	A	A
rs10410147	CEACAM8	G	A	A
rs10919563[81]	PTPRC	G	A	G
rs4750316[90]	PRKCQ	G	C	G
rs2523451	MICA	A	G	G
rs6457617[96]	HLA-DQ	C	T	C

2.5 ENVIRONMENTAL FACTORS

The auto-aggressive nature of the immune system in RA as well as in many other autoimmune disorders is affected by the genetic heritability. Likewise, environmental factors and complex interactions between genes and environment also influence the immune system to some extent. Such interactions may give rise to immune reactions that lead to chronic inflammation. Environmental factors such as smoking, diet[97], birth weight[98] and socioeconomic status[99] have been shown to increase the risk to develop

RA.

Smoking is so far the most studied environmental risk factor for RA, and was first reported in the late 1980s[100]. More recently, smoking was studied in greater detail and specific interactions between HLA-DRB1 SE alleles, smoking and ACPA-positive disease were described[38] and further confirmed[101,102]. Thus, in genetically predisposed individuals smoking may trigger specific immune responses, which will ultimately result in ACPA-positive RA. A deeper search for a possible mechanism by which smoking initiates inflammatory immune response showed that smoking induces citrullination of protein in the lungs[103], and that macrophages and other antigen presenting cells (APC) are activated by cigarette smoke[104]. Thus, it could be hypothesized that citrullinated protein are presented by antigen presented cells in the lungs following exposure to cigarette smoke and thereby promote an inflammatory response or/and brake of immunological tolerance.

2.6 CURRENT HYPOTHESIS FOR AUTOIMMUNINTY IN RA

The working hypothesis that has been followed throughout this thesis is that the adaptive immune system has a central role in the pathogenesis of RA and that both genes and environmental factors interact with the immune system and regulate immunological outcome. Environmental factors, however, have not been included in my studies.

Our current view for the development of RA can be divided in three stages:

i. Induction of immune responses - citrullination of protein in the lungs (for ACPA-positive disease) or an intake of a foreign antigen, which would be further processed and presented to T cells in genetically susceptible individuals. This stage may start in the lungs as a result of exposure to cigarette smoke or in the periphery due to activation of PAD under inflammatory conditions, or following exposure to other arthritogenic antigens.
ii. Pathogenic inflammatory responses in the joint - due to a secondary event (e.g. trauma) an unspecific arthritis develops in the joints. Influx of autoaggressive cell to the joint, production of autoantibodies and generation of immune complexes.
iii. Chronic RA - joint inflammation becomes chronic with increased influx of immune cells and inflammatory cytokine and autoantibodies production.

3 RESULTS AND DISCUSSION

The aim of this thesis work stems from the hypothesis that the adaptive immune system plays a major role in RA and contributes to its pathogenesis. Below the main findings of this work are discussed, which describe aspects of T and B cells specificities and genetic contribution to RA.

3.1 PAPER 1 – MULTIPLE ANTIBODY REACTIVITIES TO CITRULLINATED ANTIGENS IN SERA FROM PATIENTS WITH RHEUMATOID ARTHRITIS: ASSOCIATION WITH HLA-DRB1 ALLELES

Anti-CCP antibodies are highly specific for RA and are found in patients up to 10 years before disease onset [50]. These antibodies can therefore be used as a specific biological marker for RA and have been included in the new classification criteria for RA that were issued in 2010[14] and are used in the clinic for the diagnosis of RA. However, as CCP is a generic peptide it cannot be used to study true autoreactive immune responses in RA. Therefore, in this work we investigated serum-antibodies toward three RA-associated autoantigens in their native and citrullinated (cit) form: *(i)* fibrinogen, *(ii)* alpha-enolase and *(iii)* type-II collagen. Fibrinogen (fib) is a soluble plasma glycoprotein that converts to fibrin during blood coagulation. In this study antibody responses against the full-length fib protein were studied. Alpha-enolase is a glycolytic enzyme that is expressed in many tissues, and also in activated monocytes[45]; in this work the immunodominant citrullinated peptide-1 from alpha-enolase (i.e. CEP-1, aa5-21) was used[45,105]. Type-II collagen (CII) is the main structural protein of the articular cartilage. In this work one of several B cell epitopes was investigated, i.e. $C1^{III}$ a triple helical construct of aa359-369[106,107].

IgG antibody responses towards CCP and the three RA-associated antigens (fib, alpha-enolase and C1) were monitored in sera from 291 RA patients and 100 sex and age matched healthy individuals. Similarly to anti-CCP, all specific ACPAs (targeting cit-fib, CEP-1 and $citC1^{III}$) were also highly specific to RA in comparison to healthy subjects. No antibody responses toward fib or alpha-enolase in their native forms were detected, whereas as expected[26] antibodies against the native form of $C1^{III}$ were present in RA sera, but in a lower frequency than those targeting the citrullinated variant. One of the current hypotheses regarding the formation of ACPAs coming from our group is that exposure to cigarette smoke can trigger citrullination of proteins in the lungs[38,108], which would then elicit an immune response against newly citrullinated antigenic targets. IgA is the main isotype produced in mucosal tissues such as the lungs; thereby we have also studied IgA antibody responses to citrullinated proteins. IgA antibodies were found against all three citrulline antigens, yet in lower frequencies in comparison to IgGs. Interestingly, ACPA-IgA antibodies were only present in ACPA-IgG positive patients, which indirectly suggests a link between the two.

Since these antibodies target an epitope with common characteristic, i.e. citrullination, the degree of cross-reactivity between antibodies against cit-fib, CEP-1 and $citC1^{III}$ was studied. Overall the antibodies did not cross-react with each other, although low degree of cross-reactivity was found in some individuals. This suggests that the different

antibody responses against citrullinated antigens are independent and develop separately from each other. It was therefore interesting to characterize the relationship between different fine specificities and anti-CCP antibodies. Overall, we could conclude that ACPAs are confined to the anti-CCP-positive subset. CCP-high patients tend to have multiple reactivities against several citrullinated proteins in comparison to those having lower lever of anti-CCP. Some of those, however, also express multiple autoantibody reactivity. The relationships between the levels of anti-CCP antibodies and specific ACPAs are summarized in a heat map (figure 3).

It has been shown that the SE alleles (HLA-DRB1*01 and HLA-DRB1*04) associate with anti-CCP antibodies[38,109] and also control their levels (i.e. patients having two SE alleles have higher anti-CCP antibody levels than those carry one SE allele, who have higher levels from those who have none)[110]. We therefore examined the effect of HLA-DRB1*SE alleles on the levels of ACPAs fine specificities. Patients were first divided based on the number of copies of SE alleles: *(i)* no-SE, *(ii)* having one SE allele, or *(iii)* having two copies. Patients carrying two copies of the SE allele had higher ACPA levels from patients carrying one or no-SE alleles. Patients having single SE allele had higher ACPA levels from patients who do not have the SE alleles. This was true for all three examined fine specificities, demonstrating the control of the SE alleles on ACPA levels. However, for citC1^{III} there was only a trend. Next, to address which SE alleles have the strongest influence on ACPA levels we divided the patients into three groups based on their HLA-DRB1 type: *(i)* non-SE, *(ii)* HLA-DRB1*01 and *(iii)* HLA-DRB1*04, here DR*01/04 individuals were excluded from the analysis. HLA-DRB1*04 patients were found to have the highest antibody levels, whereas HLA-DRB1*01 patients displayed similar ACPA levels to the non-SE epitope group. In summary our findings show that although different ACPAs do not cross-react, HLA-DRB1*04 alleles commonly regulate ACPA levels irrespective of their specificity.

3.2 PAPER 2 – AUTOANTIBODIES TO SEVERAL CITRULLINATED ANTIGENS ARE ENRICHED IN THE JOINTS OF RA PATIENTS

Synovial fluid (SF) is found in the synovial cavities, in close proximity to the articular joint. Under inflammatory conditions excess of SF is frequently found in the articular joint, which is enriched with mononuclear cells and other cellular substances. However, the presence of ACPAs in SF has not been systematically studied in a large well-characterized RA cohort. In this study the levels of APCAs in SF were examined and systematically compared with those in sera. Also, the association of ACPAs in SF with HLA-DRB1 alleles was investigated. Two peptides from vimentin (aa2-17 and aa60-75)[111] and a vimentin-based RA-diagnostic test (i.e. mutated citrullinated vimentin – MCV)[112] were included in this study in addition to the cit-antigens studied in paper 1.

A simple comparison between the antibody levels in sera and SF demonstrated equal levels of anti-CCP and anti-MCV in both compartments. However, the total IgG levels measured in SF were approximately half of that in sera. Using the total IgG levels as a reference for comparison between the two compartments the proportions of anti-CCP and anti-MCV antibodies in SF were found to be significantly higher than in sera. That was also true for the other ACPA fine specificities that were monitored, but not for anti- TTx (tetanus toxoid) antibodies, which do not associate with the disease. This

suggests that ACPAs are locally produced or alternatively accumulate in the rheumatic joint. Indeed CD19+/CD138+ plasma cells do localize in the joint, however their specificity is still unknown[113,114].

Anti-CCP and anti-MCV antibodies highly correlated and only few patients were either anti-CCP-negative/anti-MCV-positive or vice versa, 5% and 2.9% respectively. It has been shown in a cohort of patients with early RA that anti-CCP-negative/MCV-positive represents a subset of patients with an aggressive disease course, similar to the CCP-positive group[115]. We could not confirm these findings here as most of the patients who were included in this study had an established disease and we lacked detailed clinical information

The odds ratios (OR) and 95% confidence intervals (CI) for the association of the different ACPAs in SF with HLA-DRB1 alleles were calculated. Similarly to ACPAs in sera, SF anti-citrulline antibody responses were tightly associated with HLA-DRB1*04 alleles.

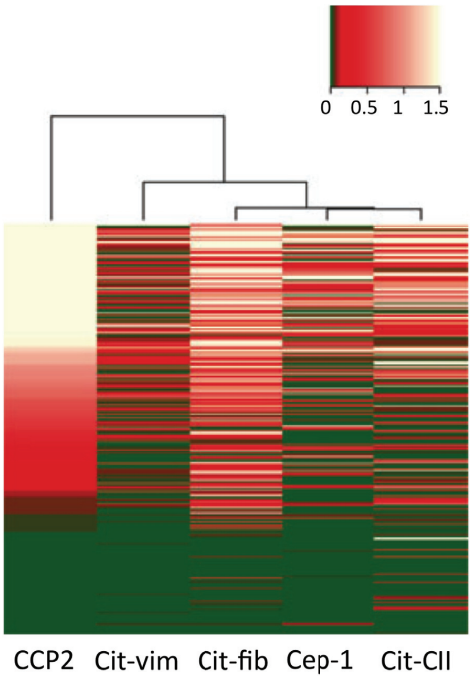


Figure 3 - Clustering of IgG RA-associated antibodies. The antibody levels against CCP and 4 RA-associated citrullinated autoantigens (vimentin, fibrinogen, alpha-enolase and CII) were measured in serum from 290 patients, each row represent one single patient. A color scale shows the relative degree of antibody levels; from low (green) to high levels (light red). Patients are sorted based on the levels of anti-CCP antibodies. Outer lines illustrate the intimacy between the different antibody responses. A similar summary of ACPAs in SF is in included in paper 2. *Amended from Wegner n. et al. immunological reviews 2010*

3.3 PAPER 3 – IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF T CELLS REACTIVE TO CITRULLINATED-VIMENTIN IN HLA-DRB1*0401 HUMANIZED MICE AND RA PATIENTS

IgG and IgA ACPAs are most likely secreted from mature B cells and plasma cells that have received T cell help. Hence, our current hypothesis is that anti-citrulline immunity in RA is T cell dependent. CD4 T cells indeed accumulate in the affected articular joints and are found both in the inflamed synovia and SF. Also, adoptive transfer experiments of T cells in animal models of RA[116,117] and a profound genetic association

with several of the HLA-DRB1 SE alleles further support this notion[116,117]. However, very little is known about autoreactive T cells in RA, their specificity and function especially with regards to citrulline immunity. Here we took the initiative to study CD4 T cell responses towards a citrullinated RA-associated autoantigen in HLA-DRB1*0401 RA patients.

All SE alleles have two subsequent positively charged amino acids at positions 71 and 72, which do not allow accommodation and presentation of positively charged peptides. Citrullination reduces the electric charge of arginine from positive to neutral, thus may allow a binding of a peptide to SE MHC in it citrullinated form rather than in its native. Hill *et al.* were the first to demonstrate that a peptide from vimentin (aa65-77) can binds three different SE alleles (DRB1*0101, DRB1*0401 and DRB1*0404) following citrullination at R_{71}, but not in its native form. The citrullination however did not affect the binding of the peptides to other non-SE alleles, demonstrating a possible role for citrullination only in binding to SE MHCs. The citrullinated version of this peptide also induced T cell recall responses in DR*04 transgenic mice. Still, the immunogenicity of the peptide was not empirically tested in human settings.

Following our discovery that citrulline immunity is predominantly associated with DRB1*04 alleles, we screened vimentin for possible HLA-DRB1*0401 binding peptides using a computer algorithm. We identified one peptide that can bind HLA-DRB1*0401 in its citrullinated form but not in its native, i.e. aa59-78. This peptide has three citrullinated sites, R_{64}, R_{69} and R_{71} and two possible binding frames, *(i)* aa59-71 and *(ii)* aa66-78. The binding capacities of all three peptides to HLA-DRB1*0401 were experimentally tested in vitro. None of the native peptides bound to DRB1*0401, while all the citrullinated did. However, when injected into DR0401-IE transgenic mice only cit-vim_{59-71} and cit-vim_{59-78} but not cit-vim_{66-78} elicited a proliferative recall T cell responses, demonstrating that cit-vim_{59-71} is the immunodominant region. Importantly, none of the corresponding native-vim peptides induced an immune response.

Following validation, we loaded cit-vim_{59-78} on MHC-class II tetramers (Tmr) and further screened for cit-vim specific CD4 T cells in immunized DR*0401-IE transgenic mice. Using Tmr we found cit-vim specific CD4 T cells only in mice that were immunized with cit-vim_{59-78}, but not with the native peptide. Next, to study citrulline-specific T cells in humans, we stained T cells from DRB1*0401 RA patients and healthy controls with cit-vim_{59-78} Tmr. Cells were first stimulated in vitro with cit-vim_{59-78} and further expanded with IL-2 before staining with Tmrs. Equal frequencies of cit-vim_{59-78} specific CD4 T cells were found in RA patients and in healthy controls; demonstrating that similarly to other autoimmune settings cit-vim autoreactive T cells also exist in healthy subjects[118-120]. This however was the first demonstration of T cells responding to citrullinated-antigen in the context of a DRB1*0401 Tmr in humans.

The finding of citrulline-specific CD4 T cells in humans encouraged us to learn more about the function of these autoreactive cells in RA. Thus, we adopted and further adjusted an assay that tested both specificity and function of T cells. We compared the function of T cells following stimulation with either vim_{59-78} or cit-vim_{59-78} in 22 RA patients and 9 healthy controls, all HLA-DRB1*0401. Fifty nine percent of the patients reacted towards cit-vim_{59-78} in comparison with 33% of the healthy controls, as evaluated by elevated levels of CD154. Also, patients' T cells secreted significantly

higher levels of TNFα and IFNγ upon stimulation with cit-vim$_{59-78}$, whereas cells from healthy controls did not.

In order to get a wider perspective on the cytokine production following stimulation with cit-vim$_{59-78}$ in RA patients and healthy controls we examined the levels of 16 cytokines in culture supernatants. Overall, patients' cells produced higher levels of cytokines in comparison to cells from healthy individuals. More specifically, the secreted levels of INFγ, IL-2 and IL-10 were significantly higher in patients and IL-1β, IL-3, IL-6, IL-9, IL-13, IL-17F, IL-23 and TNFα displayed a trend towards increased production in patients in comparison to healthy individuals. Interestingly, similar differences in the cytokine production could be observed also following stimulation with hemagglutinin (HA).

In summary, in this work we have shown that T cells from HLA-DRB1*0401 individuals can recognize a citrullinated peptide from vimentin. Patients' cells respond more vigorously to the identified antigen in comparison to T cells from healthy controls.

3.4 PAPER 4 – MULTIFUNCTIONAL T CELL REACTIVITY TO NATIVE AND GLYCOSYLATED TYPE-II COLLAGEN IN RHEUMATOID ARTHRITIS

Type-II collagen (CII) is the main structural protein in articular cartilage. Antibodies towards several native and citrullinated epitopes on CII can be found in sera and synovial fluid of RA patients with rather high specificity[121,122]. Immune responses to CII are, however, subjected to tolerization and therefore T cell responses to human CII are harder to monitor in humans than in mice[63]. Collagen induced arthritis (CIA) is a well-established model for RA. Similarly to human RA, CIA is associated with the murine MHC class-II molecules A^{q123}, and both B and T cell immunodominant epitopes are similar in mice and man.

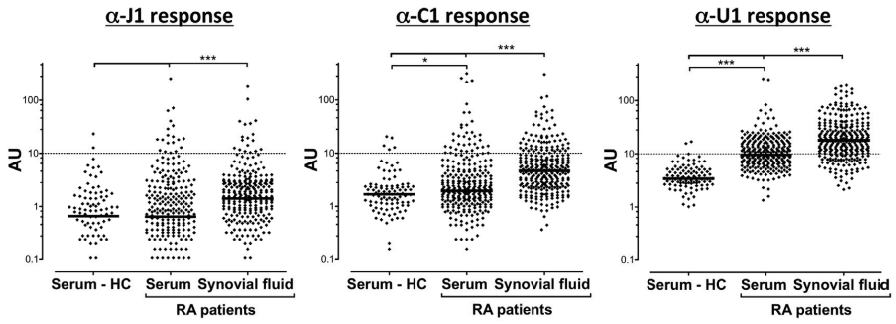
Encouraged by our findings and the methods used in paper 3 to detect T cell specificity and function we continued to examine T cell responses to the different variants of CII immunodominant epitope, i.e. aa259-273. This T cell epitope contains two lysines at positions 264 and 270 that can be hydroxylated and further glycosylated with mono- or disaccharides and thus create four different variants; *(i)* unmodified - CII-K, *(ii)* glycosylated on lysine-264 - CII-Gal264, *(iii)* glycosylated on lysine-270 - CII-Gal-270, or *(iv)* glycosylated on lysine-264 and 270 - CII-Gal264/270. These modifications are recognized by T cells and play an important role in the development of CIA in A^q- and DR4 expressing mice[52,54,55].

First, we examined whether T cells isolated from DRB1*0401 and DRB1*0404 patients' peripheral blood (PB) respond to the different CII$_{259-273}$ variants. We found that T cells responded to all four variants with simultaneous secretion of IL-17, IFNγ and IL-2. Patients demonstrated individual preferences towards different variants, but overall the responses to peptides with single-glycosylation were favored in a number of patients. Next, we compared the responses of T cell derived from both PB and SF against CII. We monitored similar T cell responses in both compartments, however SF-derived T cells secreted significantly higher levels of cytokines, in particular IFNγ.

Lastly, we examined whether T cell responses to CII were sustained or varied over time. To this aim we tested longitudinally collected cell samples from the PB of 3 RA patients. One patient showed a constant CII response over a period of 5 years, while the other two demonstrated increased anti-CII T cell responses in matters of magnitude and targeting different variants of $CII_{259-273}$. Thus, T cell responses against CII seem to continue develop overtime and may target additional variants of the $CII_{259-273}$ epitope.

This work has shown the potential importance of CII in human arthritis. Active T and B cell responses against CII occur in patients and are found in periphery and in articular joints. It is interesting that both T cells and antibody levels toward CII are increased in SF. In this work we have shown that CII-reactive T cells from SF produce higher levels of inflammatory cytokines. Likewise, the levels and the frequencies of antibodies targeting three native CII B cell epitopes (i.e. J1, C1 and U1) are elevated in SF, especially anti-U1 antibodies that were found in 77% of patients' SF (figure 4A). In fact, anti-CII antibodies in SF are present in more than 50% of ACPA-negative RA, a subset of RA of which so far little is known concerning adaptive immunity (figure 4B). Unlike ACPAs, CII-autoantibodies do not associate with HLA-DR SE alleles, or with HLA-DRB1*04. These data thus suggest that reactivity to non-citrullinated CII and citrulline immunity are regulated differently in RA patients. However, this was only examined in our cohort that includes 290 RA patients and should be further studied.

A.



B.

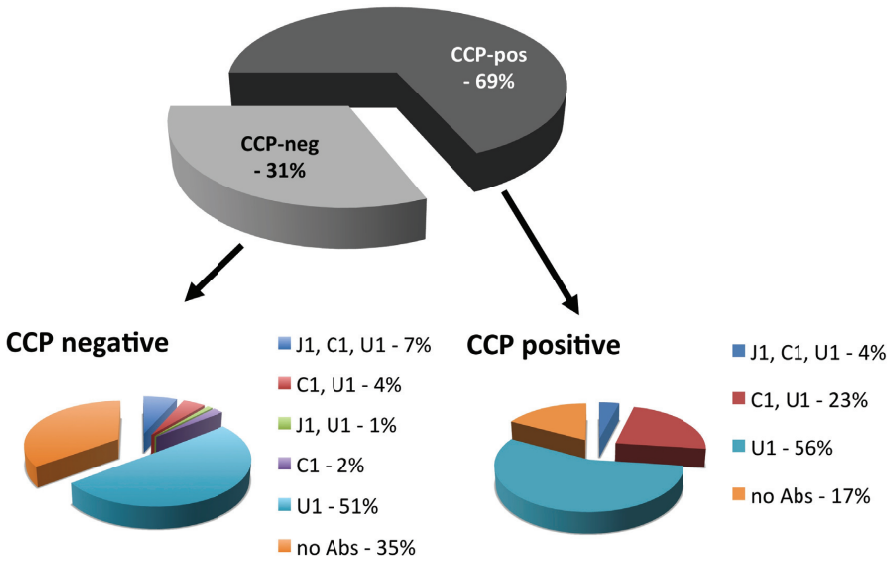


Figure 4 - levels and distribution of anti-CII antibodies in sera and synovial fluid of RA patients. (A) A comparison of the levels of anti-CII antibodies in sera from healthy control and paired sera and synovial fluid samples from RA patients. Antibody levels were measured against the following B cell epitopes on CII: J1 aa551-564, C1 aa359-369 and U1 aa494-504, from left to right respectively. (B) Distribution of anti-CII antibody responses in SF of anti-CCP negative (left) anti-CCP-positive RA patients (n=290). Dotted lines represent the cutoffs values, where all individuals above were considered positive for the specific CII epitope. * *P<0.05,* ****P<0.001*

3.5 PAPER 5 – COMMON VARIANTS IN NON-HLA-DRB1 ALLELES ASSOCIATE WITH DISTINCT ACPA FINE SPECIFITIES IN RA PATIENTS

In the previous studies that are included in this thesis we focused on HLA-DRB1, as it is the main genetic risk factor for RA known today. We have shown that it controls anti-citrulline immunity and presents antigens to autoreactive T cells. Still, additional allelic variants that associate with the risk of having RA have been revealed in recent years, in particular in the CCP-positive subset. However, it is still unknown whether these SNPs influence the formation of ACPAs and if there is distinctive genetic regulation of different ACPA fine specificities.

To study whether ACPAs are controlled by additional genetic variants apart of HLA-DRB1 we used serotype and genotype data from two independent cohorts of RA patients: *(i)* The Epidemiology Investigation of RA (EIRA) cohort, n=1362 and *(ii)* an additional 379 patients cohort (an extension of the cohort studied in papers I and II), which includes patients with established RA who are attending the Rheumatology Clinic of the Karolinska University Hospital. HLA-DRB1 type and information regarding 52 non-HLA-DRB1 RA-associated SNPs as well as ACPA status concerning CCP, cit-vimentin (aa60-75), CEP-1 and citC1^{III} were available for the EIRA cohort. Fourteen SNPs were found to be associated with the presence of anti-CCP antibodies; six exclusively associated with anti-CCP antibodies, while the remaining 8 also associated with other ACPA fine specificities. Six SNPs associated with anti-cit-vimentin antibodies and two of them exclusively linked with cit-vimentin antibody response. Five allelic variants associated with CEP-1, all were also associated with other ACPAs. Five SNPs also linked with citC1^{III} and four of them were associated also with other ACPAs, whereas one was exclusive for citC1^{III}. All genetic variants that were found to significantly associate with ACPA are shown in figure 5A.

Since anti-citrulline immunity is primarily linked with HLA-DR*04 alleles we continued to search for additional associations between ACPAs and RA-predisposing genes after subdividing our cohort in two different HLA groups: *(i)* HLA-DR*04 and *(ii)* non-HLA-DR*04. Hereby, additional SNPs were found to associate with antibodies against citrullinated antigens. CLEC4A (rs1133104) associated with anti-CCP and anti-cit-vimentin antibodies, and C5 (rs7026551) and HTR2A (rs1328674) were found in association with cit-vimentin and CEP-1 antibodies. Five additional SNPs individually associated with different ACPAs (figure 5B). In the non-HLA-DR*04 group RPA3 (rs10258735) linked with antibodies against CCP, cit-vimentin and CEP-1. CCL21 (rs2812378) associated with anti-CCP and anti-CEP-1 antibodies and REL (rs13031237) with anti-CCP and anti-citC1^{III} antibodies. Ten additional SNPs exclusively associated with the different ACPAs (figure 5C).

In order to replicate these potentially interesting findings, we examined the identified associations in an independent cohort of 379 RA patients with established disease all from Stockholm area. Four genetic variants were replicated; HLA-DQ (rs6457617) associated with antibody responses against CCP, cit-vimentin and CEP-1, while PTPN22 (rs2476601) was found to be significant in both cohorts for the presence of both anti-CCP and anti-cit-vimentin antibodies. HLA-DPB2 (rs2064476) and MICA (rs2523451) were only associated with a cit-vimentin response. The inability to replicate other allelic variants may be due to a lack of power in our replication cohort

(n=379). An indication that this may be the case is provided from the numeric data from the replication cohort where several genes display the same trend for association as in the exploratory cohort, but failed to reach statistical significance (table 4, shown in italic gray).

Figure 5A. Association of RA-predisposing genetic variants with different ACPAs (not adjusted for HLA type)

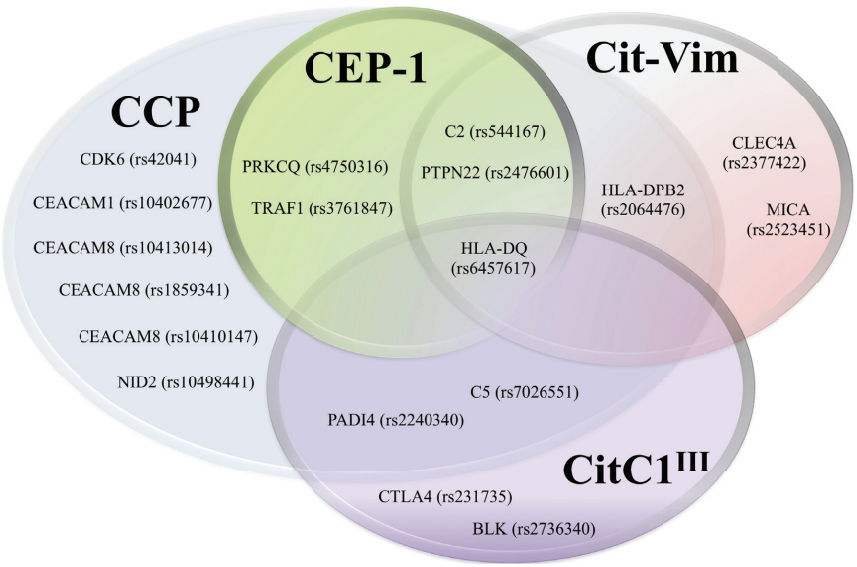


Figure 5B. Association of RA-predisposing genetic variants with different ACPAs in HLA-DR*04 patients

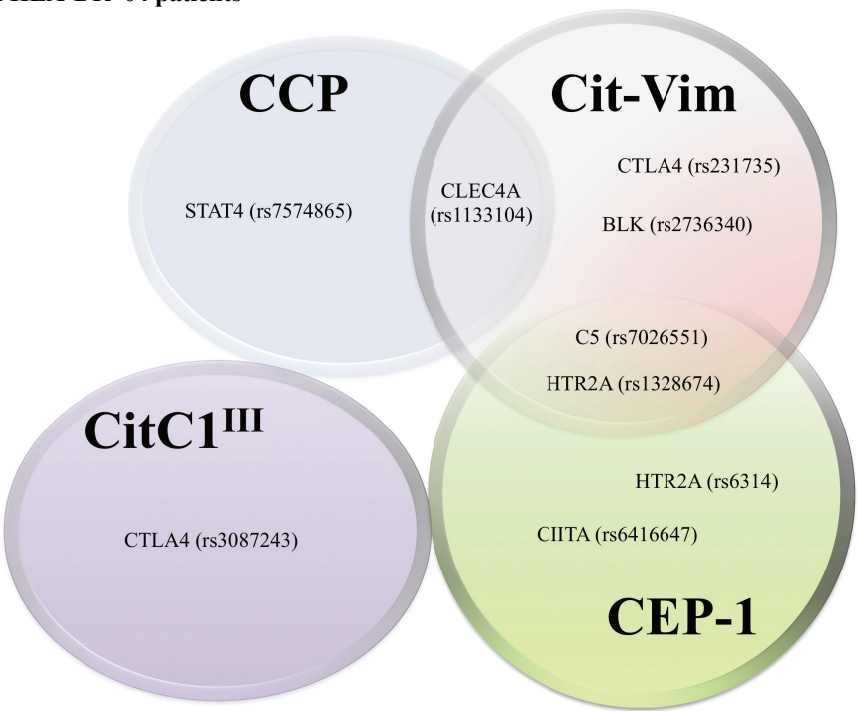


Figure 5C. Association of RA-predisposing genes with different ACPAs in patients carrying other HLA alleles rather than HLA-DR*04

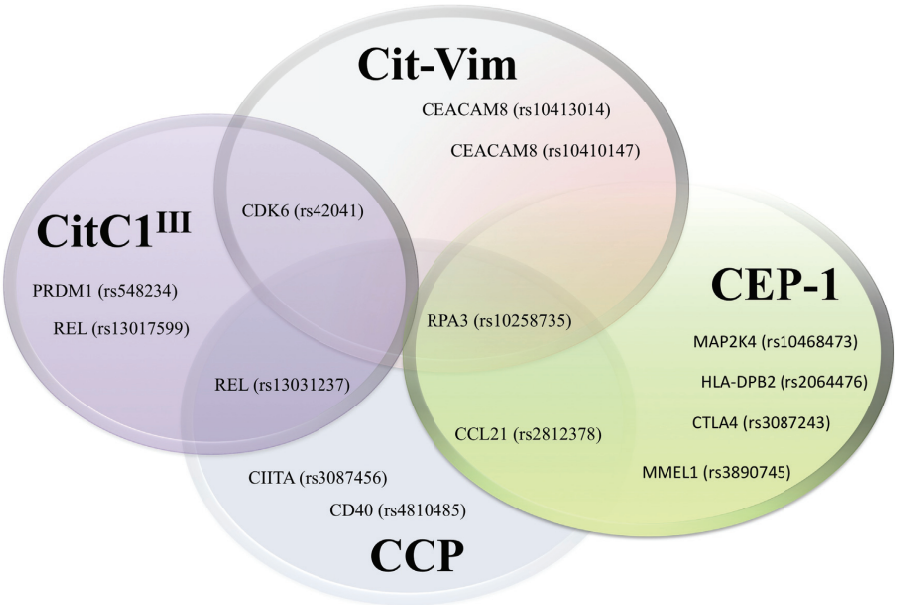


Figure 5 - The distribution of RA-predisposing genes that significantly associated with anti-CCP antibodies of other ACPAs fine specificities in the EIRA cohort. (A). Associations between the different SNPs and autoantibodies were analyzed in whole cohort (without adjustment for HLA type), or following stratification for (B) HLA-DR*04 positive and (C) non-HLA-DR*04 patients.

CCP	**Discovery cohort OR (95% CI)**	**Replication cohort OR (95% CI)**
HLA-DQ (rs6457617)	3.192 (2.267-4.545)	2.696 (1.669-4.429)
PTPN22 (rs2476601)	5.146 (1.848-22.143)	3.550 (1.003-24.411)
CDK6 (rs42041)	*1.368 (1.056-1.777)*	*1.404 (0.874-2.285)*
MICA (rs2523451)	*1.382 (0.984-1.938)*	*1.707 (1.039-2.795)*
*PRDM1 (rs548234)**	*1.350 (0.942-1.940)* **for non-HLA-DR*04*	*1.797 (0.949-3.442)* **for non-HLA-DR*04*
PADI4 (rs2240340)	*1.763 (1.161-2.688)* **for non-HLA-DR*04*	*1.627 (0.838-3.176)* **for non-HLA-DR*04*
Cit-vim	**Discovery cohort OR (95% CI)**	**Replication cohort OR (95% CI)**
HLA-DPB2 (rs2064476)	1.496 (1.100-2.040)	2.306 (1.492-3.584)
HLA-DQ (rs6457617)	2.661 (1.959-3.629)	2.146 (1.413-3.280)
MICA (rs2523451)	1.595 (1.151-2.221)	1.818 (1.162-2.851)
PTPN22 (rs2476601)	1.278 (1.011-1.615)	1.691 (1.083-2.667)
TRAF1 (rs3761847)	*1.375 (0.965-1.970)*	*1.406 (0.863-2.289)*
*CD28 (rs1980422)**	*1.962 (0.825-4.473)* **for non-HLA-DR*04*	*3.802 (0.814-29.447)* **for non-HLA-DR*04*
CEP-1	**Discovery cohort OR (95% CI)**	**Replication cohort OR (95% CI)**
HLA-DQ (rs6457617)	2.145 (1.583-2.914)	1.564 (1.037-2.365)
PTPN22 (rs2476601)	*1.369 (1.083-1.733)*	*1.475 (0.957-2.279)*
*CIITA (rs6416647)**	*1.485 (1.066-2.073)* **for HLA-DR*04*	*1.431 (0.833-2.468)* **for HLA-DR*04*
HTR2A (rs1328674)	*1.361 (0.893-2.096)*	*1.924 (0.854-4.546)*
PRKCQ (rs4750316)	*1.204 (0.945-1.534)*	*1.860 (1.220-2.849)*
CitC1^{III}	**Discovery cohort OR (95% CI)**	**Replication cohort OR (95% CI)**
HLA-DPB2 (rs2064476)	*1.306 (0.959-1.785)*	*1.386 (0.887-2.184)*
*HLA-DQ (rs6457617)**	*2.769 (1.566-4.930)* **for non-HLA-DR*04*	*1.874 (0.912-3.837)* **for non-HLA-DR*04*
CDK6 (rs42041)	*1.181 (0.933-1.495)*	*1.549 (1.012-2.372)*
MMEL1 (rs3890745)	*1.319, (0.899-1.959)*	*1.883 (0.850-4.634)*
*TNFAIP3 (rs6920220)**	*1.797 (0.960-3.507)* **for HLA-DR*04*	*3.254 (0.991-15.257)* **for HLA-DR*04*

Table 4 - Odds ratio and 95% confidence intervals (CI) for the genetic variants that show significant association in both cohorts (in black), or a trend towards significant association (marked in gray and *Italic*). Discovery cohort, EIRA (n=1362); Replication cohort, n=379.

4 CONCLUSIONS AND REMARKS

Several findings in this work are in my opinion important and may contribute to the understanding of the pathogenic mechanisms that are involved in RA, in particular ACPA-positive. Also, the finding of autoreactive T cells and the characterization of their function underlines that autoimmunity indeed drives RA, at least a subtype of the disease. Also, some of the methods applied here may be utilized to study T cell function in health and disease. Below, I highlighted the results that are in my opinion the main outcomes of this work:

i. ACPAs predominantly associate with HLA-DRB1*04 alleles, in particular *0401, rather then with other SE alleles, both in sera and synovial fluid.

ii. Antibodies against different specific citrullinated antigens display only a limited cross-reactivity between them.

iii. The levels of ACPAs are increased in the synovial fluid compared to sera.

iv. A peptide from vimentin (aa59-78) binds HLA-DRB1*0401 in it's citrullinated form but not in its native. Cit-vim$_{59\text{-}78}$ in vitro activates CD4 T cells and elicits secretion of inflammatory cytokines.

v. CD4 T cells derived from peripheral blood and synovial fluid of HLA-DRB1*04 RA patients react with several variants of CII$_{259\text{-}273}$. T cells derived from synovial fluid are hyper-reactive and produce higher levels of cytokines in comparison to those derived from peripheral blood. Also, epitope spreading of the different variants of this CII epitope occurs during the disease course.

vi. This work indirectly provides a proof of concept that autoreactive T cells can be enumerated using MHC class-II tetramers. It also presents an additional approach to detect rare CD4 T cells and to study their function.

vii. Non-HLA-DRB1 predisposing allelic variants regulate anti-citrulline immunity; some control all or most citrulline specificities that were studied here, while others exclusively associate with single ACPA fine specificity. This provides a basis for further subgrouping ACPA-positive RA.

HLA-DRB1*04 alleles represent the strongest genetic predisposition for RA, even stronger than other HLA-DRB1*SE alleles although those are also commonly found in RA patients in comparison to healthy population. In the first two papers included in this work we reported that ACPA associate with HLA-DRB1*04 alleles rather then HLA-DRB1*01 both in sera and in synovial fluid. This association was first seen in a rather small number of RA patients (n=221) and remained in the expanded cohort of 379 patients. We therefore suggest that anti-citrulline immunity shares common pathways as it is tightly linked with HLA-DRB1*04 alleles. Recently, when examining the HLA association in a larger cohort of 1362 RA patients (i.e. the EIRA cohort) the HLA-DRB1*01 locus also showed a positive association with ACPAs; suggesting that DRB1*01 alleles are also significant for the development of those antibodies, yet to a lesser extent than HLA-DRB1*04.

Citrulline immunity can be treated and studied as one general immune reaction without considering the distinct characters of its specificities. HLA is commonly linked and all

different epitopes contain citrulline. Our data suggest that antibodies against the cit-fibrinogen, cit-vimentin, CEP-1 and citC1^{III} have limited cross reactivity and therefore may represent individual and independent immune reactions with unique features. Other studies demonstrated that anti-CCP antibodies are a collection of ACPAs[124], where some antibodies toward specific citrullinated peptides cross-react[124,125]. Conformational differences and the surrounding amino acids residues in those epitopes are possibly critical for the binding of the ACPAs and may explain the differences in cross reactivity.

The limited cross reactivity between ACPAs and the fact that patients express different compositions of single or multiple antibody reactivity may indicate that additional genetic factors apart of HLA-DRB1 region control anti-citrulline immunity (here we disregard environmental factors, which may have a critical effect). We were fortunate to be able to analyse a set of additional 52 SNPs that genetically predispose to RA in the EIRA cohort and to be able to examine our findings in an independent cohort. Thirty-one allelic variants were found to associate with anti-citrulline antibody response; some linked with anti-CCP antibodies and all/most ACPAs studied here, while others exclusively associated with either anti-CCP or one antibody specificity. Among all allelic variants linked with the autoantibodies, three SNPs from the HLA region showed the strongest association both in the EIRA and the replication cohorts; i.e. HLA-DQ (rs6457617), HLA-DPB2 (rs2064476) and MICA (rs2523451). Being outside the HLA loci PTPN22 (rs2476601) was exceptional with regards to the other three replicated SNPs, and was found to significantly associate with anti-CCP and anti-cit-vimentin antibodies in both cohorts.

Interestingly, the four replicated allelic variants are all related to immune system and even specifically linked to T cells function. HLA-DQ and HLA-DP are involved in antigen presentation, similarly to HLA-DRB1. However, whether they can also present citrullinated antigens like HLA-DRB1 SE alleles is currently unknown. MICA (MHC class I-related chain A) stimulates cells via the lectin-like receptor NKG2D that is widely distributed on natural killer (NK) cells and T cells (in particular CD8 and γδ CD4)[126]. In RA MICA is expressed in synovia and can activate CD4/CD28^{null} T cells[127], which also express NKG2D[128]. CD4/CD28^{null} T cells are found in increased frequencies in RA patients and other chronic inflammatory disorders. In the light of these findings, providing an immunological explanation for MICA predisposition and regulation of ACPA is of particular interest and should be further investigated. The functional influence of the polymorphism in PTPN22, however, has already begun to be revealed. PTPN22 encodes a phosphatase (also referred to as Lyp) that inhibits TCR signal transduction. It dephosphorylates autophosphorylation sites on the protein tyrosine kinases Lck, Fyn, and Zap70 and thereby inhibits T cell activation[129]. A replacement of C-T alleles at position 1858 predispose RA and results in a single substitution of arginine to tryptophan at position 620 of the Lyp protein (Lyp 620W), which leads to a gain of function[130] (an enhanced inhibitory effect on TCR signaling). This mutation was demonstrated to alter both T and B cell function possibly by effecting TCR and BCR signaling[131,132]. The mechanisms by which this allelic variant contributes to the development of autoimmunity are still unknown.

An additional 10 allelic variants outside the HLA loci were close to show significant association with ACPAs in both cohorts and would possibly reach significance in a

larger cohort (table 4). These SNPs further emphasize the fact that the genetic regulation of immunity to citrullinated proteins is partly shared, but not identical for all different ACPAs. However it should not be excluded that environmental factors as well as interactions between genes and environment also might contribute to the shaping of autoantibody profile in RA patients.

Are the autoantibodies found in sera and synovial fluid pathogenic, and do they represent a true autoimmunity towards one or more epitopes that drive the pathogenesis? Alternatively, are they fingerprints of vigorous unspecific responses mistakenly considered to propagate and maintain chronic inflammation? Accumulating evidence suggest that ACPAs are pathogenic and display true autoimmunity[47]. In this work we found that the levels of ACPAs in the synovial fluid are proportionally higher in comparison to sera. We also showed that anti-CII antibodies are increased in levels and frequencies in synovial fluid, while in contrast the levels of anti-TTx antibodies measured in synovial fluid were significantly lower compared to sera. This suggests that ACPAs and anti-CII antibodies accumulate in the joint or are locally produced there. In the joint, the antibodies can form immune complexes with their respective citrullinated/native (arginine-containing) antigen. Such immune complexes may bind Fc receptors expressed on APC and will further drive production of inflammatory cytokines and an increased presentation of citrullinated or CII epitopes by HLA class II molecules to autoreactive T cells. T cells will then continue supporting the growing inflammatory process, which will eventually become chronic. Indeed, we demonstrated that inflammatory CII-reactive T cells are found in the joint and in the periphery of patients with established RA. T cells derived from SF were highly reactive in comparison to T cells derived from peripheral blood (PB). However, both PB- and SF-derived T cells can possibly interact with B cells and provide help for autoantibody production either in the inflammatory joint or in peripheral lymph nodes. It is intriguing that hyper-reactive CII-specific T cells are found in the synovial fluid where higher levels and proportions of anti-CII antibodies are also found. Moreover, the epitope spreading displayed by CII-reactive T cells in established disease shows that the immunity against this joint-restricted antigen continuously progresses.

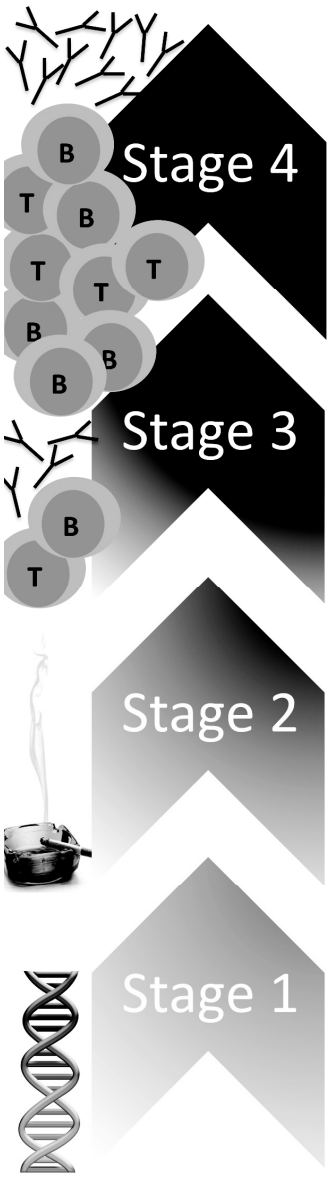
In this work we also described T cell reactivity to citrullinated vimentin in RA patients and matched healthy controls carrying HLA-DR*0401. We used computer algorithms to predict a possible peptide from vimentin that can bind HLA-DRB1*0401 allele only in its citrullinated form and not in its native, and verified it experimentally. The peptide, cit-vim$_{59\text{-}78}$, has 3 potential citrullinated sites (R_{64}, R_{69} and R_{71}) and two possible binding frames, i.e. cit-vim$_{59\text{-}71}$ and cit-vim$_{66\text{-}78}$, all display similar binding capabilities to HLA-DR*0401. However, recall T cell responses in the mouse was only detected against cit-vim$_{59\text{-}71}$ and cit-vim$_{59\text{-}78}$ but not towards cit-vim$_{66\text{-}78}$, suggesting that the critical frame is located in the first part of the peptide. Cit-vim$_{66\text{-}78}$ is very similar to the cit-vim peptide reported by Hill *et al.* aa65-77[133]. The latter bound to SE-alleles (DRB1*0101, *0401 and *0404) in its citrullinated form and induced T cell responses in similar mice that were used by us. However, there are two major differences between the two: *(i)* our peptide had citrulline at position 69, while Hill's et al. did not. *(ii)* A (large) leucine at position 70 was replaced by a (small) alanine by Hill and his colleagues, probably in order to reduce steric disturbance and to improve binding by HLA. These may explain the differences in T cell responses to these two seemingly similar peptides.

T cells specific to cit-vim$_{59-78}$ were detected in HLA-DR*0401 RA patients and healthy individuals using MHC class-II tetramers. This is a direct proof for the existence of citrulline-specific T cells in the periphery. This was further confirmed by detection of CD154 and 3 different cytokines and also provided information about the inflammatory features of these cells. Autoreactive T cells have been previously found in healthy individuals[118-120,134]. However, there is a lack of systematic comparisons between autoreactive T cells from healthy individuals and patients. An analysis of 16 cytokines in the culture supernatants following stimulation either with cit-vim$_{59-78}$ or HA showed an excessive cytokine production by patients' cells in comparison to cells derived from healthy individuals. This suggests that the patients' cells had either encountered the antigen before or are largely influenced by genetic variance and/or epigenetic modifications. The majority of the patients studied here is ACPA-positive and likely to carry allelic variants that predispose for RA. Taken as a whole, our data suggest a functional dysregulation that originates from SNPs that are favorably expressed in RA patients. The detection of rare autoreactive cells was not at all trivial and time and cell samples limitations forced us to postponed a detailed investigation of cit-vim$_{59-78}$-reactive T cells in synovial fluid. However, we strongly believe that these can also be found in the joint where they encounter their antigen and interact with other cellular compounds.

The importance of T cells in RA, and more specifically of autoreactive T cells, has long been debated and challenged. Here we supply substantial proof for the existence of autoreactive T cells in blood and synovial fluid of RA patients and further provide an insight to their distinctive hyper-reactive inflammatory functions in RA. T cell recognition of autoantigen(s) may initiate and/or sustain RA. Following antigen stimulation, theses cells can migrate to secondary lymphoid organs or to the joint where they proliferate and together with B cells, monocyte/macrophages and fibroblast initiate an uncontrolled inflammatory process that will eventually damage cartilage and bone in the joint. Having a central role in the pathogenesis of RA it is of interest to study the function and the phenotype of autoreactive T cells both in blood and in synovial fluid in order to develop future therapeutic approaches to specifically target those cells or their function. Attractive targets are CD49d (very late antigen 4, [VLA4]) that facilitates T cell trafficking and extravasation to inflamed tissues such as the RA synovium, or the chemokine receptor CXCR4 that was previously identified on synovial T cells[135]. Another target would be the interaction between activated T cells and macrophages that result in extensive TNFα production, which is a central cytokine in joint inflammation. In Summary, autoreactive T cells are found in RA[63,64,66,67,134], and express functional abnormalities that may stem from different allelic variants. T cell studies in RA ought to take a step forward using current and future technologies. RA-associated antigens must be further screened for potential T cell epitopes and the patterns of their presentation and recognition should be characterized. Only then we will have the possibility to study mechanisms and pathways used by autoreactive T cells in the periphery while trafficking, and at the site of inflammation.

Multiple susceptibility genes and environmental factors work in concert and contribute to the development of RA. Some are already known yet the mechanisms by which they drive the disease are unclear, while others are still beyond our understanding. Our work has demonstrated a role for adaptive immunity in RA and further shown that the interplay between gene and immunity may have a critical role in shaping autoimmunity

and also highlighted the allelic variants that are most significant for anti-citrulline immunity. Still, additional antigen-driven autoimmune responses in RA, e.g. anti-CII, should be studied in a similar fashion as well as the function of RA predisposing genes. A hypothetical summary of development to RA is shown in figure 6.



Establishment of RA in the joint – Recruitment of autoantibodies with multiple specificities to the joint, and formation immune complexes. Increased expression and presentation of arthritogenic antigens and production of pro-inflammatory cytokines (most notably TNF) and further activation of pathogenic T and B cells and increased autoantibody production.

A secondary event, e.g. trauma or an inflammation in the synovium. Local activation of PAD enzymes and citrullination of proteins and exposure of other epitopes like CII in the synovium and development of unspecific arthritis develops in the joints.

Environmental exposure and induction of immune response – citrullination of proteins in the lungs (for ACPA-positive disease) or an intake of a foreign antigen. Antigen-processing and presentation. T cell activation and autoantibody production

Genetic predisposition for autoantibody-positive RA – HLA-DR, -DQ, DP, MICA, PTPN22 and more.

Figure 6 – A hypothetical summary of the development of autoantibody-positive RA in genetically predisposed individuals.

5 FUTURE PERSPECTIVES

The overall aim of clinical research is to develop better means of prediction, prevention and cure. Better understanding of the patho-immunological mechanisms in RA as well as the interactions between gene, immunity and environment will create a framework, which will allow the identification of subgroups of RA patients. This will then provide new opportunities to specifically target different mechanisms and pathways in more homogenous patient groups. Several approaches for therapy are already in clinical use, other still to be explored: *(i)* Alteration of the thresholds of immune activation via blockade of stimulatory molecules and cytokines or interference of signaling cascades. *(ii)* Modulation of antigen specific cells and induction of tolerance. *(iii)* Reconstitution of the immune system with stem cells, or *(iv)* Sparing of target organs by targeting different anti-inflammatory agents, matrix metalloproteases or chemokines. In my opinion, induction of tolerance to known autoantigen(s) would be a favorable approach as it is specific and would presumably not interfere with primary immune function. Accordingly, studying specificity in RA, its initiation, regulation and progression is of particular interest.

In this work we studied T cell responses in HLA-DRB1*04 individuals towards a single peptide from cit-vim and four variants of the major CII T cell epitope. This is a first inventory, but not at all sufficient for therapeutic development. RA is most probably driven by numerous antigens, and once the disease is clinically apparent the immunologic milieu is highly inflammatory and epitope spreading may occur (as was also indicated here). Feitsma *et al.* also identified two potential T cell epitopes from citrullinated vimentin, i.e. aa26-44 and aa415-433[67], which are different than the peptide described in this work. In an attempt to find additional HLA-DRB1*0401 binding peptides from vimentin/cit-vimentin we synthesized a set of overlapping peptides that cover the whole protein in which each arginine-containing peptide has a matched citrulline-containing one. Both the strength of the interactions and the stability of the binding to HLA-DRB1*0401 were experimentally examined. Using this approach we found ten additional citrullinated peptides that bind HLA-DRB1*0401. The ability of our peptide as well as the two citrullinated peptides that were described by Feitsma *et al.* to bind HLA-DRB1*0401 was also confirmed using this approach. However, the homologous arginine-containing peptide of cit-vim$_{26\text{-}44}$ also bound to HLA-DRB1*0401. A similar screening of additional RA candidate autoantigens is of interest and would reveal many more epitopes that can be presented in the context of MHC and stimulate T cell. Peptides from alpha-enolase, fibrinogen and CII in their native and citrullinated forms are obvious choices. Current technology allows such screening to various HLA alleles. Good HLA binders, native or citrullinated, should be further used for monitoring T cell responses using similar and improved methodological approaches presented here. Not only HLA-DRB1 alleles, but also the role of HLA-DQ and -DP should be taken in consideration, as they seems to be central in immunity against citrullinated antigens.

The unique association between susceptibility genes and specific immunity is intriguing and should be further investigated in a larger well-defined material. This is not trivial though, as similar cohorts with complete genotyping/serotyping information are not commonly available. Also, population heterogeneity and methodological

differences between different research groups may introduce errors. Still, such studies will reveal similarities and distinctions between autoimmune responses and highlight the allelic variants that are the most significant for the development of specific immunity. Those variants should be also functionally investigated. Importantly, the association between gene and immunity studied here were analyzed individually for each genetic variant without considering possible interaction between the different SNPs and therefore displayed low risk for autoantibody development. We should not forget, however, that combinations of multiple susceptibility genes reside together in patients and may thus work together. Creativity is needed to define how does a set of multiple genes regulates autoimmunity and what is then the risk for disease development and/or specific immunological phenotype.

In summary, in order to find better therapeutic agents and to fit the best suitable therapy to each patient a deeper immunological understanding of RA pathogenesis must be achieved. One possible way forward to identify unique pathways for potential intervention would involve a combined investigation of genes, environment and their interactions with the immune system in health and disease.

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