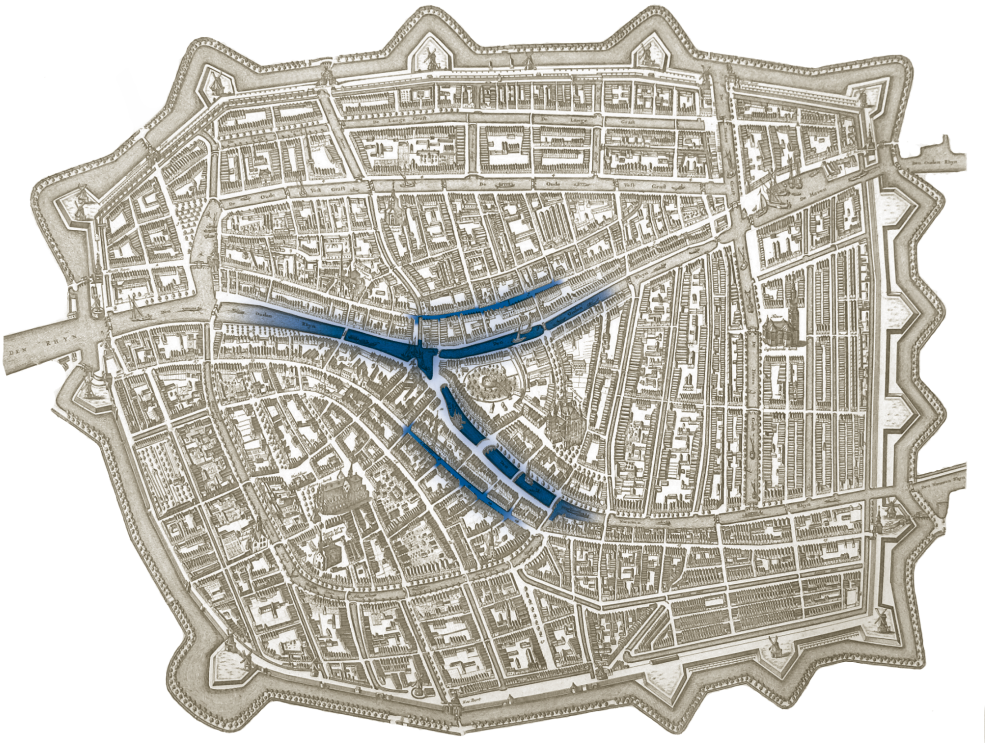
Basic Disease Mechanisms in Rheumatoid Arthritis



HANS ULRICH SCHERER

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Hans Ulrich Scherer

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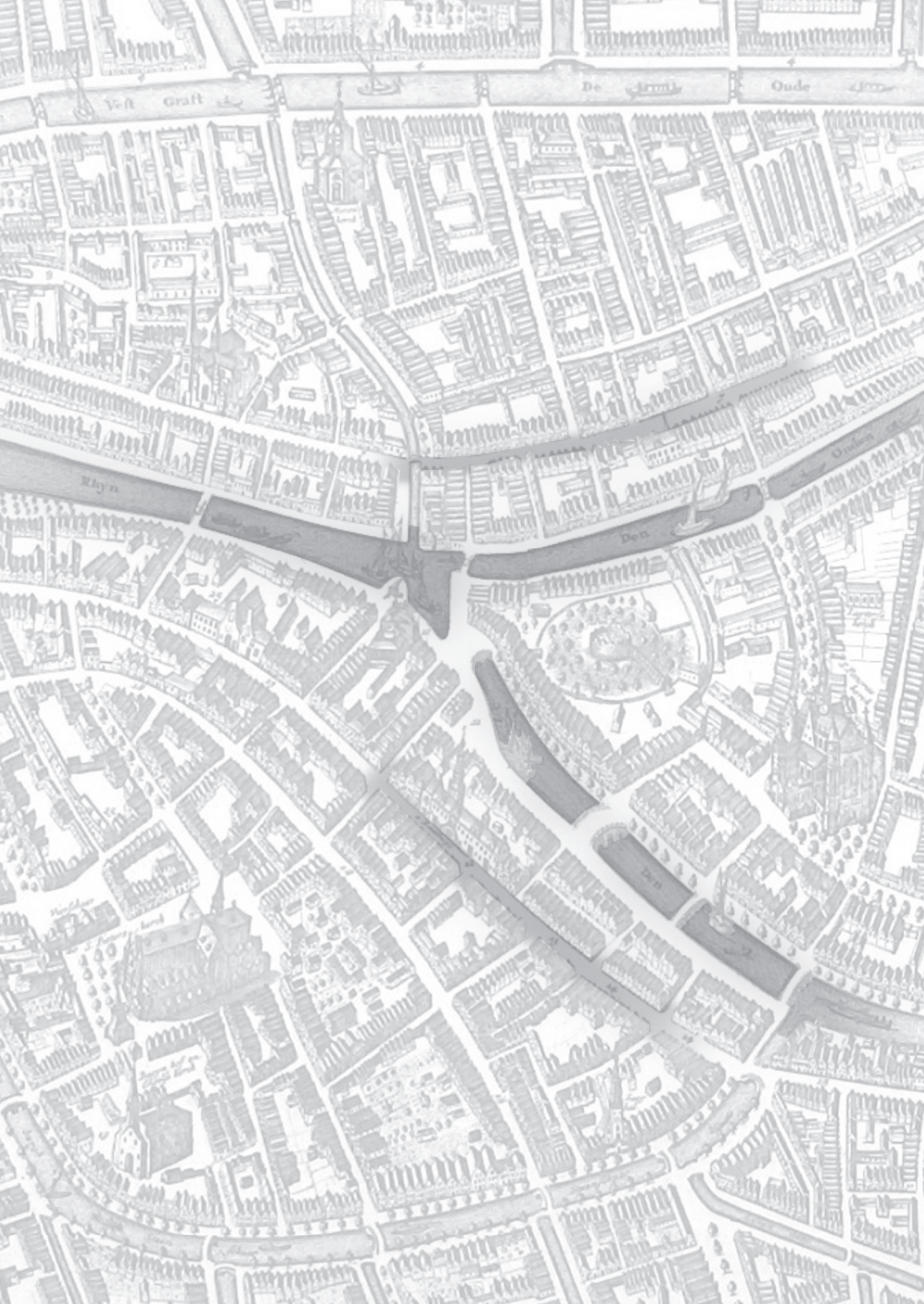
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Chapter 1

Introduction

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RHEUMATOID ARTHRITIS

Rheumatoid Arthritis (RA) is a chronic inflammatory disorder that typically affects cartilage and bone of small and middle-sized joints. Inflammatory cells invade the otherwise relatively acellular synovium, which leads to hyperplasia and formation of pannus-tissue. This infiltration causes destruction of cartilage, erosion of the adjacent bone and ultimately loss of function of the affected joint. Involvement of larger joints may also occur. Systemic inflammation, often going in parallel, can affect several organs (e.g. lungs, vessels and the hematopoietic system) and has long-term impact on organ function. Combined with inevitable side effects of yearlong anti-rheumatic medication (e.g. glucocorticoids) and the psychological burden of facing early invalidity and social instability, RA has, if insufficiently treated, important socio-economic impact and causes a reduction in life-expectancy of 7 years in average [1,2].

For the clinician, there is considerable heterogeneity in both clinical picture and course of disease. Next to the characteristic signs and symptoms of RA, overlap with other rheumatic diseases can be observed (e.g. mixed connective tissue disease (MCTD)). In addition, other autoimmune diseases (e.g. Sjögren's syndrome, autoimmune thyroiditis) may accompany RA. For reasons largely unknown, the course of disease is highly variable, ranging from mild cases with non-erosive, even sometimes spontaneously remitting disease, to severe, rapidly progressive and destructive arthritis [3]. Recent analysis of genetic risk factors and autoantibody responses together with data from clinical trials suggest, however, that the clinical entity RA might consist of pathogenetically distinct subgroups, which present with similar if not identical clinical phenotypes [4]. Different treatment strategies may need to be applied to patients within these groups.

For the immunologist, RA is considered an autoimmune disease by most, implying breakdown of immunological tolerance towards self at a given moment in a patient's life. The trigger initiating this breakdown is so far unknown [5]. The presence of autoantibodies and slowly rising C-reactive protein-levels several years before onset of clinical symptoms indicate that the inflammatory process may be well underway long before patients first consult a physician [6]. Variations between ethnic groups in susceptibility to RA, heterogeneity of disease course and variations in clinical, radiological and laboratory findings within groups strongly suggest that multiple factors, both environmental and genetic, influence onset and progression of RA, presumably with different impact during different stages of disease development. Genetic variations, autoantibodies, cellular immune responses, hormones and gene-environment interactions are among the most studied factors contributing to RA development.

AUTOANTIBODIES AND THEIR ROLE IN THE DISEASE PROCESS IN RA

An array of antibodies targeting self-antigens (e.g. collagen type II, calreticulin, cathepsin, BiP, CH65, etc.) has been described in patients with RA[7]. Demonstrating pathogenetic relevance for any of these reactivity's, however, has proven difficult, last but not least because the clinical and radiological phenotype of RA can also develop in the absence of any of the autoantibodies known so far.

Rheumatoid factor

The initial notion that mechanisms of autoimmunity might underlie RA pathogenesis came from the discovery of autoantibodies targeting the Fc-part of human IgG (so called "rheumatoid factors" (RF)) in the blood of affected patients[8,9]. RF, present mostly as IgM-RF, but detectable in subgroups of patients also as IgG- and IgA-RF, are thought to form immune complexes activating complement, which in turn leads to increased vascular permeability and the release of chemotactic factors recruiting immune-competent effector cells to the joint[10]. The mere presence of RF, however, is insufficient to initiate arthritis development, as RF are also found in infectious diseases, autoimmune diseases other than RA and in up to 15% of healthy, mostly elderly individuals. Thus, sensitivity and specificity of RF are, depending on the population studied, 60-70% and 50-90%, respectively. Despite this lack of specificity, RF are part of the former and new classification criteria for RA[11,12].

Anti Citrullinated Protein Antibodies (ACPA)

Citrullination is a process by which arginine residues in a given protein are post-translationally modified, in the presence of high calcium-concentrations, by an enzyme called PAD (peptidyl arginine deiminase). Under physiological conditions, it is believed that citrullination facilitates the degradation of intracellular proteins during apoptosis[13,14]. In 1998, two antibodies present in serum of RA patients, anti perinuclear factor (discovered in 1964[15]) and anti-keratin antibodies (first described in 1979[16]) were found to recognize a common target: citrullinated fillagrin[17,18]. This observation, together with an unprecedented specificity of citrulline-specific antibodies for RA, has placed anti-citrullinated protein antibodies (ACPA) at the center of intense research efforts. Meanwhile, citrullin-specific reactivities against several proteins (e.g. fibrinogen, collagen, vimentin, enolase, and others) have been identified, and by the use of optimized assays ACPA are now detectable in 60-70% of RA-patients, but hardly in other diseases or healthy subjects. Whether ACPA contribute to the disease process, and the possible pathogenetic mechanism of such a contribution, is matter of intense debate. A number of clinical associations and an increasing amount of experimental data, however, point in this direction.

Individuals with joint pain (arthralgia) that harbor ACPA in serum have an increased risk to develop arthritis[19]. In patients with undifferentiated arthritis (UA), the presence of ACPA increases the risk for progression to RA, and lowers the chance for remission[20]. ACPA positive RA patients suffer from more extensive joint destruction and more frequent extra-articular organ involvement than their ACPA negative counterparts[21]. Also histologically, synovial tissue differs between ACPA positive and negative patients[22]. Patients with UA benefit from treatment with methotrexate if ACPA positive, as they develop significantly less joint destruction in the first year than untreated controls, and progress to RA less frequently. For ACPA negative UA patients, methotrexate treatment was without effect on these parameters compared to placebo[23,24]. A number of genetic factors, among which the so-called shared epitope (SE-) alleles, confer risk for RA development only to ACPA positive individuals, whereas they do not seem to influence the pathogenic process in ACPA negative disease[25]. In fact, SE-alleles, a set of HLA-DRB1 molecules with a shared amino acid sequence formerly regarded as strong risk factors for RA, are risk factors only for the development of ACPA, without an independent risk effect on the development of RA itself[26].

Taken together, these observations support a model in which ACPA positive RA develops on a different pathogenetic background than ACPA negative RA[4]. The ACPA immune response broadens shortly before onset of clinical disease, with an increasing number of citrullinated epitopes recognized and more ACPA isotypes generated[27,28]. The question arises, however, as to the available scientific evidence that ACPA are the actual factors driving the disease process. This all the more, as patients can undergo complete, drug-free remission despite persistent, high ACPA serum titers.

ACPA pathogenicity

Citrullinated antigens are found in RA synovium, which is an important prerequisite for local ACPA pathogenicity[29,30]. At the same time, ACPA levels are elevated in synovial fluid as compared to serum[31]. In the mouse, antibodies to a citrullinated B-cell epitope of collagen type II (CII), which cross-react with citrullinated CII in human joints, were found to be arthritogenic[32]. Monoclonal antibodies against citrullinated fibrinogen were found to enhance arthritis in a mouse model of pre-existing collagen-induced arthritis[33], and citrullinated human fibrinogen, but not unmodified fibrinogen, was able to induce arthritis in HLA-DRB1*0401 (DR4-IE) transgenic mice[34]. In the latter experiment, no induction of arthritis was seen in wild-type mice lacking the transgene.

In the human, first evidence for ACPA-specific pro-inflammatory effects came from studies that observed stimulation of macrophages by ACPA-containing immune complexes[35]. In addition, ACPA were found to activate the complement system[36]. More recently, *in vitro* activation of basophils by ACPA of the IgE isotype was described[37]. In addition, associations were noted between IgE and FcεRI expression on synovial mast

cells, histamine levels in synovial fluid and ACPA positivity. As synovial mast cells are an important source of the proinflammatory cytokine IL-17, ACPA stimulated mast cell degranulation and cytokine production could contribute to local inflammation[38]. More recent work has demonstrated *in vitro* activation of human osteoclasts by antibodies to citrullinated vimentin, with increased bone loss in mice injected with these antibodies[39]. This latter observation, which was not noted for non-specific IgG, so far most closely links ACPA to the pathological correlate of RA: bone erosions.

In summary, both clinical associations and an increasing number of experimental data support the hypothesis that ACPA contribute to synovial inflammation and joint destruction. The observation that RA can remit despite the presence of ACPA indicates, however, that the quality rather than the quantity of the ACPA immune response determines its pathogenicity. One important aspect of the immunogenicity of antibodies is determined by Fc-linked glycans.

ANTIBODY GLYCOSYLATION AND ITS FUNCTIONAL CONSEQUENCES

Human antibodies are glycoproteins with carbohydrate structures attached to the constant and, in some cases, to the variable region of the molecule. These glycans strongly influence the *in vitro* and *in vivo* biological characteristics of an antibody, such as serum half-life, binding to Fc-receptors, complement activation and interaction with lectins[40,41]. Glycans attached to the Fc-tail of IgG-molecules have most extensively been studied.

Fc-glycosylation of human IgG

The Fc-tail of human IgG carries two complex-type N-glycans, each attached to one heavy chain at position 297 (asparagine) in the C_H2 domain of the protein backbone (Figure 1A). The sugar chains are intercalated between the heavy chains, with which they non-covalently interact at several positions. This interaction maintains the three dimensional structure of the Fc-tail, which changes conformation and loses its function once the glycans are enzymatically removed[42-44]. The glycan chains consist of a conserved biantennary, heptasaccharide core structure of *N*-acetylglucosamine (GlcNAc) and mannose residues (Figure 1B). Core fucose, additional (bisecting) GlcNAc, galactose, and a terminating sialic acid residue further modify this sequence. In total, these modifications yield more than 30 possible variants per glycan chain, of which glycoforms carrying either zero, one or two galactose residues (termed G0, G1, G2 glycoforms) are most abundantly found on human IgG (20–35%, 35%, and 16% of all glycoforms, respectively). The degree to which Fc-linked glycans on IgG are sialylated, galactosylated, fucosylated or carry bisecting GlcNAc residues in a given individual depends on various

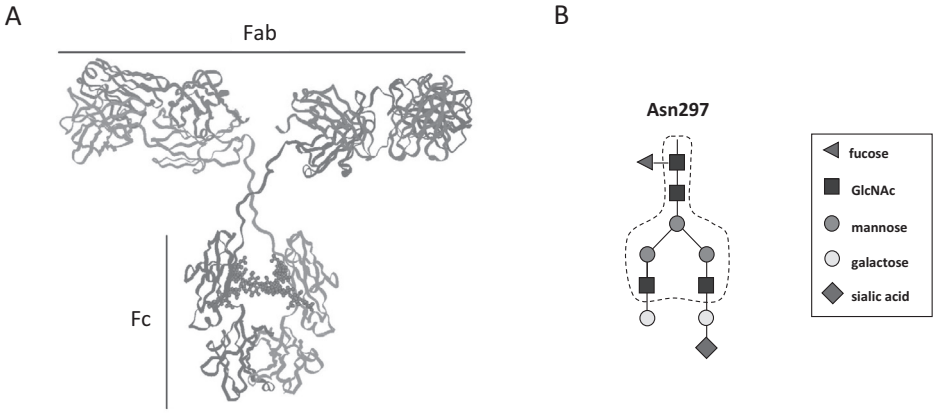

Figure 1: (A) Three dimensional structure of a human IgG1 molecule with the two heavy chains depicted in dark and light grey. The glycan chains are intercalated in between the heavy chains (modified from [48]). (B) Schematic depiction of a monosialylated glycan attached to one of the heavy chains at position 297. The dotted line shows the conserved core structure, which can be modified by fucose, galactose and sialic acid residues. Also disialylated glycoforms and glycans carrying an additional, bisecting N-Acetylglucosamine (GlcNAc) residue can be found (not shown).

factors such as age, hormonal status, and the type of immune response during which the IgG molecule is produced [45-47].

Functionally, the Fc tail interacts with Fc receptors and binds the complement component C1q. In addition, it is the target of rheumatoid factors and as such involved in immune complex formation. Absence of galactose residues (G0) on the Fc-linked glycans is associated with concomitant absence of sialic acid residues and increases the affinity of the Fc tail for activating Fc gamma receptors (FcγR) expressed by immune cells [49,50]. In addition, agalactosylated glycoforms on the Fc tail enhance the formation of IgG-containing immune complexes [51]. Mainly for these reasons, a high abundance of G0-glycoforms on IgG is thought to correspond to pro-inflammatory properties of the molecule. In contrast, presence of galactose (G2) and sialic acid residues is believed to favor anti-inflammatory effector functions. Moreover, absence of core fucose residues leads to high avidity of the Fc-tail for binding to FcγRIIIa, which is based on a unique interaction of the afucosylated Fc-glycan with carbohydrates of the receptor [52]. This interaction enhances antibody dependent cellular cytotoxicity (ADCC) by up to 100-fold, a finding exploited by glycoengineerd therapeutic monoclonal antibodies [52-54].

The *in vivo* effects of different IgG Fc-linked glycoforms can be studied using recombinant monoclonal IgG molecules of defined specificity or by employing IgG molecules purified from pooled human plasma of healthy individuals. Such polyclonal IgG preparations (termed intravenous immunoglobulin, IVIG) are known for their important anti-inflammatory effects *in vivo* and are used clinically to treat various autoimmune

diseases[55]. Using IVIG, a number of studies have proposed an important role of terminal sialic acid residues for IgG-mediated effector functions. In fact, removal of terminal sialic acid residues from IVIG-associated glycans has been reported to abrogate its anti-inflammatory activity in mouse models of autoimmunity[49,56,57]. Conversely, enrichment of IVIG for sialylated molecules enhanced this property. In fact, it has been postulated that only a minor fraction of IgG molecules (~ 1-3%) within IVIG carries terminal sialic acid residues on their Fc-linked glycans and that this small fraction might account for the therapeutic effects observed[49]. Accordingly, enrichment of the sialylated fraction by lectin affinity chromatography using Sambucus nigra agglutinin (SNA) or *in vitro* sialylation was found to result in a reduced dose requirement for the *in vivo* activity of IVIG. In line with this, a fully recombinant human IgG_1 molecule with Fc-linked glycans terminating in sialic acid-galactose linkages recapitulated the *in vivo* anti-inflammatory activity of intact IVIG and enhanced its effect by 35-fold, compared with the activity of conventional IVIG[56].

It is important to note, however, that several aspects of the studies on IVIG described above are currently under debate. This is mainly based on the observation that lectin chromatography with SNA does not enrich for Fc-linked sialylated IgG, but for IgG molecules carrying sialic acid containing glycans in the Fab portion[58,59]. In addition, the studies employed human IgG molecules to study effects in mice, indicating that it might not be possible to directly translate the findings to the human situation. Thus, although the relevance of the Fc-glycan for interaction of IgG molecules with FcγR is undisputed, the specific role of terminal sialic acid residues in this context requires further study.

In addition to FcγR mediated effects, Fc-glycans are also required for and modulate IgG-mediated complement activation[60]. Although the ability of IgG to activate complement strongly depends on the IgG subclass, C1q binding to IgG_1 and subsequent activation of the classical pathway was found to be most effective in the presence of G2-glycoforms[61,62]. Mannose-binding lectin (MBL), on the other hand, binds to exposed, terminal mannose, fucose and GlcNAc residues on agalactosylated (i.e. G0 containing) IgG molecules *in vitro*, thereby initiating the lectin pathway of the complement cascade[63]. Debate exists, however, as to the *in vivo* relevance of this finding with regard to IgG pathogenicity in autoimmune diseases, as IgG-G0 molecules in MBL-null mice (genetically deleted for MBL) did not lose their inflammatory potential in mouse models of immune-thrombocytopenia and arthritis, whereas their effects were abrogated in FcγR deficient mice[50].

IgG Fc-glycosylation in RA

In RA, early work has demonstrated aberrant glycosylation of the Fc-tail of serum IgG, which mainly lacks galactose and sialic acid residues as compared to IgG in healthy individuals[64]. This hypogalactosylation (i.e. predominance of the G0 glycoform) and,

as a consequence, hyposialylation, of the Fc-tail associates with disease activity and can revert to normal levels during effective treatment, for example with tumor necrosis factor alpha inhibiting agents[65,66]. A similar decrease in G0 content was observed in female RA-patients during pregnancy[67]. The hypogalactosylation of IgG molecules in RA is likely to be regulated on the B cell level, rather than a result of enzymatic release of galactose residues post-secretion, as reduced expression of β-1,4-galactosyltransferase, the enzyme responsible for adding galactose residues to the Fc-linked glycan, was noted in B cells of patients with RA[68].

These observations raise the question whether the disease activity dependent variation of IgG Fc-glycosylation in RA actively contributes to disease, or whether it merely reflects the inflammatory environment in which the IgG molecules are produced. Arguments for the latter concept are fuelled by the finding that hypogalactosylation of human IgG-Fc is not specific for RA, but also characterizes other autoimmune diseases and can even occur in the context of infectious diseases[69]. The hypothesis of an active modulation of disease by G0-containing IgG, however, is supported by studies on the anti-inflammatory effects of sialic acid containing IVIG described above, and by animal studies. Specifically, in a murine passive transfer model of arthritis, agalactosylated IgG induced more severe arthritis than IgG without glycan modification, indicating that Fc-linked G0 glycoforms can indeed increase inflammation in this context[67,70]. Moreover, deglycosylation of the Fc-tail abrogated arthritogenicity of monoclonal, collagen-specific murine IgG in a similar model[44]. More recent data showed that the increase in IgG G0 glycoforms in RA could be detected several years before diagnosis[71], a finding that supports, but does not prove, the concept of a pro-inflammatory effect of G0 in human RA.

In summary, glycans on the Fc tail of human IgG have a strong influence on its biological function. Intriguing aberrations of Fc-linked glycans are noted in RA. Until now, it is uncertain whether these glycosylation changes are cause or consequence of inflammation.

THE ROLE OF REGULATORY T CELLS IN RA

Regulatory (Treg) T cells represent an important mechanism by which the immune system can control the development of autoreactivity. This is crucial, as autoreactive T- and B-lymphocytes can escape the classical checkpoint of central tolerance in bone marrow or thymus, which functions as the main barrier to eliminate autoreactive lymphocytes during their development. Accordingly, severe autoimmunity including arthritis develops in the absence of regulatory T cells, both in mice and humans[72,73].

The population of CD4 expressing human Treg cells is heterogeneous; it comprises a subset with imprinted regulatory functions (“naturally occurring” Treg cells) derived

from the thymus, and a set of peripheral T cells that can acquire them ("adaptive" or "inducible" Treg cells)[74]. Both types are considered to be "regulatory" based on the capacity to effectively inhibit proliferation and cytokine secretion of effector T cells in culture. $CD4^+$ Treg cells are classically identified based on the expression of high levels of CD25 and the transcription factor FoxP3, and of low levels of the α-chain of the IL-7 receptor (CD127). The expression of FoxP3 is stable in natural Treg cells due to epigenetic imprinting, while inducible Treg cells express FoxP3 transiently[75,76]. More recently, the transcription factor and regulator of FoxP3 expression Helios was identified as phenotypic and functional marker of natural, but not adaptive, Treg cells[77]. *In vitro*, Treg cells are characterized by low proliferation rates, low production of IL-2 and by secretion of TGF-β, IL-10, IL-35, perforin and granzymes[78,79].

Regulatory T cells in RA

The role of Treg in RA pathogenesis is unclear. Unlike in the mouse, human natural and adaptive Treg are difficult to differentiate from activated T cells without regulatory functions, as markers such as CD25 and FoxP3 are inducible upon stimulation. Because of this, the number and functional integrity of regulatory T cells in RA are subject to debate[80]. In the mouse, depletion of $CD25^+$ $FoxP3^+$ Treg can enhance arthritis, while adoptive transfer of Treg can ameliorate disease[81,82]. As these experiments, together with arthritis development in Treg deficient mice and humans shows the general capacity of Treg to modulate arthritis, both functional Treg deficiency or resistance of effector cells to Treg-mediated suppression could operate in RA.

As CD25 and FoxP3 expression in human T cells cannot be equalized with suppressive function, reports on Treg in RA range from decreased numbers to increased frequencies, and from impaired to enhanced suppressive functions[83-86]. In addition, differences were reported between Treg cells in peripheral blood and synovial fluid[85]. Elegant flow cytometric studies combined with functional and epigenetic data have shown that human Treg cells can be subdivided in resting naïve and activated effector Treg cells based on the expression of CD25 and CD45RA, and in a population of FoxP3 expressing, $CD25^+$ but $CD45RA^-$ non-Treg cells[87]. This more subtle delineation of Treg cell populations, however, has not been used in most studies.

Of interest, impaired Treg cell function has been reported under the influence of TNF-α, which was reversible by anti-TNF treatment[84,88]. This observation is plausible, as Treg cells express TNFR-II, which makes them susceptible to the deleterious effects of TNF-α. In fact, treatment with TNF-antagonists gave rise to a newly generated, functionally distinct Treg-cell population that secretes TGF-β and IL-10. However, more recent data suggest that also this notion might be debatable, as TNF was also found to promote Treg cell function[80].

Taken together, it is currently unclear to what extent Treg cells are defective in RA, or why functional Treg are insufficient to control the disease.

OUTLINE OF THIS THESIS

Based on the observations and considerations described above, this thesis investigates several aspects of immunological disease mechanisms that are of relevance to the inflammatory immune response in rheumatoid arthritis. Specifically, three main research questions triggered the experiments presented and form the outline of this thesis:

1. Do regulatory T cells feature anti-inflammatory properties besides the inhibition of effector T cells, which could help explain their therapeutic effectiveness in a murine model of established arthritis?

2. Are there specific features of the ACPA immune response that could contribute to inflammation in RA, and can analysis of these features help in understanding the characteristics of ACPA producing B cells and their development?

3. Do certain genetic variants that associate with RA susceptibility contribute also to disease progression, as evidenced by the rate of joint destruction in RA?

Part I was triggered by the observation that adoptive transfer of regulatory T cells during the effector phase of murine collagen-induced arthritis significantly decreased inflammatory disease activity, without affecting the levels of circulating antibodies [82] **(chapter 2)**. This was unexpected, as effector T cells, the primary target of Treg cells, were thought to be involved primarily in the initiation phase of this otherwise antibody-driven disease model [89,90]. At the same time, this finding suggested that Treg cells possess means to dampen inflammation beyond the inhibition of effector T cell function. In the light of the role of TNF-α in RA, our studies revealed that Treg cells can express and shed a soluble receptor for TNF-α, TNFRII. This TNFR-shedding was capable of inhibiting an early, TNF-mediated inflammatory response in the mouse, which demonstrated the *in vivo* relevance of this functional aspect of Treg. Importantly, the feature of TNFR-shedding could be shown for murine as well as human Treg cells. In conclusion, the first part of the thesis demonstrates the identification of a mechanism underlying anti-inflammatory properties of regulatory T cells.

Part II is dedicated to the quality of the ACPA immune response and its potential contribution to inflammation in RA **(chapters 3 – 6)**. As described above, ACPA are detectable

in similar levels in patients with active and inactive disease[91,92], indicating that the quality rather than the quantity of the ACPA immune response determines its pathogenicity. We studied both features of the ACPA Fc tail as well as characteristics related to antigen binding via the variable region.

Based on the observation that the Fc tail of IgG molecules in RA lacks galactose and, consequently, sialic acid residues, and that Fc-linked glycans can modulate immune responses, we hypothesized that ACPA might differ from non-specific IgG molecules in their Fc glycosylation profile and thereby have the potential to enhance inflammation. To study Fc glycosylation antigen-specifically, we first developed a method for isolating ACPA from small quantities of human serum and combined it with a high throughput analysis of Fc glycopeptides by mass spectrometry **(Chapter 3)**. This methodology allowed us, for the first time, to study glycan residues linked to the ACPA Fc tail, and to compare them to those found on the Fc tail of non-specific total IgG of the same patient. When applied to serum and synovial fluid samples of ACPA positive RA patients, the analysis revealed that ACPA indeed exhibit a specific, pro-inflammatory glycan profile in that they significantly lack sialic acid and galactose residues **(Chapter 4)**. Importantly, we found differences in the Fc glycosylation profile of ACPA in serum and synovial fluid within the same patient, which was not the case for non-specific IgG. In line with the initial hypothesis, ACPA in synovial fluid were highly agalactosylated. As such, this finding represents evidence for qualitative differences of ACPA in different compartments, and indicates that ACPA producing B cells might possess specific functional characteristics, which are distinct from "conventional" B cells.

In this context, little is known on the origin and development of ACPA-specific B cells. Most B cells mature in germinal centers, where they receive help from follicular helper T cells to undergo class switch recombination and affinity maturation[93]. ACPA of all Ig isotypes have been detected in patient sera, supporting the notion that ACPA producing B cells originate from germinal center reactions[28,37]. Importantly, during conventional immune responses, only B cells with B cell receptors of high affinity for the antigen receive appropriate survival signals required to differentiate into memory B or plasma cells. To gain further insight into specific features of ACPA that might relate to aberrant B cell development, we studied the avidity of ACPA in comparison to the avidity of antibodies against recall antigens such as tetanus **(Chapter 5)**. Surprisingly, ACPA were found to be mainly of low avidity, irrespective of the degree of class switch recombination that the ACPA specific B cells had undergone. Also during the course of 5 years, we did not detect affinity maturation within individual patients. This observation supports the notion of a developmental difference between "conventional" and ACPA producing B cells, but the underlying mechanism remains unknown.

Finally, another aspect of ACPA pathology relates to the antigens recognized. The ACPA response is polyclonal and generates multiple specificities that recognize various

citrullinated proteins[94]. This has fuelled the hypothesis that certain reactivity's might be more specific for, or more relevant to the disease process than others. As most currently used detection assays use citrullinated antigens designed to detect as many ACPA positive individuals as possible, yielding high sensitivity of the assay, these do not take into account potential subgroups of patients in which the ACPA recognition profile might associate with clinical features of the disease. As destruction of the affected joint is the prominent feature of RA, we addressed this issue by analyzing whether certain fine specificities exist within the repertoire of citrullinated antigens that are specifically pathogenic by promoting enhanced joint destruction over time **(Chapter 6)**. Of interest, no fine-specificity associated with the rate of joint destruction within the ACPA positive subgroup, indicating that recognition of citrullinated antigens in itself, but not the recognition of specific citrullinated proteins, is of primary relevance to RA disease pathology. Moreover, it suggests that analysis of the ACPA recognition profile within ACPA positive individuals does not identify patients specifically at risk for progressive disease.

Part III, in keeping with risk factors for joint destruction, analyzes the contribution of genetic variants located in the 6q23 region to the rate of joint destruction in RA **(Chapter 7)**. This region had previously shown association with RA susceptibility in several studies, but the underlying mechanism for this effect, as for many genetic risk factors, remained unknown[95-97]. Of interest, the association was only found in the ACPA positive subgroup, in line with observations on other RA-associated risk factors including the shared epitope alleles. The variants are located close to the gene encoding TNFAIP3, a negative regulator of NFκB involved in TNF-receptor mediated signaling. In our study, we observed that carriers of two single nucleotide polymorphisms displayed increased joint destruction over time. This observation refines the understanding of potential effects mediated by this genetic locus and represents the first description of a risk factor outside the HLA-region that could be linked to disease outcome.

Chapter 8 provides a summary of the work presented and a discussion of the results in the context of current literature.

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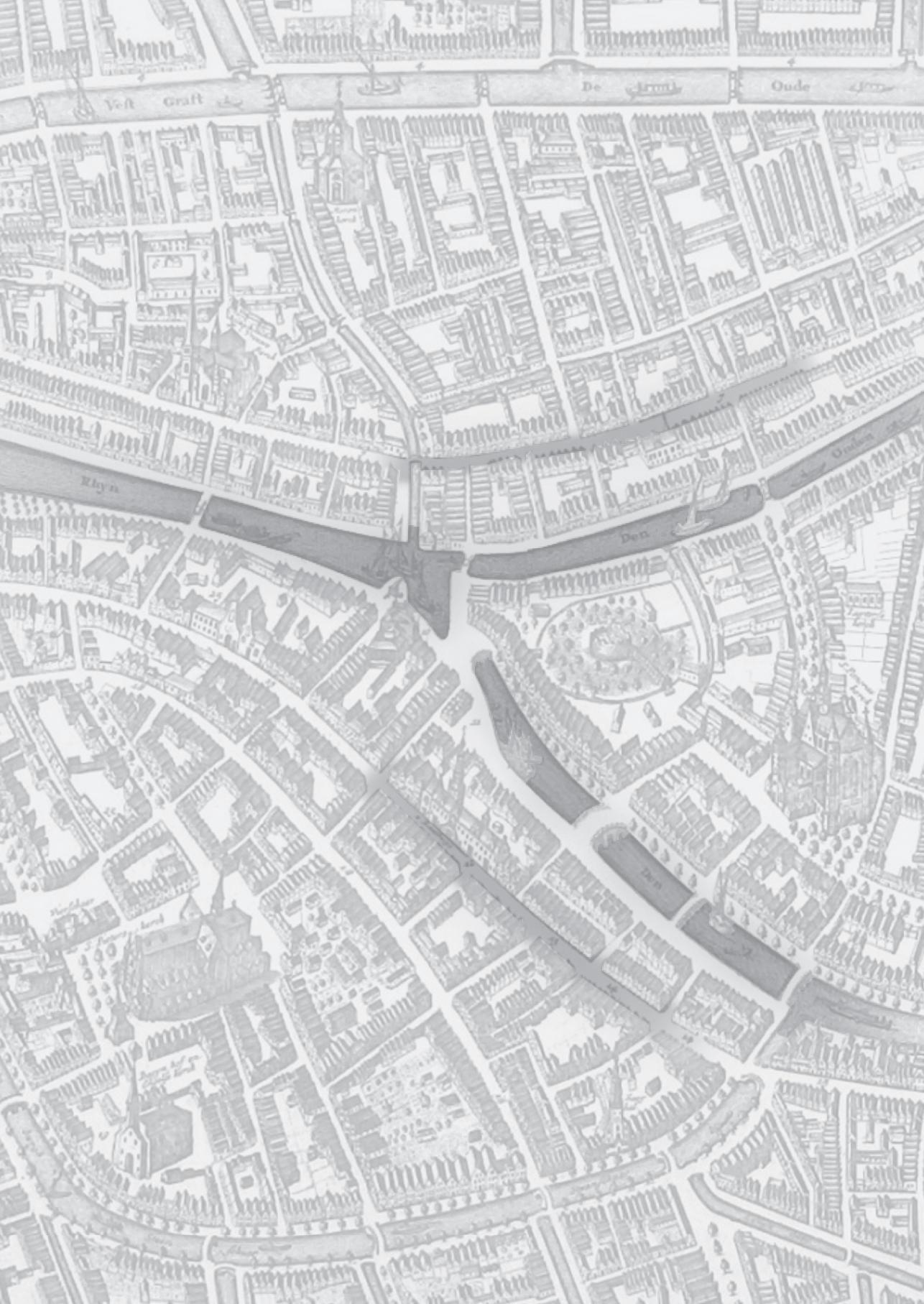
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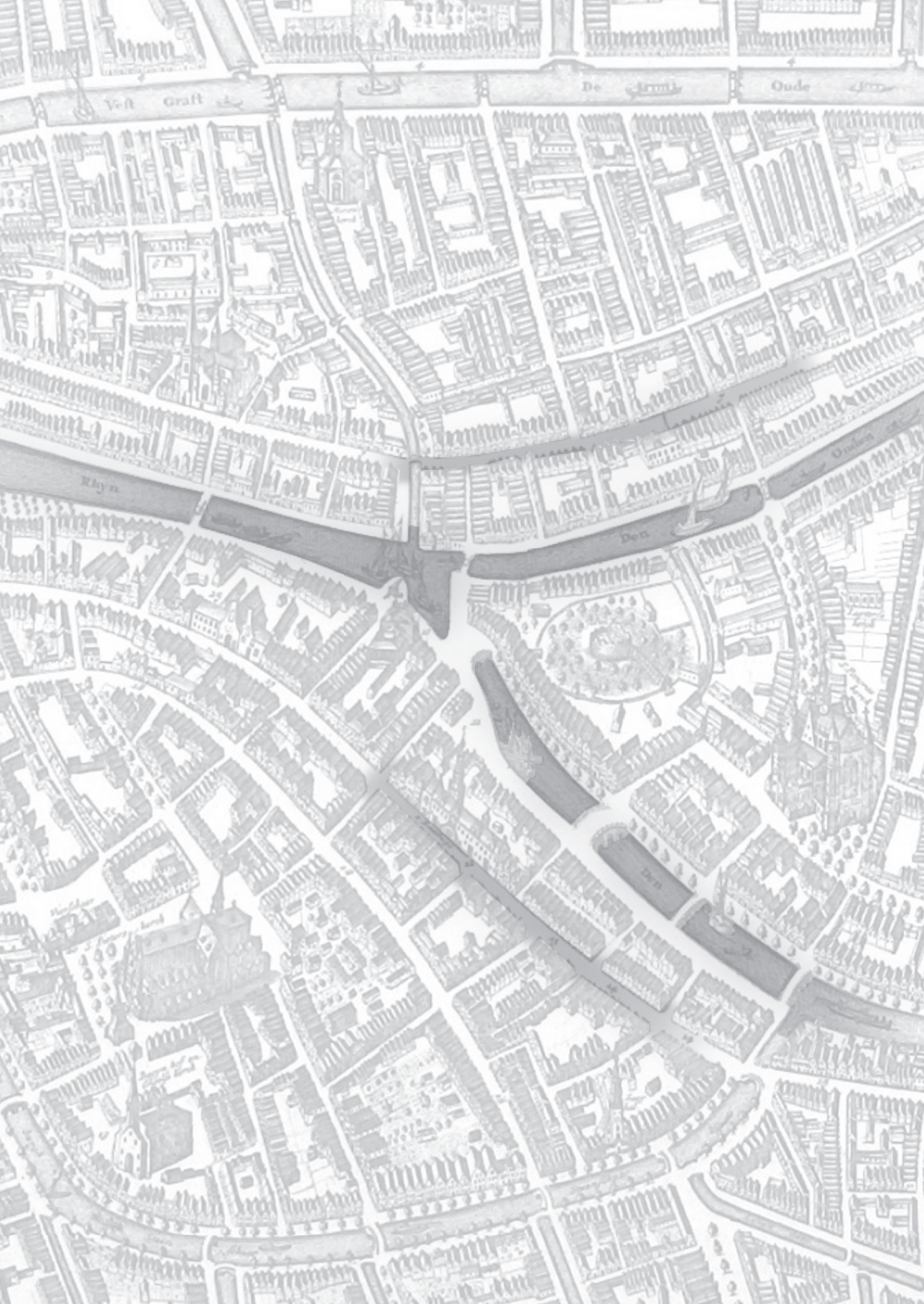
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Chapter 2

Cutting edge: TNFR-shedding by CD4+CD25+ regulatory T cells inhibits the induction of inflammatory mediators.

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ABSTRACT

$CD4^+CD25^+$ regulatory T cells (Treg cells) play an essential role in maintaining tolerance to self and non-self. In several models of T cell mediated (auto-) immunity, Treg cells exert protective effects by inhibition of pathogenic T cell responses. In addition, Treg cells can modulate T cell-independent inflammation.

We now show that $CD4^+CD25^+$ Treg cells are able to shed large amounts of TNF receptor II (TNFRII). This is paralleled by their ability to inhibit the action of TNF-α both *in vitro* and *in vivo*. *In vivo*, Treg cells suppressed IL-6 production in response to LPS injection in mice. In contrast, Treg cells from TNFRII-deficient mice were unable to do so despite their unhampered capacity to suppress T cell proliferation in a conventional *in vitro* suppression assay.

Thus, shedding of TNFRII represents a novel mechanism by which Treg cells can inhibit the action of TNF, a pivotal cytokine driving inflammation.

INTRODUCTION

Current understanding of basic processes that control immune tolerance has been fuelled by identification of $CD4^+CD25^+$ regulatory T (Treg) cells[3] as an important component of self-tolerance. $CD4^+CD25^+$ T cells have been shown to regulate peripheral self tolerance, to protect against autoimmunity and to suppress immune responses to autoantigens, alloantigens, tumor antigens and infectious agents [1-4].

Despite accumulating evidence for immunoregulatory properties of $CD4^+CD25^+$ Treg cells, the mechanism by which $CD4^+CD25^+$ Treg cells inhibit T cell-independent inflammation is not well defined. $CD4^+CD25^+$ Treg cells are anergic to TCR-stimulation *in vitro* and capable of inhibiting proliferation and cytokine production of other T cells by secretion of anti-inflammatory cytokines (e.g. IL-10 and TGF-β) and a mechanism that depends on CTLA-4 and membrane-bound TGF-β [5, 6].

We previously showed that adoptive transfer of Treg cells decreases levels of acute phase proteins such as serum amyloid P component (SAP) in mice that had been injected with CFA (unpublished observation) or that underwent total body irradiation [7]. For that reason, we hypothesized that Treg cells shed a soluble mediator that can inhibit the induction of acute-phase responses. TNF-α is one of the most prominent initiators of the acute-phase reaction which can, via the action of IL-6, promote the release of several acute-phase proteins from the liver [8-10]. Here, we describe a novel mechanism by which Treg cells can counteract the action mediated by TNF-α.

MATERIALS AND METHODS

Mice

C57BL/6 mice and TNFRII KO mice on a C57BL/6 background (B6.129S2-*Tnfrsf1b*^{tm1Mwm}/J) were maintained at LUMC animal facility in accordance with national legislation under supervision of the University's animal experimental committee.

Isolation of murine Treg cells and culture

Murine $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from spleen and LN of 6-14 week old mice by positive selection of $CD4^+$ T cells (MACS), fluorescent labeling (anti-CD4, anti-CD25) and subsequent FACS-sorting (FACS-ARIA cell sorter, BD Biosciences) on the basis of CD25 expression. Purified T cell subsets were activated in the presence of Dynabeads mouse CD3/CD28 (Dynal Biotech) and 50 IU/ml IL-2.

Isolation of human Treg cells and culture

Isolation of human $CD4^+CD25^{high}$ or $CD4^+CD25^-$ T cells from buffy coats of healthy human donors was performed as previously described [11]. FACS-sorted $CD4^+CD25^{high}$ and $CD4^+CD25^-$ cells were cultured in the presence of 1 µg/ml anti-CD28 (CLB-CD28/1, Sanquin), 5 µg/ml plate-bound anti-CD3 (OKT-3, BD Biosciences) and 100 U/ml IL-2 for up to 5 days. Metalloproteinase-inhibitor marimastat was added to cultures where indicated at a final concentration of 10 µg/ml.

Suppression assay

After 3 to 4 days of *in vitro* activation, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were cultured with equal numbers of freshly isolated splenocytes in the presence of 1 µg/ml PHA. ^{3}H-thymidine incorporation of triplicates was measured 3-4 days later. Suppression assays were performed for each sorted population of $CD4^+CD25^+$ cells to ensure the suppressive capacity of isolated Treg cells.

Flow cytometry

Murine cells were stained using mAb against CD4, CD25, CD120b (TR75-89), FoxP3 (FJK-16S, eBiosciences) or an isotype control. Human cells were stained using mAb against CD4 (RPA-T4), CD25 (2A3), CD120b (MR2-1, AbD Serotec and 22235, R&D Systems), CCR7 (3D12), HLA-DR (L243), CD45RO (UCHL1), CD45RA (HI100) and CD62L (Dreg 56). Intracellular FoxP3-staining was performed using eBiosciences FoxP3-staining kit (PCH101 or appropriate isotype control). Antibodies were purchased from BD Biosciences unless otherwise stated.

TNFRI and TNFRII secretion

sTNFRI and sTNFRII in culture supernatants were measured using standard ELISA-kits (Hycult Biotechnology for murine and R&D Systems DuoSet for human sTNFR).

Bioactivity of sTNFR

In vitro activity of sTNFR was measured using TNF-α sensitive WEHI 164 clone 13 cells as previously described [12]. *In vivo* activity was determined by injecting mice i.v. with $1x10^6$ Treg or control cells ($CD4^+CD25^-$ T cells) of either WT animals or TNFRII KO animals after 4 days of *in vitro* activation. As a control, mice were injected i.v. with 250µg Etanercept, a TNFRII-Ig fusion protein. 1 hour later mice were injected i.p. with 150µg LPS (S. typhosa, Sigma). 4 and 6 hours after LPS-injection blood samples were collected to determine serum levels of IL-6 using BD Biosciences Mouse IL-6 ELISA set.

RESULTS AND DISCUSSION

Shedding of sTNFRII by murine CD4+CD25+ Treg cells but not by CD4+CD25− cells

Adoptive transfer of $CD4^+CD25^+$ Treg cells can inhibit the induction of acute-phase responses in mice[7]. As TNF-α stimulates acute-phase responses[8-10], we hypothesized that $CD4^+CD25^+$ Treg cells could directly inhibit TNF-α. FACS-analysis revealed that $CD4^+CD25^+$ Treg cells, as opposed to $CD4^+CD25^-$ T cells, strongly express TNFRII (data not shown), leading us to predict that Treg cells may be able to shed sTNFRII. Therefore, we activated purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations *in vitro* and analyzed supernatants of these cultures for presence of sTNFR. While no sTNFRI could be observed (data not shown), sTNFRII was detectable in culture supernatants of $CD25^+$ cells from day 1 onwards (Figure 1). No TNFR-shedding was noted in the presence of IL-2 only (data not shown), indicating that TCR-triggering is required for TNFR-shedding.

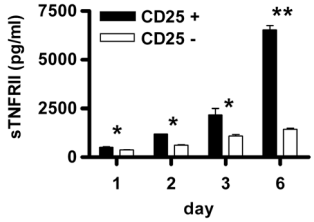

Figure 1: $CD4^+CD25^+$ T cells shed TNFRII

Levels of soluble TNFRII in cell culture supernatants. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were cultured with anti-CD3/anti-CD28 coated beads and IL-2. Supernatant was analyzed for the presence of TNFRII by ELISA (* $P<0.05$, ** $P=0.0001$). Depicted is one representative experiment out of 3.

Although sTNFRs were also detected in cultures of activated $CD4^+CD25^-$ cells, this T cell subset produced far less sTNFR. $CD4^+CD25^-$ T cells proliferate more vigorously than $CD4^+CD25^+$ Treg cell populations, resulting in 8-10 times higher cell numbers after 6 days of culture. When corrected for cell numbers, $CD4^+CD25^+$ Treg cells produced approximately 50 times more sTNFR than their $CD4^+CD25^-$ counterparts on a per cell basis (data not shown).

CD4+CD25+ Treg cell-derived sTNFRII inhibits the action of TNF-α in vitro

We next wished to examine the biologic activity of Treg cell-derived sTNFRII. For this purpose we performed a bioassay using TNF-α sensitive WEHI cells[12]. Survival of WEHI cells was measured after incubation with ranging amounts of rTNF-α in the presence or absence of culture supernatants derived from activated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Supernatant from $CD4^+CD25^-$ T cells induced ~ 50% WEHI cell death without addition of rTNF-α, reflecting increased shedding of TNF-α by activated

$CD25^-$ effector T cells as compared to $CD4^+CD25^+$ Treg cells. TNF-α-induced death of WEHI cells was largely prevented, however, when, next to titrated amounts of rTNF-α, culture supernatant of $CD4^+CD25^+$ Treg cells was added to the wells. (Figure 2A). To confirm that the inhibition of cell-death was indeed mediated by sTNFRII, we next isolated $CD4^+CD25^+$ Treg cells from TNFRII KO mice. No inhibition of cell-death was observed after addition of culture supernatant from activated $CD4^+CD25^+$ cells derived

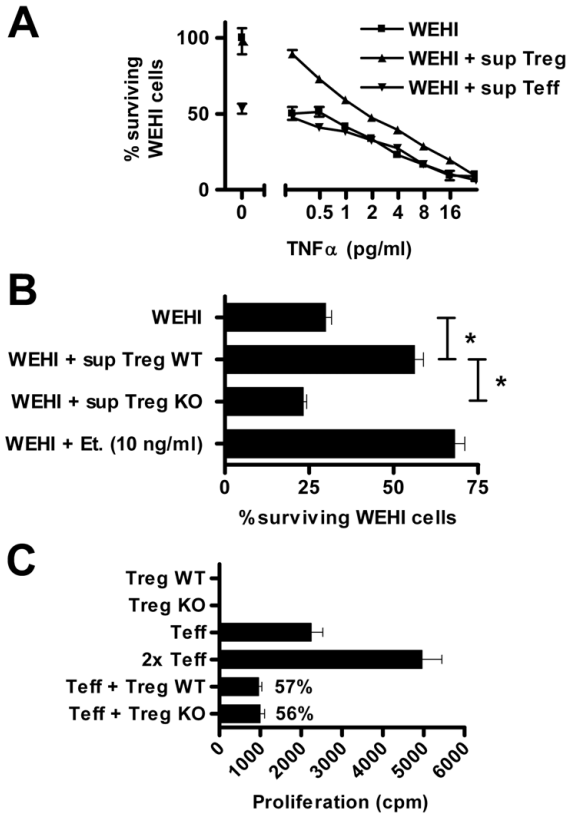

Figure 2: Biological activity of Treg cell-derived sTNFRII.
(A) sTNFRII in the supernatant of $CD4^+CD25^+$ T cell cultures can prevent TNF-α induced cell death. $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were activated in vitro for 4 days. Supernatant was added to cultures of WEHI cells in the presence of varying amounts of rTNF-α. Depicted is the percentage of WEHI cells surviving the culture for 20 hours. (B) TNFRII shed by Treg cells is required to prevent TNF-α induced cell death. $CD4^+CD25^+$ cells from naïve WT or TNFRII-KO mice were isolated and supernatant of cell cultures was used in the WEHI cell bioassay in the presence of 0.5 pg/ml rTNF-α (* $P<0.001$; Et. = Etanercept). Representative data from 11 independent experiments are shown in (A) and (B). (C) $CD4^+CD25^+$ cells from TNFRII KO and WT mice are equally capable of suppressing effector T cells. A conventional T cell suppression assay was performed as control for Treg activity using $CD4^+CD25^+$ T cells from WT or TNFRII KO mice as suppressor cells. Percentages depict percent inhibition of proliferation. Representative data from 9 experiments are shown.

from TNFRII KO mice (Figure 2B). Nonetheless, these cells were at least as potent as $CD4^+CD25^+$ cells from control WT animals in a conventional *in vitro* T cell suppression assay, indicating that $CD4^+CD25^+$ cells from TNFRII KO animals were bona-fide Treg cells with the ability to suppress effector T cells (Figure 2C). Addition of Etanercept, a soluble TNFRII-Ig fusion protein used clinically to treat rheumatoid arthritis, had comparable effects on the survival of WEHI cells (Figure 2B). These data indicate that the ability of Treg cells to inhibit T cell proliferation is unaffected in TNFRII KO mice and further show that Treg cell-derived sTNFRII is functional as it is able to prevent the action of TNF-α *in vitro*.

$CD4^+CD25^+$ Treg cell-derived sTNFRII modulates LPS-induced IL-6 production in vivo

TNF-α modulates the kinetics of IL-6 expression following LPS-injection in mice [13,14]. IL-6, in turn, induces the expression of acute phase proteins. Thus, we reasoned that the reduction of the acute phase response observed previously [7] could be due to Treg cell derived sTNFRII. To analyze this possibility, we injected mice i.p. with LPS one hour after injection of $CD4^+CD25^+$ Treg cells from either WT or TNFRII KO mice. Serum IL-6 levels were analyzed at different time-points following LPS-injection.

At 4 and 6 hours after LPS-injection, treatment with Etanercept significantly reduced the amount of IL-6 produced in response to LPS, confirming that, indeed, TNF-α is involved in the induction of IL-6 following LPS injection (Figure 3). Likewise, adoptive transfer of $CD4^+CD25^+$ Treg cells isolated from WT animals significantly decreased

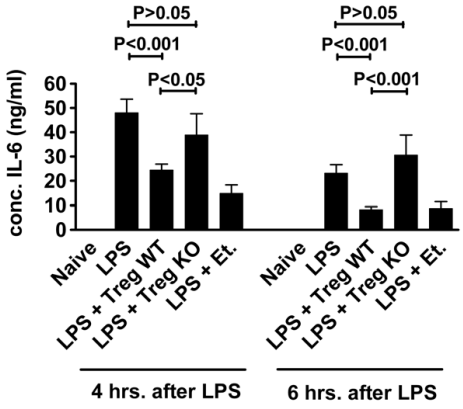

Figure 3: Treg cells inhibit LPS-induced IL-6 production through TNFR-shedding.
$CD4^+CD25^+$ cells were isolated from either WT animals or TNFRII-KO animals and activated in vitro for 4 days as described. $1x10^6$ cells were injected into mice receiving 150 µg of LPS i.p. one hour later. Serum IL-6 levels were determined 4 and 6 hours after LPS-injection. One representative experiment out of 4 is shown (Et. = Etanercept).

IL-6 production. In contrast, $CD4^+CD25^+$ Treg cells isolated from TNFRII KO animals lacked this ability, indicating that Treg cell derived TNFRII is involved in the inhibition of the IL-6 response following injection of LPS. Together, these data show that $CD4^+CD25^+$ Treg cells can inhibit the action of TNF-α and dampen inflammation by releasing sTNFRII.

TNFR-shedding by human $CD4^+CD25^{high}$ T cells

Given the potential implications of our findings with murine Treg for the treatment of TNF-mediated inflammatory disorders, we next investigated whether TNFR-shedding would also be a feature of human Treg.

We first analyzed TNFRII-expression on freshly isolated human $CD4^+$ T cells. TNFRII-expression was found to be highest on $CD4^+CD25^{high}$ T cells, but could also be detected on $CD4^+CD25^{intermediate}$ and on a minor fraction of $CD4^+CD25^-$ T cells (Figure 4A). In line with this, TNFRII-expression was highest on, but not exclusively confined to, $CD4^+FoxP3^+$ T cells. Phenotypic analysis using markers relevant for T cell function revealed that $CD4^+TNFRII^+$ T cells were largely $CD45RO^+CD45RA^-$ (Figure 4B). While $CD4^+TNFRII^-$ T cells uniformly expressed CCR7, no HLA-DR and were mostly $CD62L^{high}$, $CD4^+TNFRII^+$ T cells exhibited a more heterogeneous expression of these markers. Interestingly, FoxP3-expression was restricted to $TNFRII^+$ T cells. Thus,

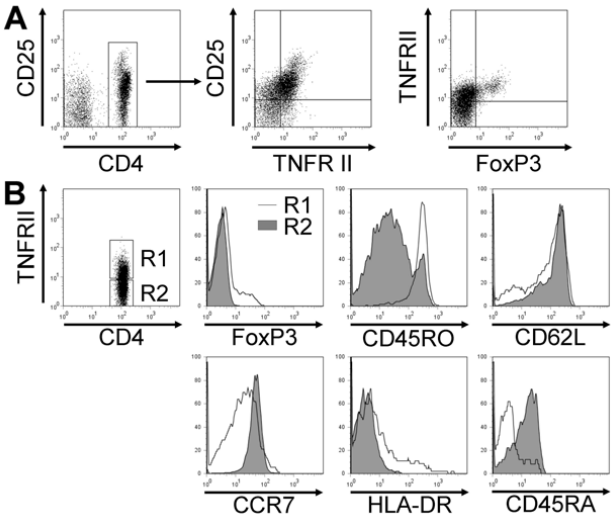

Figure 4: Phenotype of TNFRII-expressing human $CD4^+$ T cells.
(A) Cell surface expression of TNFRII on freshly isolated human PBMC from healthy donors. TNFRII expression is highest on $CD4^+CD25^{high}$ cells that coexpress FoxP3. (B) Phenotypical analysis of $CD4^+TNFRII^+$ (open line) vs. $CD4^+TNFRII^-$ (filled line) T cells. Gating was based on an appropriate isotype control. Data are representative of 4 independent experiments.

$CD4^+CD25^{high}FoxP3^+$ T cells that display both effector and central memory markers [15] constitutively express TNFRII and have in part lost expression of homing receptors CD62L and CCR7.

Similar to the experiments performed in mice, $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were subsequently purified by FACS-sorting and activated *in vitro* for up to 5 days. TCR-stimulation induced strong upregulation of TNFRII surface expression on $CD4^+CD25^{high}$ and, to a lesser extent, on $CD4^+CD25^-$ T cells during 5 days in culture (Figure 5A). Cultures were performed in the presence or absence of marimastat, an inhibitor of metalloproteinases such as TNF-α converting enzyme (TACE, ADAM17), the enzyme responsible for cleavage of TNFRs from the cell surface. Inhibition of TNFR-shedding by marimastat led to a strong accumulation of TNFRII on the cell surface of $CD4^+CD25^{high}$ T cells as determined by an increase in mean fluorescence intensity (MFI) of the TNFRII-staining. Activation of $CD4^+CD25^-$ T cells under the same conditions, however, led to an only weak increase in TNFRII-MFI, indicating lower shedding activity. In line with this observation, large amounts of sTNFRII were detectable in culture supernatants of $CD4^+CD25^{high}$ T cells, with much lower levels being produced by $CD4^+CD25^-$ T cells (Figure 5B). sTNFRI was almost undetectable in cultures of both cell types (data not shown). The difference in TNFRII-levels was even more prominent when adjusting TNFRII-levels in supernatants for cell counts, taking into account the stronger proliferation of $CD4^+CD25^-$ T cells. In addition, calculation of the amount of TNFRII shed from day to day revealed that shedding activity of $CD4^+CD25^-$ T cells reached a peak between day 3 and 4, whereas TNFRII-shedding of $CD4^+CD25^{high}$ T cells still increased (Figure 5C). It is unlikely that the sTNFRII-levels determined originate from activated effector T cells contaminating the $CD4^+CD25^{high}$ T cell population, as FACS-sorting and subsequent activation of $CD4^+CD25^{intermediate}$ T cells led to substantially lower amounts of sTNFRII in culture supernatants than activation of $CD4^+CD25^{high}$ T cells (data not shown). Interestingly, surface staining with HLA-DR of $CD4^+CD25^{high}$ T cells from two donors activated in the presence or absence of marimastat revealed that $CD4^+CD25^{high}HLA\text{-}DR^+$ T cells had substantially higher shedding capacity than $CD4^+CD25^{high}HLA\text{-}DR^-$ T cells (Figure 5D). Within the Treg cell compartment, HLA-DR has previously been described to define a population with enhanced suppressive ability. Our data indicate that the TNFR-shedding capacity differs between these two subsets. This might be relevant for the *in vivo* function of Treg cells and further emphasizes that human Treg cell populations are composed of functionally distinct subsets [16].

In summary, we here describe a new mechanism of action of $CD4^+CD25^+$ Treg cells. Our results are in line with recent observations describing that TNF-α can transiently silence the suppressive activity of Treg cells through signaling via TNFRII [17, 18]. Interestingly, suppressive function was found to be restored after several days [18]. Our findings

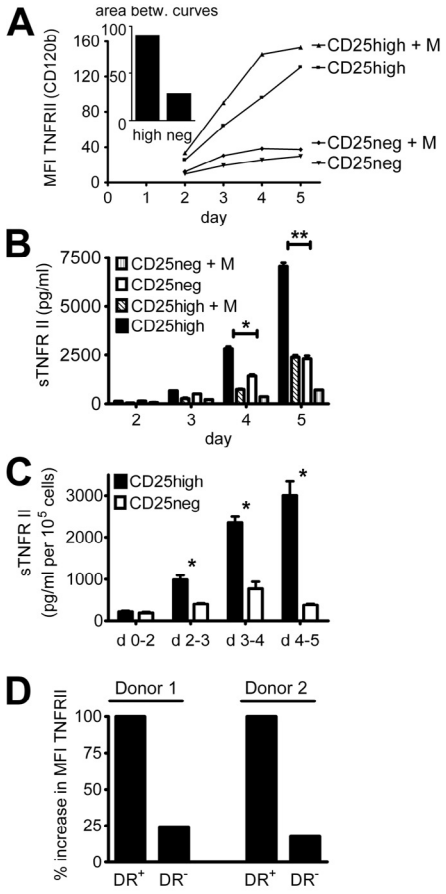

Figure 5: Characteristics of TNFRII-shedding by human $CD4^{+}CD25^{high}$ T cells.
(A) Mean Fluoresence Intensity (MFI) of TNFRII-staining after culture in the presence or absence of marimastat (Mst.). The inset depicts the increase in MFI on marimastat-treated cells $CD25^{high}$ or $CD25^{-}$ cells as the area between the respective curves. (B) Levels of sTNFRII in supernatants of cells after activation (* $P<0.05$, ** $P<0.01$). (C) Levels of sTNFRII produced from day to day adjusted for cell count. Cells were manually counted at each time point in duplicates. The amount of sTNFRII produced from one day to the next was divided by the average cell number present in the wells at these time points using the formula: (conc. sTNFRII day_{x+1} – conc. sTNFRII day_{x}) / (1/2 * (cell-count ($x10^5$) day_{x} + cell count ($x10^5$) day_{x+1})) (* $P<0.05$). Results presented in A-C are representative of 5 independent experiments. D) Increase in MFI of TNFRII-staining on $CD4^{+}CD25^{high}$HLA-DR^{+} and $CD4^{+}CD25^{high}$HLA-DR^{-} T cells from two separate donors after 5 days of activation in the presence and absence of marimastat. Increase in MFI of HLA-DR^{+} cells was normalized to 100 in order to account for overall differences in HLA-DR expression levels between donors.

showing that TNFRII is shed several days after activation of Treg cells fit well with these observations, as shedding of TNFRII would allow Treg cells to counteract the action of TNF-α, thereby circumventing its inhibitory effect on Treg cell function. This way, Treg

cells could regain their suppressive ability to regulate the function of effector T cells and, at the same time, suppress the effects of TNF-α, a crucial mediator of acute and chronic inflammation.

We have previously shown that $CD4^+CD25^+$ T cells can be used effectively in the treatment of collagen induced arthritis (CIA), a model for systemic arthritis in mice [7, 19]. CIA is primarily an antibody driven disease [20, 21], the role of T cells is, most likely, restricted to the provision of help to B cells that produce collagen type II (CII) specific antibodies. As $CD4^+CD25^+$ Treg cells are able to reduce arthritis severity in the effector phase of the disease without affecting circulating anti-CII antibodies, it is likely that the shedding of sTNFR by adoptively transferred Treg cells is involved in the inhibition of arthritis.

Finally, our data obtained with human Treg cells indicate that the mechanism we show in mice is essentially similar in the human setting. Although constitutive TNFRII-expression is not confined to Treg cells, human $CD4^+CD25^{high}$ T cells exhibit a much stronger and more sustained shedding activity upon activation when compared to $CD4^+CD25^-$ T cells. This is of potential interest in the context of TNF-mediated autoimmune diseases such as rheumatoid arthritis, in which TNFRII-Ig fusion proteins are used effectively in therapeutic settings. Together, these data provide a rationale for the therapeutic use of Treg cells in systemic autoimmune diseases.

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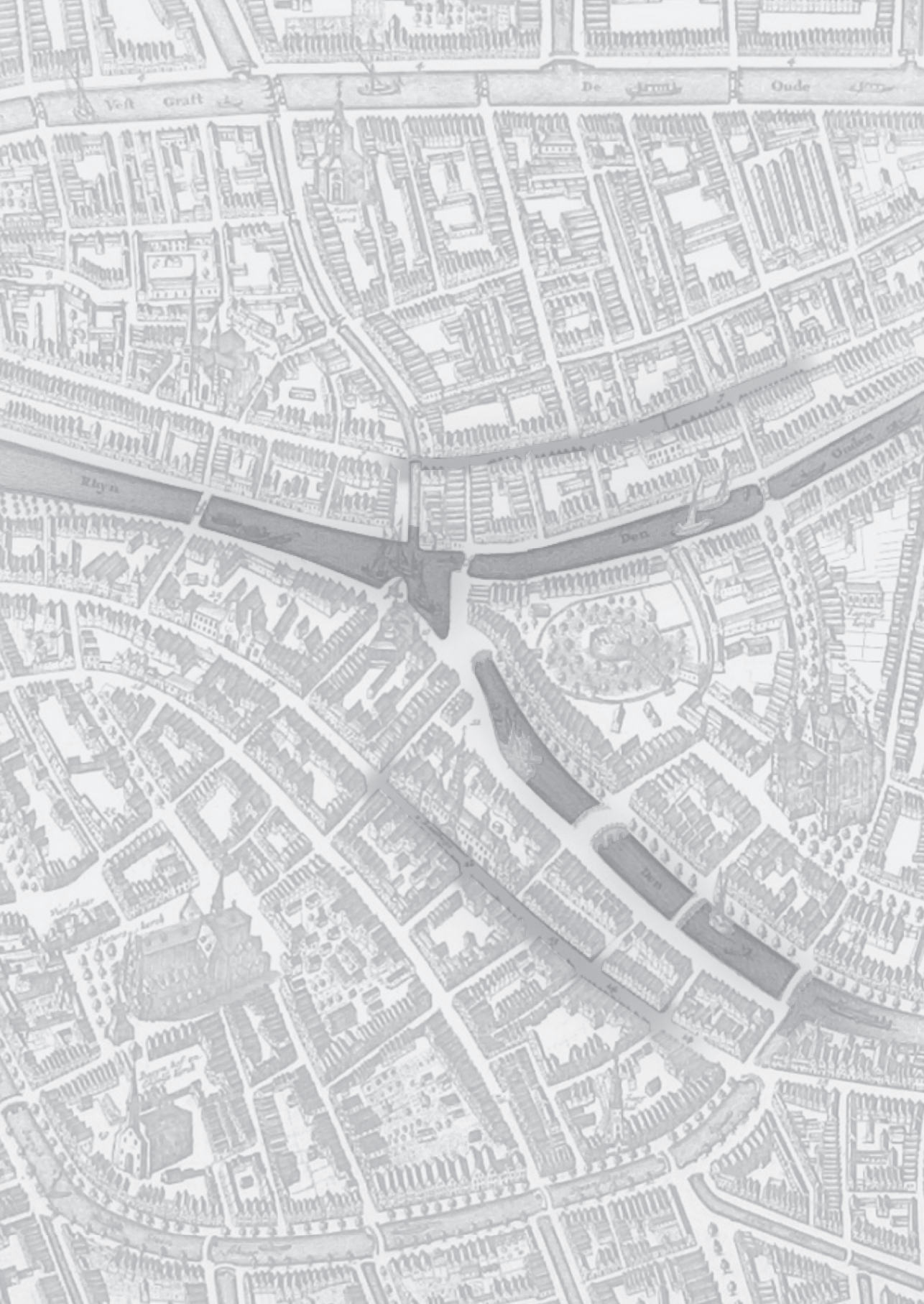
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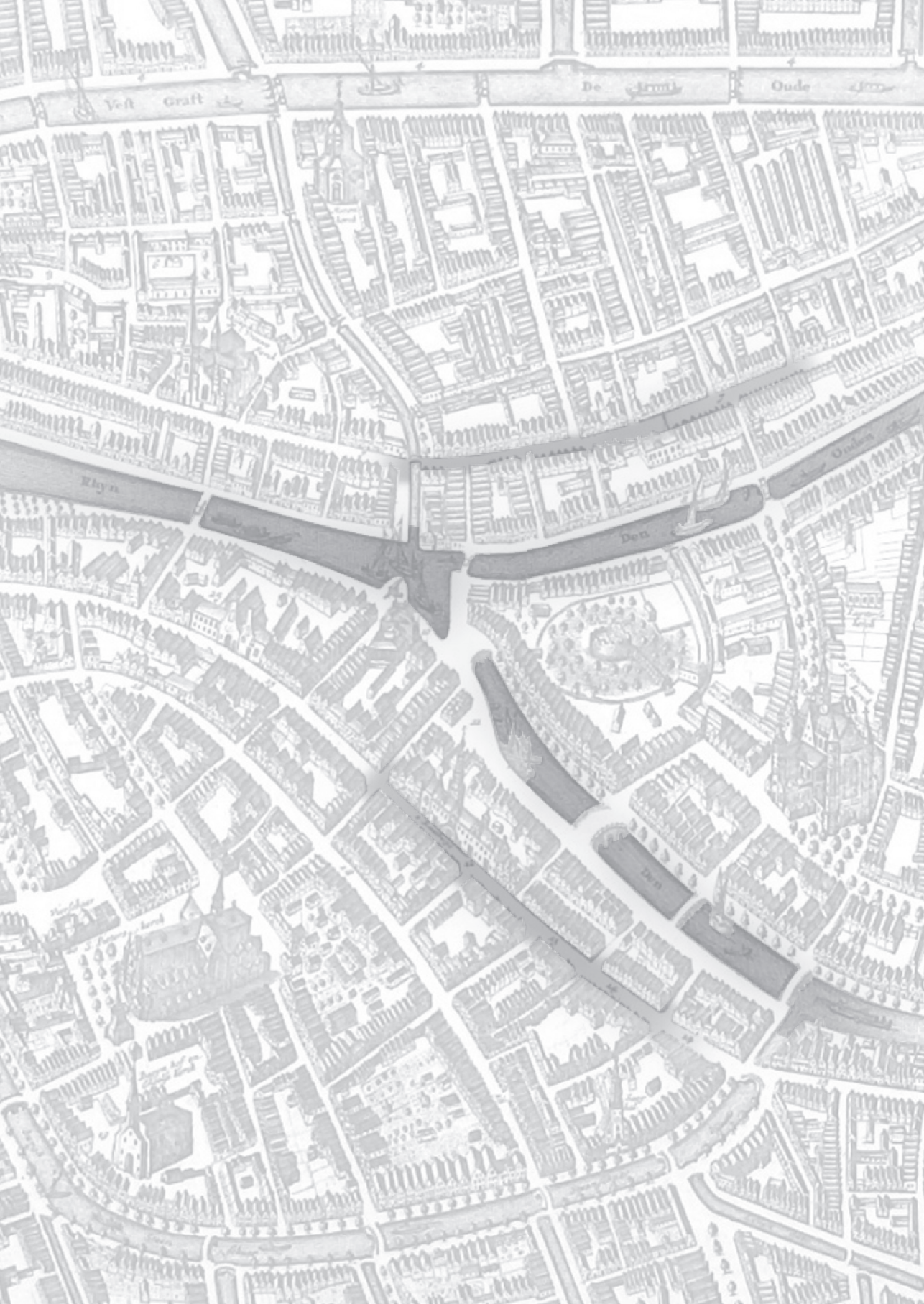
Part II

Characteristics of the immune response to citrullinated antigens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Chapter 3

Immunoglobulin 1 (IgG1) Fc-glycosylation profiling of anti-citrullinated peptide antibodies from human serum.

Scherer HU, Wang J, Toes RE, van der Woude D, Koeleman CA, de Boer AR, Huizinga TW, Deelder AM, Wuhrer M.

Proteomics Clin Appl. 2009 Jan;3(1):106-15.

ABSTRACT

In several autoimmune disorders, including rheumatoid arthritis (RA), autoantibodies are thought to be the driving force of pathogenicity. Glycosylation of the Fc-part of human immunoglobulins is known to modulate biological activity. Hitherto, glycosylation of human IgG-Fc has been analyzed predominantly at the level of total serum IgG, revealing reduced galactosylation in RA. Given the pathogenic relevance of autoantibodies in RA, we wished, in the present study, to address the question whether distinct Fc-glycosylation features are observable at the level of antigen-specific IgG subpopulations. For this purpose, we have developed a method for the micro-scale purification and Fc-glycosylation analysis of anti-citrullinated peptide antibodies (ACPA). ACPA represent a group of auto-antibodies that occur with unique specificity in rheumatoid arthritis patients. Their presence associates with increased inflammatory disease activity and rapid joint destruction. Results indicate that ACPA of the IgG1 subclass vary considerably from total serum IgG1 with respect to Fc-galactosylation, with galactosylation being higher on ACPA than on serum IgG1 for some patients, while other patients show higher galactosylation on serum IgG1 than on ACPA. Using this method, studies can be performed on the biological and clinical relevance of ACPA glycosylation within rheumatoid arthritis patient cohorts.

INTRODUCTION

Human immunoglobulin G (IgG) occurs in four subclasses, which share a conserved N-glycosylation site in their Fc region. Notably, Fc-N-glycosylation is very different from Fab N-glycosylation, the latter occurring in the variable regions of approximately 20% of the polyclonal IgG population from human serum. Fab N-glycans show a high degree of galactosylation and sialylation whilst Fc-N-glycans are predominantly non-sialylated and truncated structures (i. e., biantennary N-glycans lacking galactose residues). This difference has been found for IgGs from the human circulation [1,2] as well as for monoclonal IgGs expressed in mammalian cell culture [3-6].

Fc-glycosylation varies much between individuals: Fc-N-glycan galactosylation shows a pronounced age dependency, with a rather low degree of galactosylation for young children, a maximum of galactosylation around age 25, and a decrease in galactosylation at higher age [7-9]. During pregnancy the degree of IgG Fc-galactosylation increases and drops again after delivery [10-12], and various inflammatory diseases including rheumatoid arthritis (RA) and tuberculosis are associated with decreased galactosylation of IgG [10,12,13].

Fc-glycosylation modulates various biological activities of IgG: first, fucose-deficient IgG1 is binding with higher affinity to the Fcγ receptor IIIA than its core-fucosylated counterpart [14,15]. Sialylation does likewise influence the binding of the various IgG subclasses to different activating and inhibitory Fcγ receptors [16]. This modulation of the interaction with Fcγ receptors by IgG Fc-glycosylation features strongly influences the efficacy of IgGs in antibody dependent cellular cytotoxicity (ADCC) assays [14,17-20]. Second, in a mouse model, sialylation of the Fc-part N-glycan has been shown to be involved in the anti-inflammatory properties of human IgG [16], which seems to be at the basis of the beneficial effects of donor IgG (intravenous immunoglobuline; IVIG) for the treatment of various inflammatory and autoimmune diseases. In this study Kaneko *et al.* showed in a mouse model that a change in total IgG Fc-glycosylation characterized by an altered sialylation is underlying the pro- versus anti-inflammatory activity of antibodies [16].

Most studies on IgG glycosylation have analyzed N-glycans released from total human serum IgG, which includes Fc N-glycans and Fab N-glycans. IgG glycosylation, however, is different for the Fc-part and the Fab part [1-6], and varies between IgG subclasses [12]. Only recently, Mehta *et al.* have for the first time analyzed IgG glycosylation at an antigen-specific level [21], while all the other studies have not addressed the occurrence of possible specific glycosylation features of antigen-specific subpopulations of IgG.

In order to obtain a deeper insight into antibody glycosylation, we decided to analyze the Fc N-glycans of antigen-specific subpopulations of serum IgG. We chose for analyzing the Fc-glycosylation of anti-citrullinated peptide antibodies (ACPA), which are

autoantibodies of predominantly the IgG1 subclass that occur in RA-patients with high specificity[22-24]. For this purpose, we have established a micro-scale capturing assay for ACPA from serum followed by monitoring of Fc-glycosylation at the glycopeptide level in an IgG-subclass specific manner. Our results clearly indicate that ACPA IgG1 differs from total serum IgG1 in the degree of galactosylation of the Fc N-glycan.

MATERIALS AND METHODS

IgG purification from total human serum

Protein A-Sepharose beads (GE Healthcare, Eindhoven, The Netherlands) were washed three times with 10 volumes of PBS. 15 µl of beads per well were applied to a 96-well filter plate (Multiscreen Solvinert, 0.45 µm pore-size low-binding hydrophilic PTFE; Millipore, Billerica, MA). The volume was brought to 150 µl with PBS, and 2µl of serum was applied per well. The plate was sealed with tape and incubated on a shaker for 1 h. The beads were washed 5x with 200 µl PBS under vacuum (approximately 100 mbar). After washing 2x with 200 µl water, immunoglobulins (IgG1, IgG2, and IgG4) were eluted with 100 µl of 100 mM formic acid (p. a. for mass spectrometry; Merck, Darmstadt, Germany) into a V-bottom microtiter plate (Nunc, Roskilde, Denmark). Samples were dried by vacuum centrifugation.

Purification of anti-citrullinated peptide antibodies (ACPA)

Sera for the isolation of ACPA were chosen based on their reactivity against a second generation citrullinated peptide antigen (CCP2) in a commercially available ELISA system (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). For purification, sera of ACPA-positive and ACPA-negative RA-patients were diluted 1:10 in dilution buffer (provided with the ELISA kit) and incubated in CCP2-coated ELISA plates for 1 hour at 37°C. Supernatants were discarded and plates were washed 2x with PBS followed by 2x washing with 25 mM ammonium bicarbonate (Merck, Darmstadt, Germany). ACPA were then eluted using three different approaches:

1. Elution with sodium-isothiocyanate: After washing, samples were incubated with 100 µl 5M sodium-isothiocyanate for 15 min at room temperature. Eluates were collected from the ELISA plate and immediately dialyzed (dialysis membranes with molecular weight cut-off 12-14 kD, cat.-no. 551300; Thermo Fisher Scientific, Waltham, MA) over night at 4°C against PBS. ACPA IgG was purified from PBS using Prot A beads (see 2.1) and subjected to trypsin treatment (2.4) and Fc-glycosylation analysis (2.5).
2. In-plate tryptic digest: After washing, 30 µl of 25 mM ammonium bicarbonate buffer containing 200 ng of sequencing grade modified trypsin (Promega, Leiden, The Netherlands) was added to each well of the ELISA-plate. Plates were incubated for

30 min on a shaker at room temperature followed by over night incubation at 37°C. Samples were subjected to Fc-glycosylation analysis (2.5).

3. Elution with 100 mM formic acid: After washing, 50 µl 100 mM formic acid was added to each well to achieve antibody elution. Plates were incubated for 15 min at room temperature. Eluates were transferred to 96 well V-bottom microtiter plates, dried in a vacuum centrifuge, and subjected to trypsin treatment (2.4) and Fc-glycosylation analysis (2.5).

For comparison of the methods, the isolation procedures 2 (in-plate tryptic digest) and 3 (formic acid elution) were performed in parallel on one plate in order to avoid inter-assay variation.

Enzyme-linked immunosorbant assay (ELISA)

Detection of human IgG: Human IgG was detected using an antibody-based sandwich ELISA system. In brief, 96 well flat-bottom microtitration plates (Maxisorb; Nunc, Copenhagen, Denmark) were coated with polyclonal rabbit anti-human IgG (Dako A0423; Glostrup, Denmark) diluted 1:5000 in coating buffer (50mM sodium bicarbonate buffer pH 9,6) for 2 hours at 37°C. Plates were blocked with PBS containing 1%BSA for 30 min at 37°C. Samples and standard (pooled normal human serum) were added to the wells diluted in PBS containing 1%BSA and 0.05% Tween and incubated for 30 min at 37°C. Biotinylated goat F(ab')2 anti-human IgG diluted 1:40000 (Biosource International AHI1309, Camarillo, CA) in PBS containing 1%BSA and 0.05% Tween was used for detection. Plates were subsequently incubated with poly-horse radish peroxidase labeled with streptavidine (diluted 1:750 in PBS containing 1%BSA and 0.05% Tween) (Sanquin M2051, Amsterdam, The Netherlands) and bound IgG was visualized using 2,2'-azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma). Color signal was measured at an optical density of 415nm using a conventional ELISA reader. Plates were vigorously washed between each step with PBS containing 0.05% Tween 20. Antibody dilutions were optimized by serial titrations for detection of the standard IgG prior to performance of the assay.

Detection of ACPA: ACPA were detected using a second generation ELISA-kit (Immunoscan RA Mark 2; Euro-Diagnostica) according to the manufacturer's instructions.

IgG digestion with trypsin

A 20 µg aliquot of trypsin (sequencing grade; Promega, Leiden, The Netherlands) was dissolved in 4 ml of 25 mM ammonium hydrogencarbonate. Within 1 min after preparation, 40 µl of this mixture was added per well to the dried purified antibodies. Samples were shaken (1 min), incubated overnight at 37°C, and stored at -20°C until usage.

Nano-LC-ESI-ion trap-MS

Prior to analysis, microtitration plates containing tryptic digests of IgG were subjected to centrifugation (10 min at 3000 g). 0.2 µl aliquots of trypsinized Protein A eluates (corresponding to IgG1, IgG2, and IgG4 from 10 nl of serum) and 2 µl aliquots of trypsinized samples obtained from the various ACPA purification procedures were applied to a reverse-phase column (C_{18} PepMap 100Å, 3 µm, 75 µm x 150 mm; Dionex/LC Packings, Amsterdam, the Netherlands) using an Ultimate nano-LC, a Famos autosampler, and a Switchos trap-column system (Dionex/LC Packings). The column was equilibrated at room temperature with eluent A (0.1% formic acid in water and 0.4% acetonitrile) at a flow rate of 150 nL/min. After injection of the samples, a gradient was applied to 25% eluent B (95% acetonitrile, 5% water containing 0.1% formic acid) in 15 min and 70% eluent B at 25 min followed by an isocratic elution with 70% eluent B for 5 min. The eluate was monitored by UV absorption at 215 nm.

The LC system was coupled via an online nanospray source to an Esquire HCT ultra ESI-IT-MS (Bruker Daltonics, Bremen, Germany) equipped with an electron transfer dissociation module (PTM Discovery System™) and was operated in the positive ion mode. For electrospray (1100-1250 V), capillaries (360 µm OD, 20 µm ID with 10 µm opening) from New Objective (Cambridge, MA, USA) were used. The solvent was evaporated at 165°C employing a nitrogen stream of 7 L/min. Ions from *m/z* 600 to *m/z* 1800 were registered. For glycosylation profiling, the mass spectrometer was used in the MS mode. The HPLC method resulted in a resolution of the glycopeptides based on the peptide moiety with IgG1 glycopeptides eluting first, followed by IgG4 and IgG2 glycopeptides. Moreover, glycopeptides with neutral glycan moieties tended to elute earlier than glycopeptides with antennae sialylation, as described before [12]. For both the neutral and the acidic glycopeptides of each IgG subclass, average mass spectra were generated over a 1 min elution range.

RESULTS

Purification of anti-citrullinated peptide antibodies (ACPA) was achieved using commercially available ELISA plates which contain immobilized citrullinated peptide antigens. Sera from rheumatoid arthritis patients that were previously tested as ACPA-positive or ACPA-negative (controls) were diluted in PBS and added to the ACPA ELISA plate wells in order to allow binding to the immobilized citrullinated peptide antigens. Unbound serum IgG as well as other serum components were washed away, and IgGs bound to the ACPA plates were analyzed using three different procedures, as schematically presented in Figure 1.

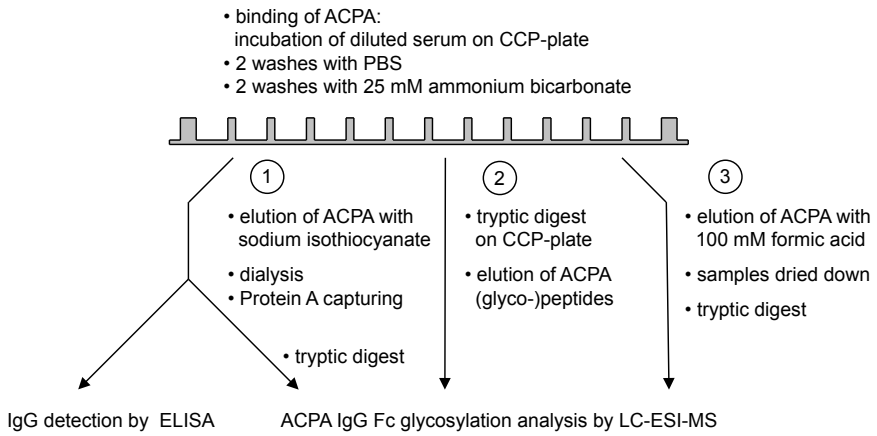


Figure 1: Schematic representation of the three strategies which were evaluated for purification of anti-citrullinated peptide antibodies (ACPA).

In a first attempt, antibodies bound to the ACPA plates were eluted using sodium isothiocyanate. For this experiment sera were used from four ACPA-positive rheumatoid arthritis patients and four ACPA-negative rheumatoid arthritis patients, as determined by ELISA (Fig. 2A). Sodium isothiocyanate was removed by dialysis against PBS, and the amount of captured IgG was quantified for the 8 sera using an IgG-ELISA (Fig. 2B). The results showed the presence of captured IgG for the 4 ACPA-positive RA patients. For the ACPA-negative RA-patients no IgG was detected after dialysis, i. e., no unspecific co-purification of non-ACPA IgGs was detected. These results indicate the successful purification of ACPA with high specificity using an affinity capturing approach at the ELISA plate format. Virtually no background level of unspecifically bound IgG was detected.

The obtained amounts of ACPA were sufficient for Fc-glycosylation analysis by nano-LC-ion trap-MS at the tryptic glycopeptide level (Fig. 3B) using a recently described method [12]. The results obtained for patient RA10 are shown in Fig. 3B. Next to ACPA IgG1 glycosylation profiles, the Fc-glycosylation profiles of IgG1 from total serum were registered after Prot A capturing and trypsin treatment (Fig. 3B). IgG1 Fc glycopeptides were identified based on their specific tryptic peptide moiety (E_{293}EQYNSTYR_{301}), which allows to distinguish them from glycopeptides of other IgG subclasses based on the resulting glycopeptide masses and elution positions [12]. Interestingly, the obtained glycosylation profile of ACPA IgG1 differed from the profile obtained for total IgG1 of the same patient (Fig. 3A): whilst total IgG1 exhibited a typical rheumatoid arthritis glycosylation profile with a low degree of galactosylation (high G0; glycoform at *m/z* 878.7), ACPA of RA10 showed a much higher degree of galactosylation, with G1 as the major glycoform (*m/z* 932.8). Moreover, sialylation was slightly higher in ACPA

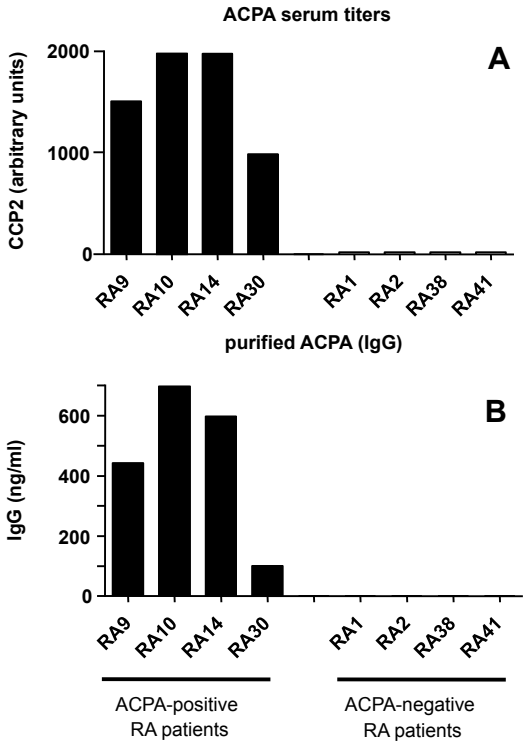


Figure 2: Demonstration of ACPA purification by ELISA.
Eight sera from rheumatoid arthritis (RA) patients were assayed for ACPA by ELISA, which revealed 4 ACPA-positive and 4 ACPA-negative RA patients (A). ACPA were purified following strategy 1 (Fig. 1), followed by the measurement of IgG in the eluates by ELISA (B). The results show that ACPA were successfully purified, and no background levels of non-ACPA IgG were detected.

compared to total serum IgG, with a relatively higher signal for the core-fucosylated biantennary monosialylated glycoform (triple protonated species at *m/z* 1083.8).

In a next step we wished to confirm that the glycosylation profiles obtained from the eluates of the capturing plate were reflecting total ACPA. We wanted to be sure that specific ACPA glycosylation features were not missed due to the possible depletion for high-affinity ACPA which might not be eluted from the capturing plate with the applied sodium isothiocyanate treatment. We addressed this point by comparing the elution profiles obtained by strategy 1 (Fig. 3B) with ACPA glycosylation profiles obtained by tryptic digest of the captured IgG1 directly on the capturing plate (strategy 2). This comparison was performed for 4 sera from ACPA-positive RA patients and for 2 sera from ACPA-negative RA patients. No IgG glycopeptides were registered for the ACPA-negative RA patients, whilst the Fc-glycosylation profiles of the ACPA-positive patients obtained by strategy 2 (on-plate tryptic digest; shown for patient RA10 in Fig. 3C) were very similar to those obtained by strategy 1 (sodium isothiocyanate elution; shown for patient RA10

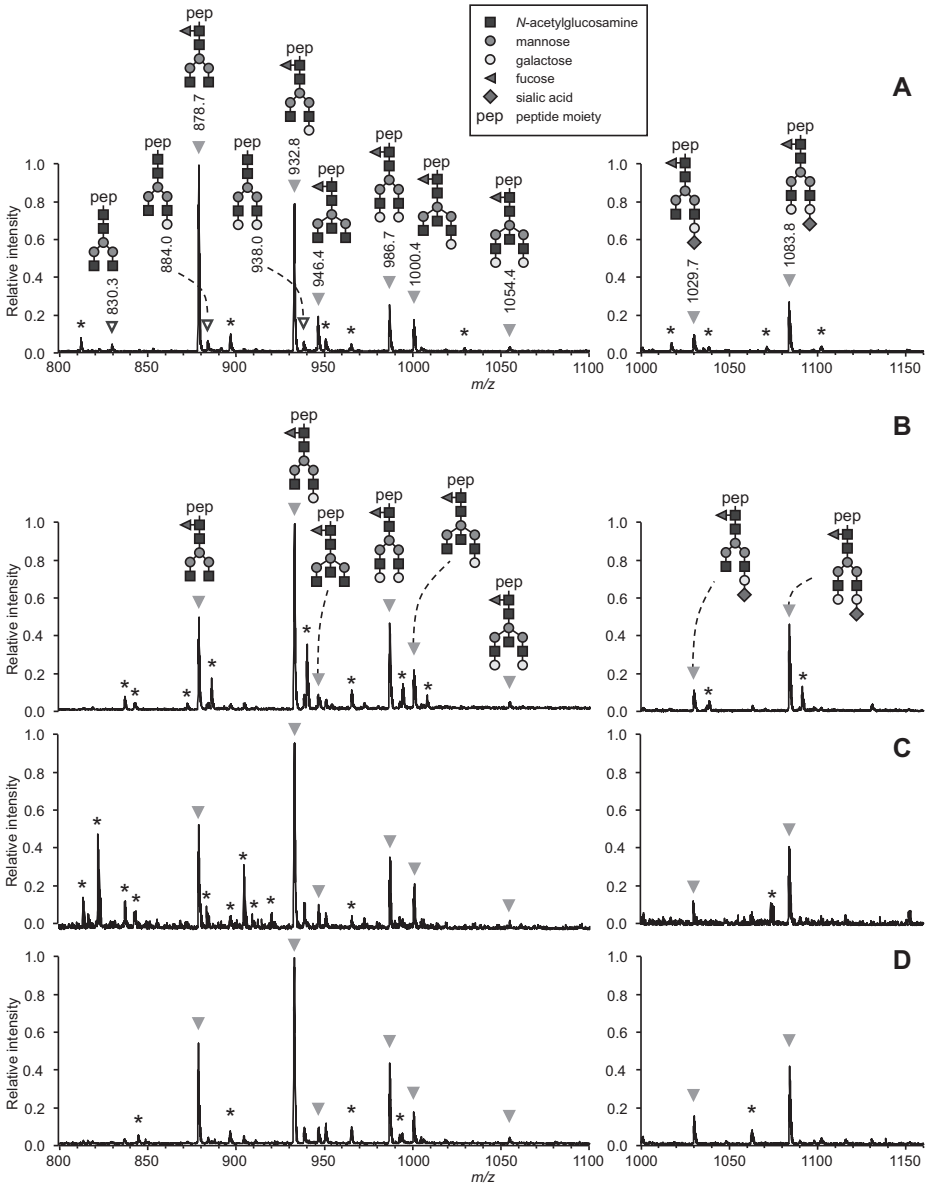


Figure 3: IgG Fc-glycosylation analysis of total serum IgG1 of patient RA10 (A) and of ACPA of the same patient purified following strategy 1 (B), strategy 2 (C), and strategy 3 (D).
Nano-LC-MS signals are shown for the glycopeptides with neutral N-glycan chains (left panels) and acidic N-glycan chains (right panels). Triple protonated signals were registered throughout. Signals representing a fucosylated glycoform are labeled with filled triangles, whilst signals of non-corefucosylated glycoforms are labeled with open triangles. *, non-glycopeptide signal or irrelevant adduct.

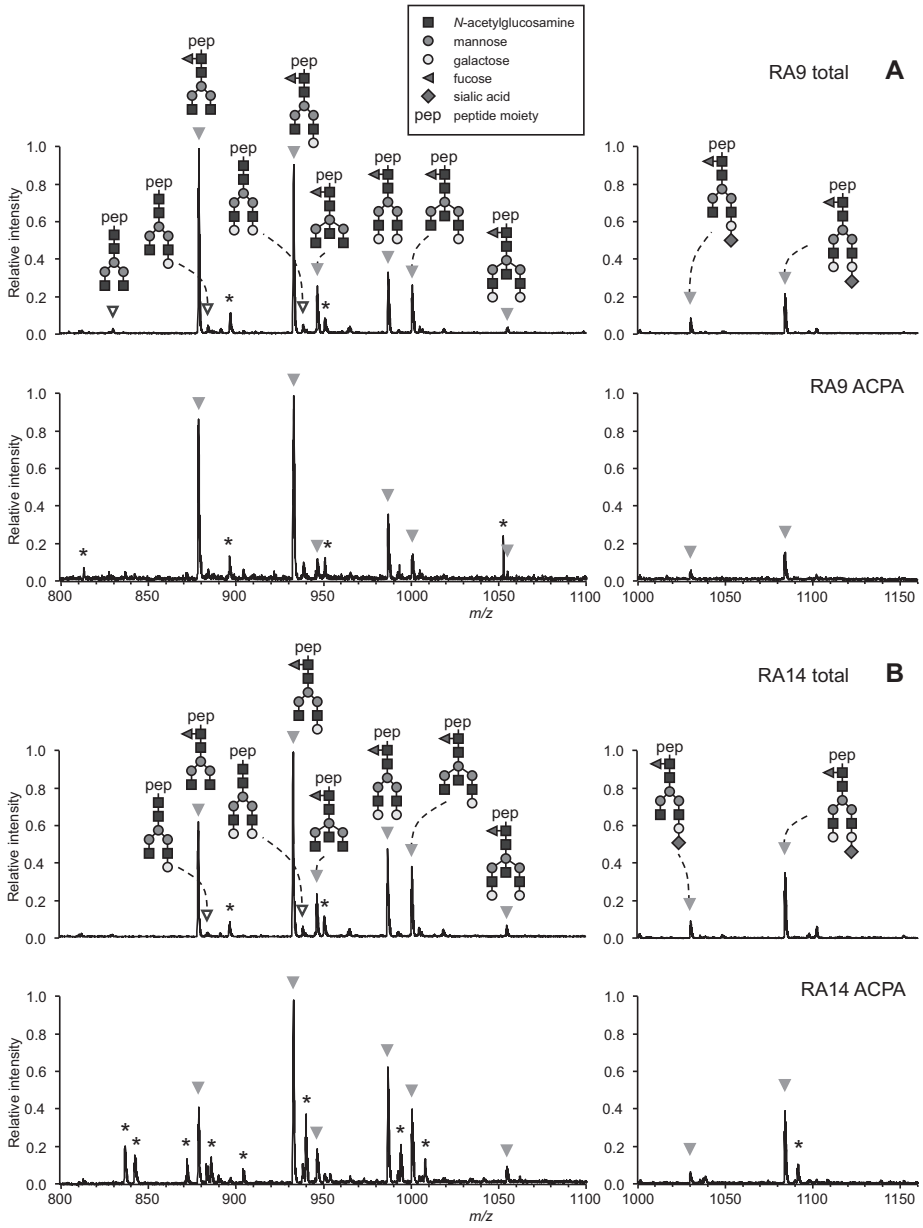
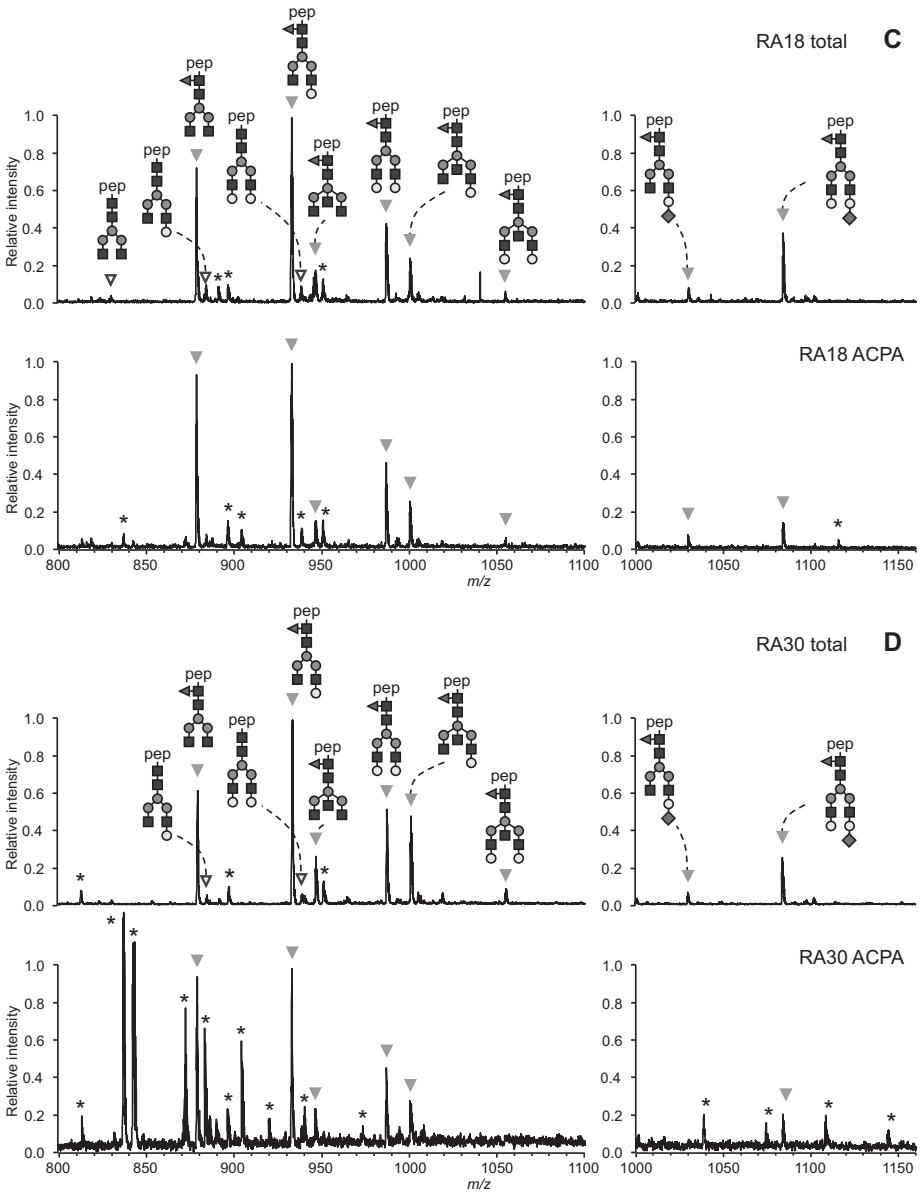


Figure 4: IgG Fc-glycosylation analysis of total serum IgG1 and ACPA IgG1 of patients RA9 (A), RA14 (B), RA18 (C), and RA30 (D).

Nano-LC-MS signals are shown for the glycopeptides with neutral N-glycan chains (left panels) and acidic N-glycan chains (right panels). Triple protonated signals were registered throughout. Signals representing a fucosylated glycoform are labeled with filled triangles, whilst signals of non-corefucosylated glycoforms are labeled with open triangles. *, non-glycopeptide signal or irrelevant adduct.



in Fig. 3B). The rationale for using strategy 2 was that all ACPA adsorbed to the capturing plate would be hydrolyzed by trypsin, and the observed glycosylation profiles would reflect with great certainty total ACPA Fc-glycosylation. Because the obtained ACPA Fc-glycosylation profiles were virtually identical, both on-plate tryptic digest and sodium isothiocyanate elution are suitable for ACPA Fc-glycosylation profiling.

While the direct tryptic treatment on the ACPA capturing plate provided a sensitive and rapid manner for ACPA Fc-glycosylation profiling, these samples showed a rather high level of background signals. Therefore, we decided to evaluate another, simple method, which involves ACPA elution with 100 mM formic acid, drying of the samples in a vacuum centrifuge, and tryptic digestion of ACPA (strategy 3, Fig. 3D). The obtained ACPA Fc-glycosylation profiles were again very similar to the one obtained following strategies 1 and 2 (shown for patient RA10 in Fig. 3D). Due to its simplicity and the good quality of the data, strategy 3 was selected for further analysis of ACPA Fc-glycosylation.

In addition, the Fc-glycosylation profiles of total IgG1 (upper panels) and ACPA IgG1 (lower panels) were analyzed for 4 additional ACPA-positive RA patients (Figure 4), which are patients RA9, RA14, and RA30 (all included in Fig. 2), as well as patient

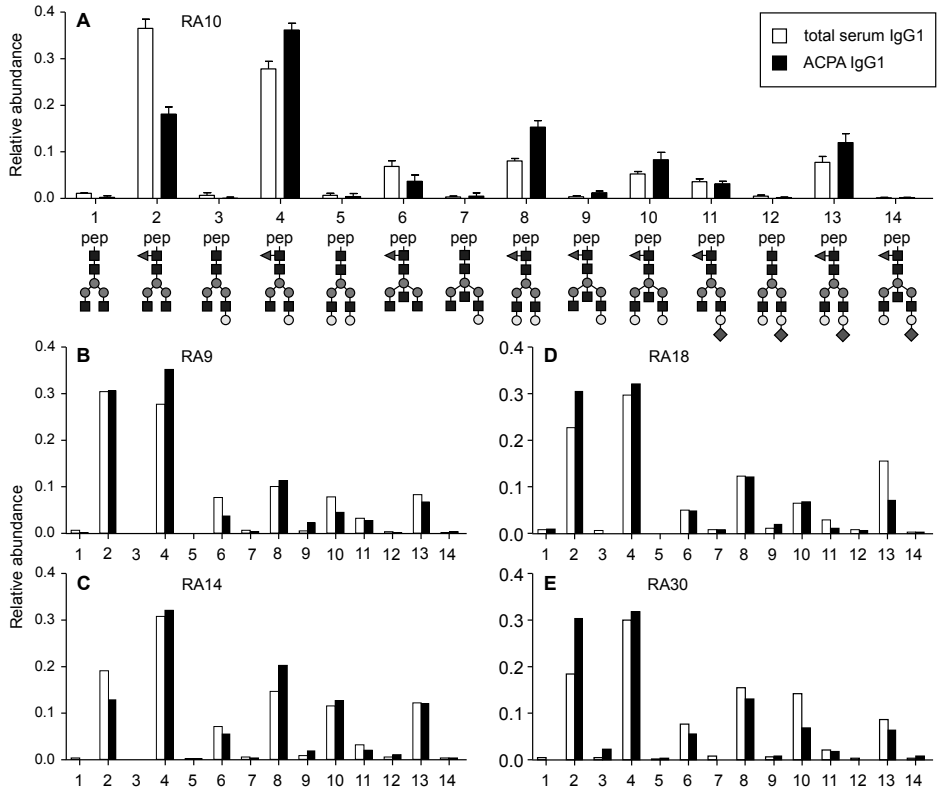


Figure 5: Relative expression levels of glycoforms of IgG1 from total serum and ACPA for 5 RA patients.

Relative intensities are given for the triple-charged ions of the various glycoforms (Fig. 3A and B). Total serum IgG1 and ACPA IgG1 for patient RA10 were measured three times and seven times, respectively, and average relative intensities as well as standard deviations are given for the fourteen glycoforms which were registered (A).

RA18 (not included in Fig. 2). The glycosylation data for these four patients are represented in histograms (Fig. 5B-E), together with the data for patient RA10 (Fig. 5A). A comparison of the total IgG1 Fc-glycosylation profiles for the five patients showed marked differences. The major differences were in the degree of galactosylation (upper panels of Fig. 4A-D). Moreover, some differences in sialylation were observed. Notably, the ACPA Fc-glycosylation profiles observed for the 4 RA patients (lower panels Fig. 4A-D) differed from the profiles obtained for total IgG1. RA10 showed a much higher degree of galactosylation for ACPA compared to total IgG1 (Fig. 3; Fig. 5A). RA9 and RA14 likewise showed a higher degree of galactosylation, though less prominent. RA30, in contrast, showed a rather high degree of galactosylation for total IgG1 and a much lower galactosylation of ACPA. ACPA of RA18 likewise showed a lower degree of galactosylation than total serum IgG1 of RA18. Next to the differences in galactosylation, some small variations in sialylation were observed between ACPA and total serum IgG1.

In conclusion, ACPA Fc-glycosylation profiles can be monitored efficiently with the protocol established in this study. The observed glycosylation patterns show differences in galactosylation and sialylation between individual RA patients. Moreover, these first analyses indicate that ACPA glycosylation may be very different from total IgG1 glycosylation.

DISCUSSION

In several autoimmune disorders autoantibodies are thought to be of crucial pathogenetic relevance. Understanding their mode of action might greatly enhance our perception of disease pathogenesis. We here describe an assay which allows the detailed analysis of IgG Fc-glycosylation at the level of autoantigen-specific IgG subpopulations from human serum. The assay makes use of a commercially available ELISA plate with covalently attached citrullinated peptide antigens, which allows the capturing of anti-citrullinated peptide antibodies (ACPA). ACPA are auto-antibodies that are often of the IgG1 subclass and are highly specific for rheumatoid arthritis (RA)[22-24]. ACPA are continuously produced by B-cells, and have been implicated in disease pathogenesis[25,26]. Antibodies are eluted from the capturing plate using a low pH step, followed by trypsin cleavage and profiling of the Fc glycopeptides by nano-LC-ESI-ion trap-MS. This method was found to be highly specific, as no background IgG signals could be detected for RA-patients which were judged to be ACPA-negative on the basis of ELISA results. First results obtained for a limited set of ACPA-positive RA patients indicate differences between ACPA IgG1 Fc-glycosylation and total IgG1 Fc-glycosylation. In particular, ACPA IgG1 appear to be remarkably different from total serum IgG1 with respect to galactosylation, with some patients showing higher galactosylation for ACPA than for

total serum IgG1 and other patients showing higher galactosylation on serum IgG1 than on ACPA. Moreover, some difference between ACPA and total serum IgG1 was found in the degree of sialylation. The latter difference may possibly represent a secondary effect: in the IgG glycosylation process in the Golgi, the attachment of less galactoses means less acceptor structures for sialyl transferases, which may explain the reduced sialylation to a large extent. Notably, for fucose and sialic acid, which are known to exhibit acid-labile glycosidic bonds, no hydrolysis was observed with the applied elution conditions: for a monoclonal antibody, no differences were observed in the degree of Fc glycopeptide sialylation and fucosylation with and without treatment with 100 mM formic acid followed by vacuum centrifugation (data not shown). Hence, the registered values for fucosylation and sialylation are not expected to be influenced by hydrolysis during sample preparation.

ACPA Fc-glycosylation does not appear to feature a particularly high percentage of G0 glycoforms. Hence, the high degree of G0 found for IgGs of many RA patients does not seem to be caused by a very high degree of G0 on ACPA. It may be speculated, therefore, that the low degree of Fc-galactosylation in IgG of RA patients may occur rather on a broad population of IgGs with various antigen specificities and might not be the result of particularly high G0 of RA-associated autoantibodies. However, to be able to draw firm conclusions on the specific features of ACPA glycosylation and its biological and clinical information content, ACPA Fc-glycosylation will have to be analyzed in future studies within large clinical cohorts.

The establishment of a sensitive method for the analysis of ACPA Fc-glycosylation is an important step towards analyzing and understanding IgG glycosylation at an antigen-specific level. With the large variation shown in these first analyses, ACPA fulfill an important prerequisite for a biomarker candidate (large spreading of values which reflects information content). Analyses in various clinical RA cohorts will be performed to evaluate the diagnostic potential of ACPA glycosylation and reveal whether it indeed represents a marker for disease activity or disease progression / prognosis.

Recently Metha *et al.*[21] have analyzed IgG heavy chain glycosylation of anti-alpha-Gal IgGs from serum of hepatitis C virus-infected persons with fibrosis and cirrhosis and have found increased levels of galactose-deficient glycoforms (IgG-G0) compared to anti-alpha Gal IgGs from healthy controls. Anti-Gal IgGs and ACPA are hitherto the only antigen-specific IgGs studied for Fc-glycosylation. Efforts should be made to analyze the glycosylation of IgGs directed against other auto-antigens, xeno-antigens, vaccines, and infectious agents. For this purpose more assays for the Fc-glycosylation analysis of other antigen-specific IgG subpopulations will have to be established in the future. The application of these assays to large study populations should provide a deeper insight into the complexity and regulatory potential of IgG glycosylation at the antigen-specific level, which may represent important, hitherto neglected, immunological parameters. Based on

the results of Kaneko *et al.* [16], it will be interesting to compare Fc-glycosylation profiles for different phases of antibody responses and B-cell development. Next to population studies, more functional studies in animal models will be necessary to understand the regulatory events which make B-cells produce antibodies with a certain Fc-glycosylation profile. In addition, the effects of the differences in IgG Fc-glycosylation on ADCC and complement-mediated cytotoxicity need further attention. These combined efforts should provide us with a deeper understanding of antibody-mediated immunological effects and may reveal new rationales for modulating the immune response in various diseases.

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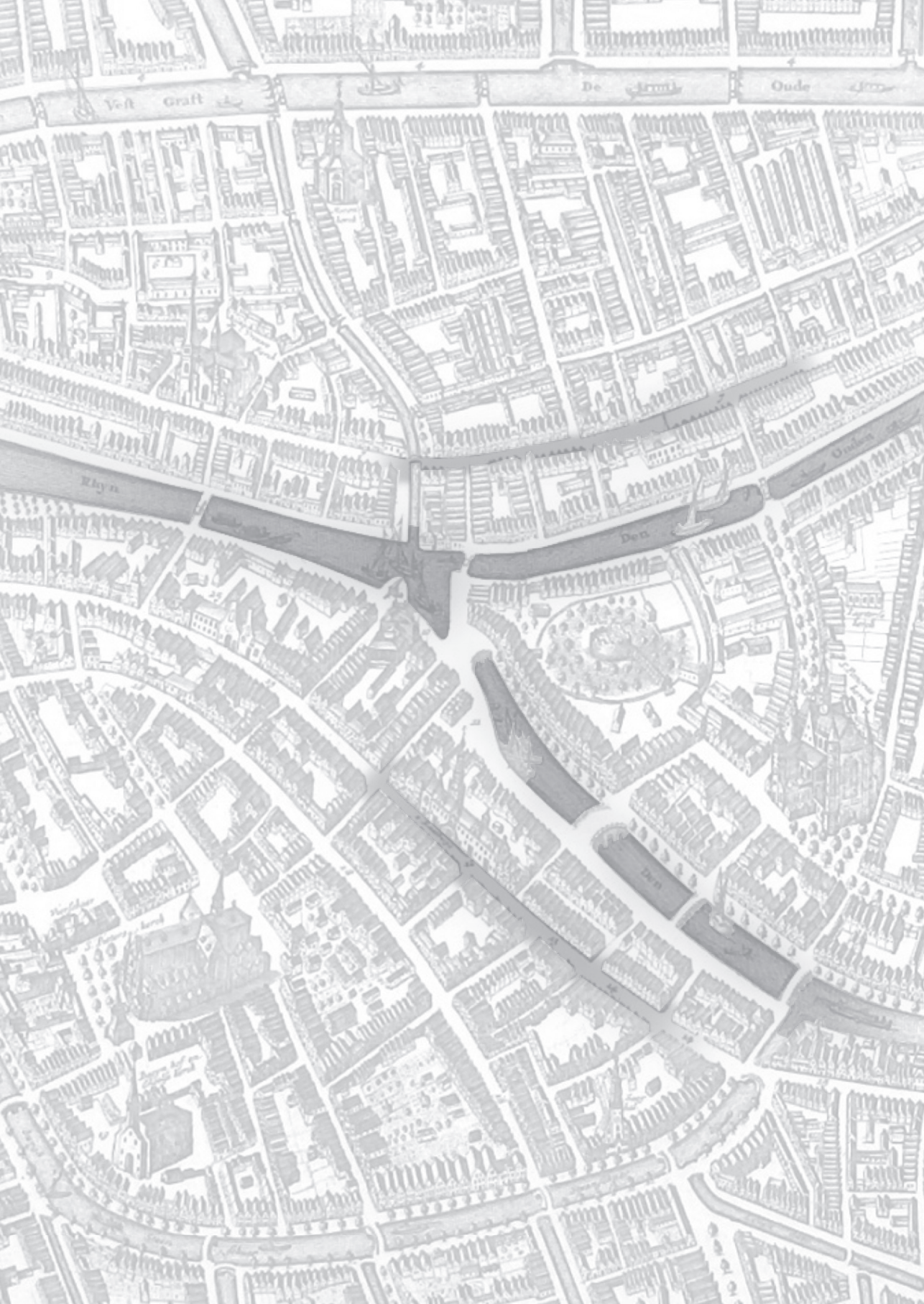
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Chapter 4

Glycan profiling of anti-citrullinated protein antibodies isolated from human serum and synovial fluid.

Scherer HU, van der Woude D, Ioan-Facsinay A, el Bannoudi H, Trouw LA, Wang J, Häupl T, Burmester GR, Deelder AM, Huizinga TW, Wuhrer M, Toes RE.

Arthritis Rheum. 2010 Jun;62(6):1620-9.

ABSTRACT

Objective: Anti-citrullinated protein antibodies (ACPA) exhibit unique specificity for RA. Whether and how ACPA contribute to disease pathogenesis, however, is incompletely understood. The Fc part of human IgG carries two N-linked glycan moieties which are crucial for the structural stability of the antibody and modulate its binding affinity to Fcγ receptors and its ability to activate complement. We have purified ACPA from serum and synovial fluid and analyzed Fc glycosylation profiles in a specific manner.

Methods: ACPA were isolated by affinity purification using cyclic citrullinated peptides as antigen. IgG_1 Fc glycosylation was analyzed by mass spectrometry. ACPA glycan profiles were compared to glycan profiles of total serum IgG_1 obtained from 85 well-characterised patients. Glycan profiles of paired synovial fluid and serum samples were available from 11 additional patients.

Results: Compared to the pool of serum IgG_1, ACPA IgG_1 lack terminal sialic acid residues. In synovial fluid, ACPA are highly agalactosylated and lack sialic acid residues, a feature that was not detected for total synovial fluid IgG_1. Moreover, differential ACPA glycan profiles were detected in RF-positive versus -negative patients.

Conclusion: ACPA IgG_1 exhibit a specific Fc-linked glycan profile which is distinct from total serum IgG_1. Moreover, Fc glycosylation of ACPA differs markedly between synovial fluid and serum. As Fc glycosylation directly affects the recruitment of Fc-mediated effector mechanisms, these data could further our understanding of the contribution of ACPA to disease pathogenesis.

INTRODUCTION

Antibodies relevant to tissue pathology in autoimmune diseases are identified based on antigen binding specificity of the variable region. Only very few autoantibodies, however, mediate pathology by direct interaction with the antigen. In most other cases, the constant region (Fc part) determines antibody-mediated effector functions such as complement activation, antibody-dependent cellular cytotoxicity (ADCC) and engagement of activating or inhibitory Fc receptors. These Fc-mediated effects are influenced by the Fc part's amino acid sequence (i.e. antibody isotype and sub-class) and by Fc-linked carbohydrate structures. The latter are located in the Cγ2 domain of the heavy chain in close vicinity to amino acids that interact with Fc receptors and the complement system. Accordingly, Fc-linked carbohydrate structures have recently received increasing attention, as modification of Fc-linked glycan residues of therapeutic antibodies has been shown to strongly influence the antibodies' therapeutic potential [1-6].

The Fc part of human immunoglobulin G (IgG) carries two N-linked, highly heterogeneous glycan moieties interposed between the heavy chains. These carbohydrate structures are crucial for the stability of the antibody and influence its biological activity [7-9]. Attached to amino acid Asn297 of each heavy chain is a conserved, biantennary glycan backbone of N-acetylglucosamine (GlcNAc) and mannose residues, which is modified by core fucose, additional N-acetylglucosamine, galactose and terminating sialic acid residues to yield a large variety of glycoforms (Figure 1). The most prevalent glycoforms

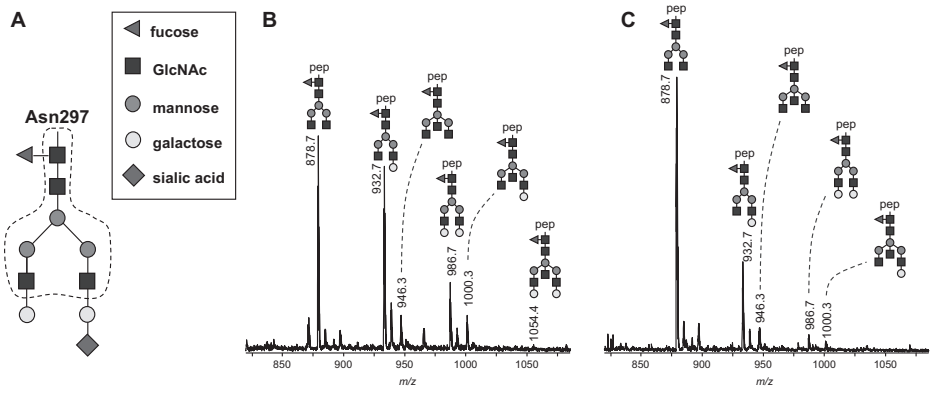

Figure 1: (A) Schematic depiction of a monosialylated glycan chain linked to Asn297 of the IgG heavy chain and legend for symbols used in this article (box). The dotted line indicates the conserved sequence of glycan residues. An additional sialic acid residue can be attached to the second galactose residue, and an additional N-acetylglucosamine residue ('bisecting GlcNAc') to the central mannose (not depicted). (B, C) Typical mass-spectrometry profiles of IgG_1 tryptic glycopeptides obtained after isolation of ACPA from serum (B) and synovial fluid (C). Sum mass spectra of the elution range of the IgG_1 glycopeptides with neutral glycans are shown [34]. pep: peptide moiety.

are characterized by presence or absence of galactoses, so called G0 (no galactose), G1 (one galactose) and G2 (two galactoses) glycoforms, which make up around 20-35%, 35% and 16% of all IgG Fc-linked glycoforms in healthy subjects, respectively[9]. Lack of galactose residues results in a concomitant lack of terminal sialic acid residues and increases the affinity for activating Fcgamma receptors (FcγR)[10,11]. In contrast, presence of the fully processed glycan structure terminating in sialic acid residues has anti-inflammatory effects[3,4]. Presence of an additional, bisecting GlcNAc residue and absence of core fucose residues was found to enhance ADCC through high-avidity interaction with FcγRIIIa[1,12-14]. Hydrolysis of the entire glycan backbone by glycosidase treatment or mutational deletion of the entire Asn297 N-linked glycosylation site leads to a change of the Fc part's quartenary structure. This yields a non-immunogenic antibody unable to interact with FcγR and, consequently, to elicit significant cytokine release[5,6,8]. Of interest, immune complexes containing such aglycosylated IgG fail to be eliminated rapidly from the circulation[15].

In line with these structural observations, both human and murine studies indicate a strong impact of Fc-linked glycan variants on the pathogenic potential of IgG antibodies in inflammatory disorders such as rheumatoid arthritis. Early studies have demonstrated a predominance of IgG-G0 glycoforms in sera of RA patients that correlates with disease activity and reverses to normal levels in patients who undergo (spontaneous) remission[16-19]. Removal of galactose residues from murine IgG by β-galactosidase treatment resulted in increased arthritogenicity of transferred IgG in a murine arthritis serum transfer model[20]. Likewise, pregnancy-induced remission in the pristane-induced arthritis model was paralleled by a decrease of IgG-G0 glycoforms, while the postpartum period was accompanied by a respective increase in agalactosylated glycoforms[21]. While these studies have narrowed the focus on the presence or absence of galactose residues, more recent studies indicate that not a lack of galactose residues itself, but rather the concomitant absence of terminal sialic acid residues may be responsible for the enhanced inflammatory activity exerted by G0 glycoforms[3,4]. Indeed, a receptor specifically recognizing IgG Fc-linked sialic acid residues has newly been described on a population of murine regulatory splenic macrophages, a cellular subset that may be involved in downregulation of inflammatory responses[22]. While these latter findings await confirmation in the human system, relevance of sialic acid residues for modulating immune responses is demonstrated by the finding that intravenous immunoglobulin preparations (IVIG) completely lose their immunosuppressive capacity upon removal of sialic acid residues by neuraminidase treatment[3,4]. Together, these findings underline the importance of IgG Fc-linked glycan structures for antibody-mediated inflammatory responses.

Anti-citrullinated protein antibodies (ACPA) are highly specific autoantibodies for a subgroup of rheumatoid arthritis patients that suffer from severe erosive disease. A body

of evidence points to a crucial role for ACPA in disease pathogenesis, but the pathogenic potential of ACPA and the mechanism by which ACPA could cause tissue pathology are ill-defined. ACPA are present in serum years before the onset of clinical symptoms, ACPA production has been detected in synovial membrane explants, and increased ACPA levels were found in synovial fluid as compared to serum [23-25]. ACPA-positive RA patients suffer from more severe disease than patients without a citrulline-specific immune response, and presence of ACPA favours the development of overt RA in patients with undifferentiated arthritis [26-29]. Intriguingly, however, ACPA levels do not or only moderately correlate with disease activity, and depletion of $CD20^+$ B cells improves clinical symptoms while ACPA levels are only moderately affected [30,31]. The observation that ACPA can be present without signs of inflammation, both before and during disease, raises the possibility that the quality rather than the quantity of the ACPA immune response influences disease. So far, studies have focussed on ACPA fine-specificity and isotype usage, but data on ACPA Fc-linked glycan residues have not been obtained. In light of the strong influence of Fc-linked glycans on the inflammatory potential of antibodies, we have recently developed a technique that allows analysis of Fc-linked glycans in an antigen specific manner [32]. We have now used this technique to analyze Fc glycosylation of IgG_1-ACPA in serum and synovial fluid of early arthritis patients in order to further characterize the immune response to citrullinated antigens.

PATIENTS AND METHODS

Serum and synovial fluid samples

Serum samples of 85 clinically well-defined, ACPA-positive arthritis patients participating in the Leiden Early Arthritis Clinic (EAC) were collected following informed consent of study participants and study-approval by the local institutional review board. The Leiden EAC is a population-based inception cohort that includes patients with self-reported symptom duration of ≤ 2 years [33].

Paired serum and synovial fluid samples were collected from 11 additional ACPA-positive rheumatoid arthritis patients. 7 samples originated from the outpatient clinic of the Department of Rheumatology and Clinical Immunology at Charité Hospital in Berlin (Germany), 4 samples were collected at the respective outpatient clinic of the Department of Rheumatology of Leiden University Medical Center (The Netherlands).

ACPA positivity was determined based on reactivity of sera against a second generation cyclic citrullinated peptide (CCP2) in a commercially available ELISA system (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands). Serum and synovial fluid samples were aliquoted and stored at -80°C until further use.

Isolation of ACPA and total IgG from serum and synovial fluid samples

ACPA were isolated from total human serum and synovial fluid as previously described[32]. In brief, sera and synovial fluid samples were incubated in CCP2-coated ELISA plates (Immunoscan RA Mark 2; Euro-Diagnostica, Arnhem, The Netherlands) for 1 hour at 37°C. Supernatants were discarded and plates were washed thoroughly. Bound antibodies were eluted from the plates by adding 100mM formic acid (p.a. for MS; Merck Darmstadt, Germany) for 15 min at room temperature. Eluates were collected in 96-well V-bottom plates, dried in a vacuum centrifuge and subjected to tryptic digest by adding 200ng trypsin (sequencing grade; Promega, Leiden, The Netherlands) in 40µl ammonium bicarbonate to each well followed by incubation at 37°C overnight. Digested samples were stored at -20°C until further use. Purity of eluted ACPA was verified by subjecting ACPA-negative control sera to the same isolation procedure. Non-trypsinized eluates of ACPA-negative samples were tested for the presence of human IgG by ELISA. None of the ACPA-negative eluates contained detectable amounts of IgG, indicating that only citrulline-specific IgG-molecules were eluted from the CCP2-plates (data not shown; for methodological details see[32]).

Purification of total IgG was achieved by incubating serum or synovial fluid samples with Protein A-sepharose beads (GE Healthcare, Eindhoven, The Netherlands) in 96-well filter plates (Multiscreen Solvinert, 0.45 µm pore-size low-binding hydrophilic PTFE; Millipore, Billerica, MA) on a shaker for 1 hour. Beads were thoroughly washed and bound IgG-molecules (IgG_1, IgG_2 and IgG_4) were eluted into a 96-well V-bottom plate using 100mM formic acid. Samples were dried by vacuum centrifugation, digested with trypsin and stored at -20°C until further use[32,34].

Fc glycoslylation analysis

Analysis of Fc-linked N-glycans was performed as previously described[32,34]. In brief, trypsinized samples obtained from the ACPA and total serum IgG isolation procedures were applied to a RP column (C18 PepMap 100 Å, 3 µm, 75 µm x 150 mm; Dionex/LC Packings, Amsterdam, The Netherlands) using an Ultimate3000 nanoLC (Dionex/LC Packings). The LC system was coupled via an online nanospray source to an Esquire HCTultra ESI-IT-mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electron transfer dissociation module (PTM Discovery System™) and was operated in the positive ion mode. Ions from *m/z* 600 to *m/z* 1800 were registered. The HPLC method resulted in resolution of the glycopeptides based on the peptide moiety with IgG_1 glycopeptides eluting first, followed by IgG_4 and IgG_2 glycopeptides. Glycopeptides with neutral glycan moieties tended to elute earlier than glycopeptides with antenna sialylation. Average mass spectra were generated over a 1 min elution range for both the neutral and the acidic glycopeptides of each IgG subclass.

As the most frequent isotype in ACPA-positive RA patients is IgG_1, only IgG_1 Fc-linked N-glycans were analyzed in this study. Furthermore, it is important to note that also the Fab-fragment of IgG-molecules can carry N-glycans[35]. Most previous studies have analyzed N-glycans released enzymatically from total human serum IgG, which results in a mixture of both Fc-linked and Fab-linked N-glycans. However, Fab-linked glycoforms can differ considerably from Fc-linked N-glycans, and vary between IgG subclasses[36]. In our approach, the specific mass of the Fc peptide portion obtained after tryptic cleavage of the IgG molecule allows to exclusively analyze Fc-linked N-glycans, whilst Fab-linked glycopeptides exhibit a heterogeneous group of signals that do not interfere with Fc glycosylation analysis at the glycopeptide level. Due to the exclusive analysis of Fc-linked N-glycans, the total number of glycoforms detected was lower than that of previous reports, and some glycoforms were not detected at all.

Statistical analysis

In order to account for measurement variability, the relative peak intensity of the glycoform containing one galactose residue (G1) identified by mass spectrometry was deliberately set to 1, and peak intensities of all other glycoforms were normalized accordingly. Frequencies of individual glycoforms are reported as percent of the total number of detectable glycoforms based on this normalization procedure. Differences in frequencies of glycoforms obtained from different samples were evaluated for statistical significance using the non-parametric Kruskal-Wallis test (in case of unpaired samples) followed by Dunn's post hoc test to adjust for errors introduced by multiple testing. Dunn's test generalizes the Bonferroni adjustment procedure for multiple testing to maintain the probability of a type-I error at $\leq 5\%$. For glycoforms of paired serum/synovial fluid samples groups were compared using the non-parametric Mann-Whitney rank-sum test. This is indicated where appropriate. P-values <0.05 were considered statistically significant. Data were analyzed using SPSS version 16.0.2 and GraphPad Prism Software 4.0.

RESULTS

Fc-linked galactosylation of ACPA IgG_1 isolated from human serum

Specific Fc glycosylation profiles of ACPA IgG_1 and respective profiles of total serum IgG_1 were obtained from 85 ACPA-positive Dutch early arthritis patients (mean age 52.6 ± 14.4 years; 69.4% female). Based on previous observations reporting a predominance of agalactosylated glycoforms in patients with rheumatoid arthritis, we first analyzed frequencies of IgG_1 Fc-linked galactose residues (Figure 2). A median of 25.4% (IQR 19.8-32.3) of serum IgG_1 molecules were found to lack galactose residues, consistent with previous reports (Figure 2A)[16]. ACPA IgG_1 contained slightly higher frequencies

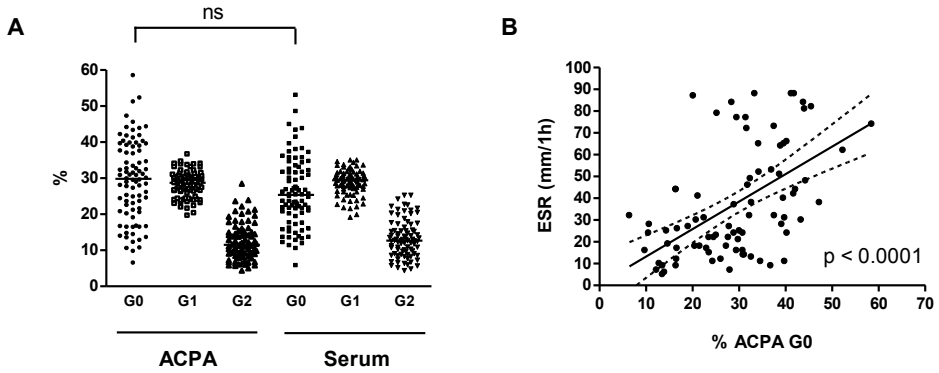

Figure 2: Fc galactosylation of ACPA and serum IgG_1.
(A) Frequencies of G0-, G1- and G2-glycoforms for ACPA and serum IgG_1 in percent of all detected glycoforms. Lines represent medians (B) Correlation between the frequency of ACPA-G0 glycoforms and erythrocyte sedimentation rate (ESR). Broken lines represent the 95% confidence interval of the linear regression (solid line).

of G0-glycoforms as compared to total serum IgG_1 (29.8% (IQR 20.9-38.8)), a nominal difference that did not remain significant after correction for multiple testing. Almost two thirds (62.4%) of patients carried ACPA IgG_1 that exhibited higher G0-frequencies than their respective serum IgG_1. Patients of whom these ACPA were isolated did not differ significantly in age (data not shown), but did have slightly higher ESR than those with ACPA G0-frequencies below those of serum IgG_1 (median ESR 32.0 mm/1h (IQR 21.5 - 65.0) vs. 24.5 (IQR 15.5 - 40), $p = 0.06$). This is in line with a linear correlation between the frequency of ACPA G0-glycoforms and ESR (Spearman $\rho = 0.54$; $p<0.0001$; Figure 2B). A similar correlation, although weaker, was found for G0-glycoforms of serum IgG_1 (Spearman $\rho = 0.38$; $p<0.0003$) and has been reported previously [19]. Importantly, the frequency of ACPA G0-glycoforms did not correlate with ACPA-titres ($p = 0.28$; data not shown). Furthermore, no significant difference was noted between ACPA IgG_1 and total serum IgG_1 with respect to the presence of G1- or G2-glycoforms. Taken together, we observed a trend towards more agalactosylated glycoforms on ACPA IgG_1 than on serum IgG_1 that did not reach statistical significance at the available sample size.

ACPA-specific Fc-linked sialylation and fucosylation patterns

Given the reported impact of sialic acid and core fucose residues on the biological activity of antibodies, we next determined the frequency of IgG_1 Fc-linked terminal sialic acid and core fucose residues (Figures 3 & 4). Absence of sialic acid residues increases the affinity for activating FcγR, while presence of sialic acid accounts for anti-inflammatory effects, as has been shown for IVIG [3,10]. The frequency of sialic acid residues attached to ACPA IgG_1 was found to be significantly reduced as compared to serum IgG_1 for both

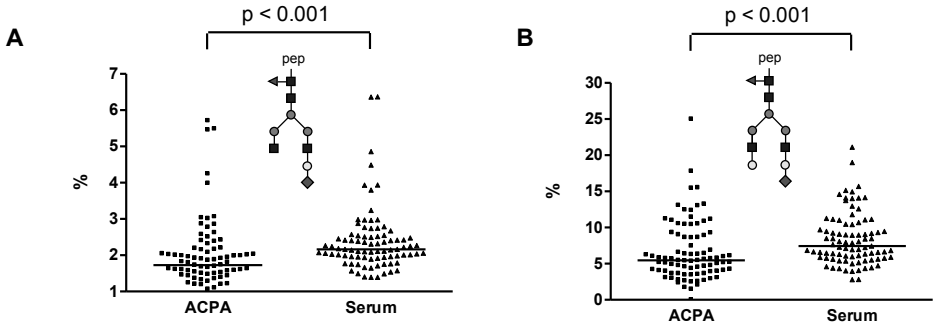

Figure 3: ACPA-specific Fc-linked sialylation patterns.
Frequencies of monosialylated G1- (A) and G2-glycoforms (B) for both ACPA and serum IgG_1. No Fc-linked disialylated glycoforms were detected. Lines represent medians.

G1- (Figure 3A; ACPA median 1.73% (IQR 1.36-2.16), serum median 2.16% (IQR 1.94-2.53); p<0.001) and G2-glycoforms (Figure 3B; ACPA median 5.47% (IQR 3.94-8.96), serum median 7.43% (5.82-10.44); p<0.001). As terminal sialic acid residues require galactose residues for linkage, sialic acid can only be detected on G1- or G2-, but not on G0-glycoforms. We did not detect glycoforms carrying two sialic acid residues in our samples.

Fucosylation strongly influences ADCC by modulating the Fc part's binding avidity to FcγRIIIa. In the absence of core fucose residues ADCC is significantly increased [12, 14, 37]. The vast majority of IgG_1-antibodies analyzed here contained core fucose residues with no significant overall difference between ACPA IgG_1 and total serum IgG_1 (Figure 4; ACPA median 93.9% (IQR 92.5-95.3); serum median 94.0% (IQR 92.1-94.9), p=0.66).

Overall, these data indicate that the glycosylation profile of ACPA IgG_1 differs considerably from total serum IgG_1 in the degree of sialylation.

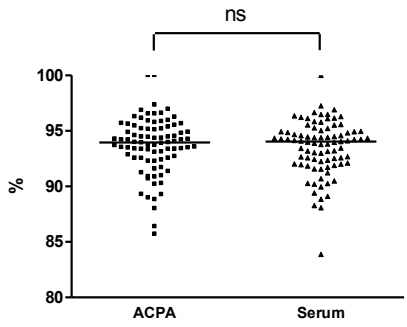

Figure 4: ACPA-specific Fc-linked fucosylation patterns.
Overall frequency of fucosylated ACPA (G0+G1+G2) versus fucosylated serum IgG_1 (G0+G1+G2) glycoforms. Lines represent medians.

Fc-linked glycosylation of ACPA isolated from synovial fluid

RA is primarily characterized by synovial inflammation and progressive joint destruction. ACPA are thought to be involved in this process. However, the origin of ACPA circulating in serum is still unknown, and it remains to be determined to what extent serum ACPA contribute to synovial inflammation in the joint. In fact, ACPA titres in synovial fluid (SF) exceed those in serum, and accumulating evidence suggests that ACPA can also be generated locally by plasma cells within the synovial membrane[24]. So far, however, qualitative differences between serum ACPA and ACPA in SF have not been determined. In order to investigate whether Fc glycosylation differs between ACPA in SF and ACPA circulating in serum, we isolated ACPA from serum and synovial fluid of 11 paired samples. ACPA IgG_1 glycosylation profiles were compared to profiles of total IgG_1 isolated from the same compartments. In contrast to ACPA in serum, ACPA isolated from SF were found to be highly agalactosylated (Figure 5A; serum-ACPA: G0 median

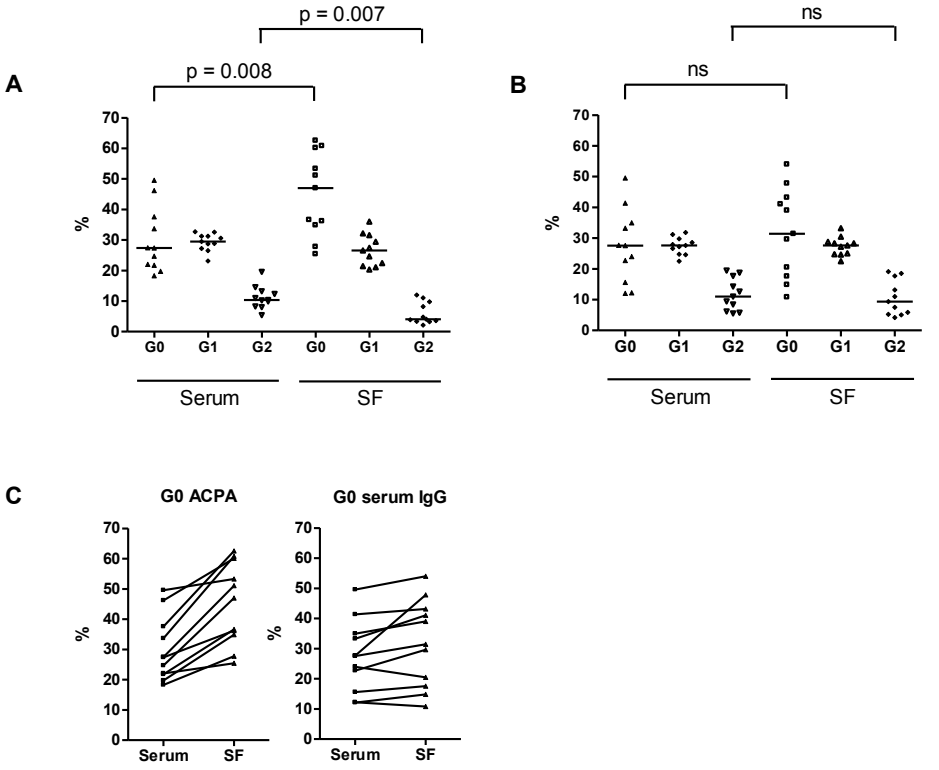

Figure 5: ACPA-specific Fc glycosylation in serum versus synovial fluid.
G0-, G1- and G2-glycoforms of ACPA (A) and total serum IgG_1 (B) isolated from serum and synovial fluid of 11 paired samples. Lines represent medians. (C) ACPA and total serum IgG_1 G0-frequencies per individual patient in serum and synovial fluid. Significance levels were determined by comparing individual groups using the non-parametric Mann-Whitney rank sum test.

27.4% (IQR 21.7-37.6), SF-ACPA: G0 median 47.0% (IQR 34.9-60.1), Mann-Whitney p = 0.008). This finding was specific for ACPA IgG_1, as no such predominance of G0-glycoforms was observed for the pool of total synovial fluid IgG_1 (Figure 5B; serum IgG_1: G0 median 27.5% (IQR 15.6-35.0); SF-IgG_1: G0 median 31.4% (IQR 17.6-43.2), Mann-Whitney p = 0.5). Increased frequencies of ACPA G0-glycoforms in synovial fluid as compared to serum were observed in all 11 patients analyzed (Figure 5C). In contrast, G0-frequencies of total synovial fluid IgG_1 were higher than in serum for some patients, but lower in others. Due to the lack of galactose residues, SF-ACPA also exhibited a significantly lower degree of sialylation (data not shown). All glycoforms examined in SF exhibited a high degree of fucosylation (up to 100%) with no significant difference between ACPA and total IgG_1 (data not shown).

In summary, ACPA IgG_1 in synovial fluid differ from ACPA IgG_1 in serum in terms of Fc glycosylation. Synovial fluid ACPA are highly agalactosylated and lack terminal sialic acid residues. This differential glycan profile appears to be a specific feature of ACPA, as no significant difference in Fc glycosylation was observed for total IgG_1 isolated from the same compartments.

Differential ACPA IgG_1 Fc glycosylation profile in RF-positive and -negative patients

Previous studies indicate that specificity and positive predictive value of ACPA for RA are increased in the presence of rheumatoid factors (RF)[25]. Individuals with both ACPA and IgM-RF are significantly more likely to develop RA than those who test positive for ACPA only, suggesting that ACPA and RF directly or indirectly interact[38,39]. Intriguingly, RF binding sites include the Cγ2 domain of the Fc part of human IgG which is in close proximity to amino acid Asn297 to which the carbohydrate chains are attached. Therefore, it has been suggested that Fc glycosylation could influence RF-binding to IgG-Fc, and indeed fractions of RF exhibiting high affinity for agalactosyl IgG have been identified in RA patients[40,41]. Based on these observations, we were interested in investigating whether ACPA Fc glycosylation differs between IgM-RF-positive and -negative patients (Figure 6). We observed a significant predominance of ACPA IgG_1 G0-glycoforms in RF-positive (n = 61; G0 median 31.6% (IQR 24.4-39.3)) as compared to RF-negative patients (n = 24; G0 median 19.1% (IQR 13.7-31.3), Figure 6A). This RF-dependent difference was only observed for ACPA, but not for total serum IgG_1 (Figure 6B). In order to exclude that the observed difference could be due to the co-elution of agalactosylated RF bound to ACPA during the ACPA isolation procedure, we also isolated ACPA from RF-negative/ACPA-positive sera after mixing them with RF-positive/ACPA-negative sera. Fc glycan profiles of ACPA isolated from these mixing experiments did not differ from the profiles obtained without additional RF (data not shown), making it unlikely that our results are confounded by RF bound to ACPA.

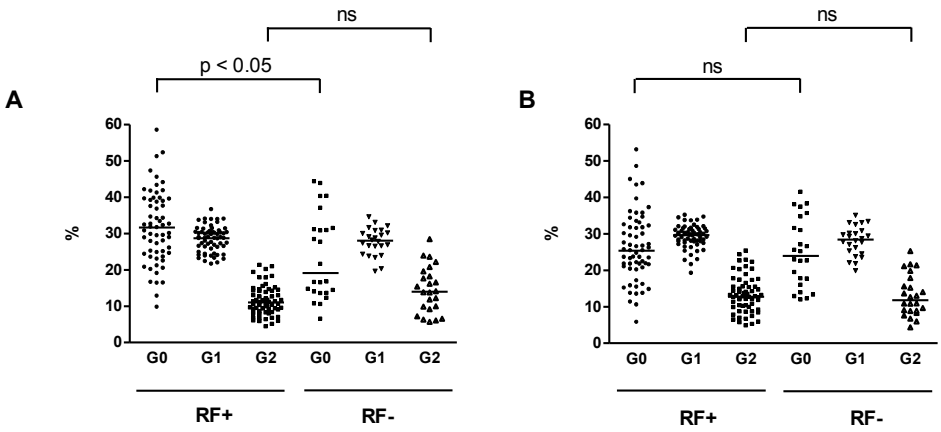

Figure 6: Differential ACPA Fc glycosylation in RF+ versus RF- patients.
Galactosylation profiles for ACPA (A) and total serum IgG_1 (B) molecules in IgM-RF-positive and -negative patients. Lines represent medians.

We also noted a significant lack of the sialylated ACPA-linked G2-glycoform in RF-positive patients (RF-positive: median 5.2% (IQR 3.6-6.5), RF-negative: median 8.6% (4.4-11.1), p = 0.038), while the sialylated G1-glycoform was equally frequent in both subgroups (data not shown). No difference within these subgroups was observed for core fucosylation. Of note, RF-positive and -negative subgroups did not differ significantly in age or the degree of ESR-/C-reactive protein elevation (data not shown).

DISCUSSION

N-glycans are crucial determinants of IgG Fc-mediated antibody effector functions. Modification of the Fc-linked carbohydrate backbone by addition of galactose, sialic acid, fucose and N-acetylglucosamine residues has differential impact on Fc-mediated immune responses both *in vivo* and *in vitro* [10-14,20]. Serum IgG molecules of RA patients have long been known to lack Fc-linked galactose residues when compared to serum IgG molecules of age-matched healthy controls, but whether antibodies of defined antigenic specificity differ in their degree of Fc glycosylation remained undetermined in these patients. Given recent insights in RA pathogenesis based on the identification of anti-citrullinated protein antibodies, we sought to determine whether these RA-specific autoantibodies exhibit specific Fc glycosylation profiles that would help to elucidate their role in disease pathogenesis.

Comparing Fc-linked glycosylation profiles of ACPA IgG_1 to the pool of total serum IgG_1 in early arthritis patients, we found a significant lack of sialic acid residues on ACPA IgG_1 molecules. Furthermore, we observed a non-significant trend towards a

lower degree of ACPA-galactosylation which could result in an additional reduction in sialic acid residues. This finding points to an increased inflammatory potential of ACPA IgG_1 and is in line with the recent observation that sialic acid residues rather than galactose residues determine the anti-inflammatory activity of human IgG[3]. Sialylated IgG molecules have reduced affinity to activating FcγR and, at least in the mouse, have been described to bind to a specific receptor on regulatory macrophages in the spleen[22]. Likewise, a complete lack of Fc-linked sialic acid residues favours the pathogenic potential of IgG as demonstrated by the loss of anti-inflammatory activity of IVIG upon treatment with neuraminidase[3]. Thus, ACPA IgG_1 circulating in human serum exhibit, based on functional data from murine studies, a more pro-inflammatory Fc glycosylation profile than the pool of total serum IgG_1.

With regard to core-fucosylation, we found that ACPA IgG_1 G0-glycoforms were highly fucosylated, an observation previously reported for total serum IgG G0-glycoforms of RA patients when compared to healthy controls[42,43]. Based on *in vitro* studies this high core fucose content is associated with a low potential to induce ADCC[12], but the functional relevance for RA pathogenesis is currently unclear and warrants further study.

Extending our ACPA-specific glycan analysis to synovial fluid we observed both a reduction in ACPA IgG_1-linked galactose residues as well as concomitant absence of sialic acid residues. This lack of galactose and sialic acid residues was specific for ACPA, as no such difference in Fc glycosylation was observed for the pool of total IgG_1. Although this finding is suggestive of an increased inflammatory potential of SF-ACPA as compared to serum ACPA, we cannot conclude that this ACPA Fc glycosylation profile is cause rather than consequence of inflammation. Despite this limitation, our observations demonstrate for the first time that ACPA in serum and in synovial fluid are not only quantitatively but also qualitatively different. Fc-linked glycan residues are unlikely to be enzymatically modified post-secretion in SF, as such modifications would be independent of antigen specificity and also affect the pool of total IgG in SF. Therefore, differential ACPA Fc glycosylation in SF as compared to serum suggests that SF-ACPA originate predominantly from specific B cell subsets in synovial tissue. Such ACPA-specific B cells could be under the influence of local cytokines that regulate glycan processing in plasma cells susceptible to the respective cytokine signal. Local ACPA-production has been postulated previously and is in line with the histological presence of germinal centers in synovial tissue of RA patients[23,24,44]. RA B and T cells are known to exhibit reduced enzymatic activity of β-1,4 galactosyltransferase, the enzyme responsible for adding galactose residues to terminal GlcNAc residues in N-glycans[45,46]. Whether even more complex differences in N-glycan processing exist among B cell subsets in different compartments in RA is unknown.. In the context of RA and other autoimmune disorders such as SLE, it would be of particular interest to investigate whether newly generated and long-lived plasma cells differentially process Fc-linked N-glycans.

We noted differential ACPA Fc glycosylation profiles in IgM-RF-positive versus -negative patients. Serum-ACPA of RF-positive patients lacked galactose residues when compared to ACPA isolated from RF-negative patients. This differential Fc glycosylation profile was only observed for ACPA but not for total serum IgG, the latter being in line with previous results[47]. Our finding is intriguing, as it fuels the hypothesis of an interaction between ACPA and RF. ACPA associate with RA independently of RF, but the risk to develop RA increases markedly (>30 fold) if RF are additionally present. In contrast, IgM-RF alone do not seem to associate with RA in the absence of ACPA[38]. RF binding sites have been mapped to the Cγ2 and Cγ3 domains of human IgG. Not all RF recognize the same antigenic determinant, but at least one binding site involves Asn297 which carries the Fc-linked N-glycans[41]. Thus, it has been postulated that Fc glycosylation can influence RF-binding affinity, and indeed RF with high affinity to agalactosylated IgG were repeatedly identified in RA patients[40,41]. Moreover, IgG aggregrates in synovial fluid of RA patients were found to contain high amounts of agalactosylated IgG, suggesting that a lack of galactoses facilitates association of IgG molecules[48]. It is conceivable that lack of galactose residues leads to a conformational change in IgG-Fc structure[7] which could favour high affinity RF binding and even reveal novel epitopes that promote the generation of high affinity RF. So far, binding activity of IgG-RF was found to increase with decreasing RF Fc galactosylation (and -sialylation), compatible with the idea that Fc-glycosylation influences "antigenicity" of the Fc-tail[49]. Whether RF directly or even preferentially also bind to ACPA that lack galactose residues cannot be concluded from our data, but our data suggest that the IgG recognized by RF exhibits a similar glycosylation profile than the one detected on ACPA. Thus, it is intriguing to speculate that ACPA G0-glycoforms help in the generation of RF with high affinity for agalactosylated IgG, thereby facilitating immune complex formation and complement activation.

Finally, as others before, we observed a strong correlation between the frequency of serum IgG-G0 glycoforms and ESR as well as age (data not shown). This association was even more pronounced for ACPA-G0 glycoforms (Figure 2). Of note, we accounted for these correlations in our subgroup analyses, rendering it unlikely that our results are influenced by these confounding factors.

In summary, we describe a detailed analysis of Fc-linked glycosylation profiles specific for ACPA. Fc glycosylation profiles differ between antibodies of different antigenic specifities and between antibodies isolated from different compartments. These data enhance our understanding of the citrulline-specific immune response and may open up novel strategies for therapeutic interventions.

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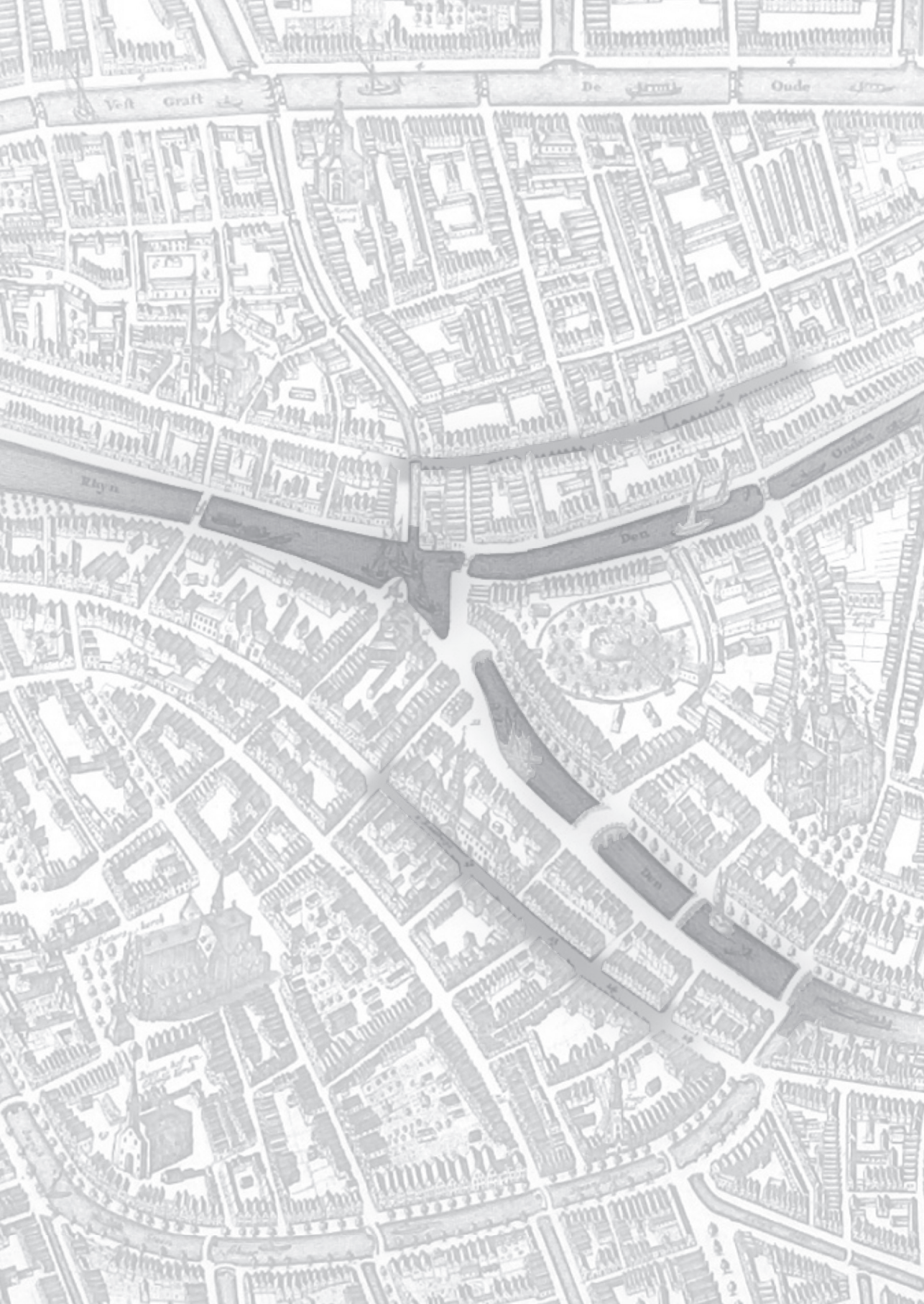
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Chapter 5

Anti-citrullinated protein antibodies have a low avidity compared with antibodies against recall antigens.

Suwannalai P, Scherer HU, van der Woude D, Ioan-Facsinay A, Jol-van der Zijde CM, van Tol MJ, Drijfhout JW, Huizinga TW, Toes RE, Trouw LA.

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ABSTRACT

Objectives: Anti-Citrullinated Protein Antibodies (ACPA) are highly specific for Rheumatoid Arthritis (RA) and have been implicated in disease pathogenesis. Recent ongoing evidence indicates that the ACPA-response broadens before precipitation of full-blown RA as indicated by a more extensive isotype usage and an increased citrullinated epitope recognition profile. Nonetheless, the evolution of the ACPA-response is still poorly understood and might intrinsically differ from the protective responses against pathogens.

Methods: We analyzed the avidity and the avidity maturation of ACPA in relation to the avidity of antibodies against recall antigens.

Results: The avidity of ACPA was significantly lower compared to the avidity of antibodies to the recall antigens Tetanus Toxoid (TT) and Diptheria Toxoid (DT). Moreover, ACPA did not show avidity maturation during longitudinal follow-up and ACPA avidity was relatively low also in patients that displayed extensive isotype switching.

Conclusions: These observations indicate that the natural evolution of ACPA differs from the development of antibodies against recall antigens. Moreover, these data indicate that ACPA avidity maturation and isotype-switching are disconnected whereby extensive isotype switching occurs in the setting of restricted avidity maturation. Intrinsic differences between RA specific autoantibody system and protective antibody responses against pathogens could be of relevance for designing novel B cell targeted therapies for RA.

INTRODUCTION

Rheumatoid Arthritis (RA) is a chronic autoimmune disease that mainly affects the joints. The identification of anti-citrullinated protein antibodies (ACPA) represents an important breakthrough in the field of RA[1,2]. ACPA recognize post-translationally modified proteins, in which arginines have been modified into citrullinated residues by peptidylarginine deiminase (PAD) enzyme during inflammation. Therefore it is hypothesized that citrullination of proteins in the joint may create epitopes that can serve as targets of ACPA, ultimately leading to inflammation and arthritis. Indeed several observations implicate ACPA in disease pathogenesis as it has been shown that the presence of ACPA predicts the emergence and outcome of RA[3-5]. Moreover, ACPA have been implicated in disease pathogenesis by the observations that ACPA can induce and aggravate arthritis in mice[6,7] and can activate human immune effector mechanisms, such as triggering of cellular Fc receptors[8] and activation of the complement system[9].

During a B cell response, isotype switching and affinity maturation typically occurs in the germinal center. Following somatic hypermutation, different B cell clones will compete for antigen on follicular dendritic cells (FDC). The B cells expressing surface immunoglobulins with a higher avidity will acquire the signals necessary for survival and proliferation. As a result, the total avidity of the immune response increases because low avidity B cells will not be stimulated and will eventually disappear from the population.

By definition, antibody affinity is the strength of interaction between a single antigen binding sites and soluble monovalent antigens in solutions. However, in reality antibodies are multivalent and contain 2 (IgG) to 10 (IgM) antigen binding sites. In addition, also the antigens are often multivalent and/or nonsoluble. Therefore antibody avidity which is defined as the overall binding strength of polyclonal antibodies to a multivalent antigen provides a better measure for the strength of antibody responses.

Extensive information has been obtained regarding the avidity maturation of antibody responses against recall antigens, mostly following vaccination[10-12]. Interestingly, in the mouse it was recently described that B-cells producing arthritogenic antibodies are relatively short lived plasmablasts that are different from the long lived plasma cells typically producing protective antibodies[13].

Given the implicated role of ACPA in RA, we have now investigated ACPA-avidity and avidity maturation in arthritis patients in relation to avidity of recall antigens.

MATERIALS AND METHODS

Patients

Sera of 92, ACPA positive, patients with early arthritis were selected from the early arthritis clinic (EAC), an inception cohort of recent onset arthritis previous described [14]. Sera for longitudinal analysis were selected from available samples at baseline, 1 year and 5 year follow-up. Characteristic of patients in our cohort were as follows: 65.2% are female, age at inclusion is 51.7(17.0-82.3) years, disease duration is 7.71(0.2-36.13) months, 80.4% are RF positive and 66.3% have erosion at baseline. These individuals were not vaccinated with TT and DT for the purpose of this study. We also studied ACPA avidity at baseline during active disease and at remission in 5 RA patients with complete drug free remission. From 15 randomly selected RA patients we analyzed paired serum and synovial fluid samples. Disease duration of these patients is 8.27 (1-21) years. The collection and use of patient samples was approved by the local medical ethics committee in compliance with the Helsinki declaration.

Avidity assays for ACPA and recall antigens

To determine the avidity of ACPA IgG and IgG antibodies against recall antigens we used elution enzyme-linked immunosorbent assay (ELISA) assays [15,16].

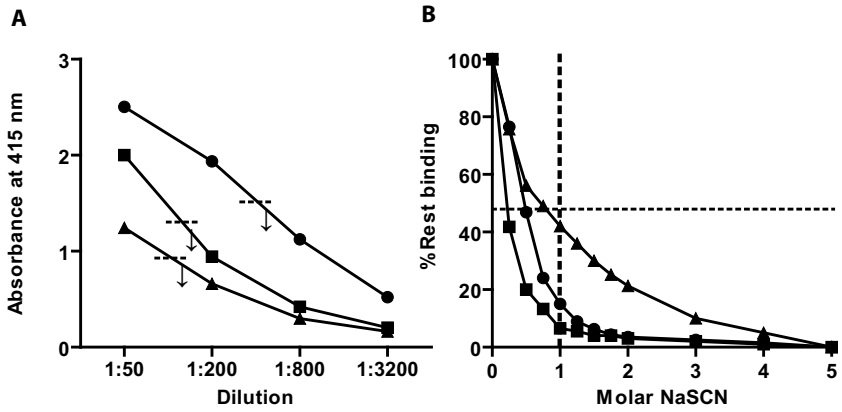


Figure 1: Characteristics of the avidity assay.
(A) The appropriate serum dilution for the avidity assay was determined by performing a dose response study. The serum dilution was chosen as to obtain a 50% of maximal binding (arrows). Three representative samples for anti-CCP2 reactivity are presented.
(B) The relative Avidity Index (AI) is defined as the ratio of the amount of residual antibodies bound to the coated antigen after NaSCN elution (indicated by the vertical dotted line) relative to the amount of binding antibodies in the absence of NaSCN, expressed as percentage. The horizontal line indicates the 50% elution profile which is used to calculate the concentration NaSCN that is necessary to elute 50% of the antibodies. Three representative samples for anti-CCP2 are presented.

For ACPA the appropriate serum dilution was first determined by performing a titration using a CCP2 ELISA (Immunoscan RA Mark 2, Euro-Diagnostica, Arnhem, The Netherlands) with minor modifications, now using 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic-acid substrate. The serum dilution at which the response was 50% of maximum was considered 'optimal' and a minimal dilution we used is 1:25. In addition, after developing the plate for 1 hour the absorbance at 415 nm had to be between 0.5-2.0 to allow optimal detection (figure 1A).

To determine the avidity of the anti-CCP2 antibodies, plates were incubated with the appropriate serum dilutions in PBS-Tween 1% BSA (PTB), for 1 hour at 37°C. After washing, the wells were incubated with increasing concentrations of the chaotropic agent sodiumthiocyanide: NaSCN at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4 and 5M, for 15 minutes at RT. The wells were washed and bound antibodies were detected using HRP-labeled goat-anti-human IgG (DAKO, Denmark). The amount of antibody bound to the plate without elution and the amount that resisted elution by NaSCN were determined relative to a standard curve.

No difference in avidity was detected where ACPA avidities were measured on commercial plates or on in house plates coated with CCP2 peptide (data not shown).

The "relative avidity index" (AI) was calculated[17]. The AI is defined as the ratio of the amount of residual antibodies bound to the coated antigen after NaSCN (1M) elution to the amount of binding antibodies in the absence of NaSCN, expressed as percentage (figure 1B).

$$AI = \frac{\text{remaining antibodies at 1M NaSCN (AU/ml)}}{\text{binding antibodies at 0M NaSCN (AU/ml)}} \times 100$$

To determine the avidity and levels of antibodies against citrullinated human fibrinogen (cit Fib) we used a plate bound assay as described before[18] with minor modifications.

To determine the avidity of antibodies against modified citrullinated vimentin (anti MCV) we used a commercial ELISA (ORGENTEC Diagnostica GmbH, Mainz, Germany).

To determine the avidity of anti-TT IgG and anti-DT IgG we used an in-house ELISA[19,20]. In short, plates were coated with 100 ul/well of TT (1.5 Lf/ml in 0.05 M carbonate buffer, pH 9.6) or DT (0.75 Lf/ml in carbonate buffer, pH 9.6) both from RIVM (Bilthoven, The Netherlands).

Avidity of anti cit Fib, anti MCV, anti-TT IgG and anti-DT IgG antibodies were determined as for anti-CCP2 antibodies.

In all cases, conditions used for each of the assays were optimized to allow maximal antibody binding. The buffers used for coating antigens are washed away before avidity measurement and thus will not impact on the avidity measurement. After the antibodies have bound to their different antigens, the plates are washed with the same buffer and

eluted with elution buffer, and subjected to a similar detection of the residual antibody binding to the plate. In this way, this assay allows the comparison of avidities of antibodies binding to different antigens [12].

Since the presence of Rheumatoid Factor (RF) could potentially influence our observations, we analyzed the occurrence of RF in the low and high avidity groups. No correlation between RF positive status and ACPA avidity was observed (data not shown). In addition, we measured avidity of RF IgM and IgG in three patients and observed also for RF a low avidity compared to avidity of anti-TT and anti-DT (data not shown).

Isotype measurements were performed as described elsewhere [21].

Statistical analysis

Differences between groups were analyzed with the Mann-Whitney test or ANOVA, and correlation was determined by the Spearman's correlation coefficient with GraphPad Prism 4.0 software (GraphPad Inc, San Diego, CA, USA) or SPSS for Windows (release 16.0, SPSS, Chicago, IL, USA). In all tests, $P < 0.05$ was considered significant.

RESULTS

The avidity of ACPA is low compared to the avidity of antibodies against recall-antigens

As the avidity of antibodies towards antigens can have important implications for the biological effects mediated, we wished to determine the avidity of the ACPA response in RA patients and compared the avidity of ACPA IgG to the avidity of IgG against recall antigens.

In an initial group of 8 patients we analyzed the avidity of antibodies directed against three citrullinated antigens; the CCP2 peptide and two citrullinated proteins; cit-Fib and MCV, as well as the avidity of antibodies against the T cell dependent - recall protein antigens TT and DT. As shown in Figure 2A we observed, within the same patients, a low avidity for antibodies directed against all three different citrullinated antigens and a high avidity for antibodies against recall antigens TT and DT. To study this phenomenon in a larger cohort we focused on CCP2 and cit-Fib versus TT and DT. As shown in Figure 2B, in the cohort as a whole, the avidity of anti-CCP2 antibodies is generally low (median 19%), with most patients displaying an avidity with an AI lower than 40% (Figure 2B). Next we also analyzed the avidity of antibodies directed against cit-Fib, an entire citrullinated protein, similar results were obtained. In sharp contrast, the avidity of antibodies against the recall antigens TT and DT (medians 74% and 61%, respectively) are of high avidity (Figure 2B), with most individuals displaying an AI of more than 40%. This is significantly higher than the avidity of ACPA ($P<0.0001$). Thus our data show that these ACPA are of low avidity as compared to antibodies against recall antigens.

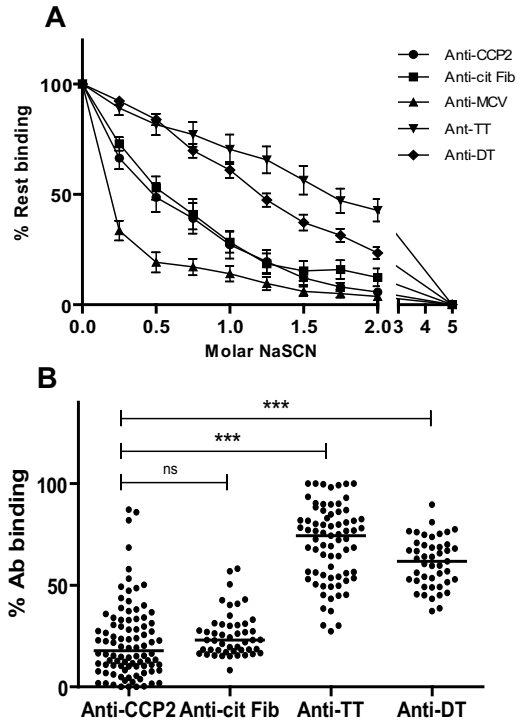


Figure 2: The avidity of ACPA IgG is lower than the avidity of IgG against recall antigens.
(A) Avidity of ACPA IgG, anti-CCP2, anti-cit Fib and anti-MCV were compared to the avidity of IgG against recall antigens TT and DT in 8 patients. The data of the elution profiles are presented as mean +/- SEM. (B) Avidity of ACPA IgG, anti-CCP2 and anti-cit Fib, were compared to the avidity of IgG against the recall antigens TT and DT in 67 patients and is expressed as relative AI. The avidity of ACPA IgG is significantly lower than the avidity of IgG to recall antigens (P-value < 0.0001).

ACPA IgG avidity in sera represents overall avidity of ACPA

To control for the possibility that the overall avidity of ACPA ,as detected in serum, is influenced by retention of high avidity ACPA in the inflamed joint, we next analyzed ACPA avidity of patients during active disease and compared it to the avidity of ACPA of the same patients during complete drug free remission (N=5). We did not observe an increase in the overall avidity of ACPA indicating that ACPA avidity is not influenced by disease activity and that there is no evidence for a retention of high avidity antibodies in the inflamed joint (figure 3A). Likewise, we have analyzed if there is a difference in the avidity of ACPA IgG between patients that are only IgG positive to patients that are also positive for IgA and / or IgM ACPA. There was no difference in the IgG ACPA avidity depending on the presence of either IgM or IgA ACPA (data not shown). Furthermore we purified IgG from serum that was positive for IgG, IgA and IgM ACPA and compared the ACPA IgG avidity from serum to that of the purified IgG and observed a similar

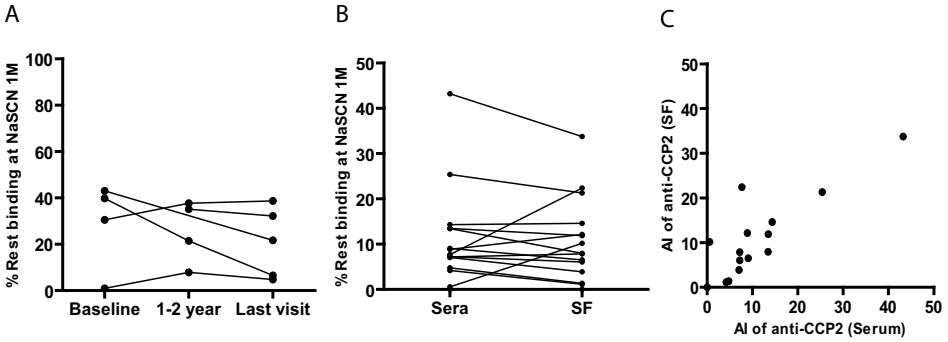


Figure 3: Avidity of ACPA IgG in serum is not influenced by disease activity and is similar to ACPA IgG in synovial fluid.

(A) Comparison of the avidity of ACPA IgG in serum measured in RA patients during active disease at baseline to complete absence of disease activity at drug free remission. The avidity of ACPA measured in serum is not influenced by disease activity. (B) Avidity of ACPA in sera compared to avidity of ACPA in paired samples of synovial fluid. (C) Correlation between avidity of anti-CCP2 in sera (x-axis) and in paired synovial fluid (y-axis).

avidity. Together these data indicate that the other isotypes had no impact on the avidity measurement of ACPA IgG (data not shown).

We also compared the avidity of anti-CCP2 in synovial fluid to the avidity of anti-CCP2 in paired sera. Avidity of anti-CCP2 in synovial fluid was comparable to avidity of anti-CCP2 in sera (Fig.3B,C). Together, these data indicate that the avidity of ACPA as measured in serum does provide a good representation of the overall ACPA avidity.

The diversity of avidities of ACPA differs from the avidity diversity of recall antigens

So far we have depicted the avidity as avidity index (AI), a measure of the percentage of antibody still bound after elution with 1M NaSCN. However, since it is possible that elution profiles differ while having a similar AI, we also analyzed the elution profiles. Different antibody elution patterns for ACPA in comparison to IgG against recall antigens within one subject were observed (figure 4). For example, as presented in figure 4, the avidity of anti-CCP2 IgG present in the sample analyzed displayed a rather homogenous response, with over 80% of the antibodies eluting in the first 3 elution steps (Figure 4A). In contrast, a wide distribution of avidities is observed for IgG against TT (Figure 4B). Interestingly, the antibody response to recall antigens displays a wider distribution of avidity, whereas the avidity distribution of the ACPA response is, in general, narrow (Figure 4C). The absence of heterogeneity within the avidity-profile of ACPA, further indicates limited avidity maturation of the ACPA response.

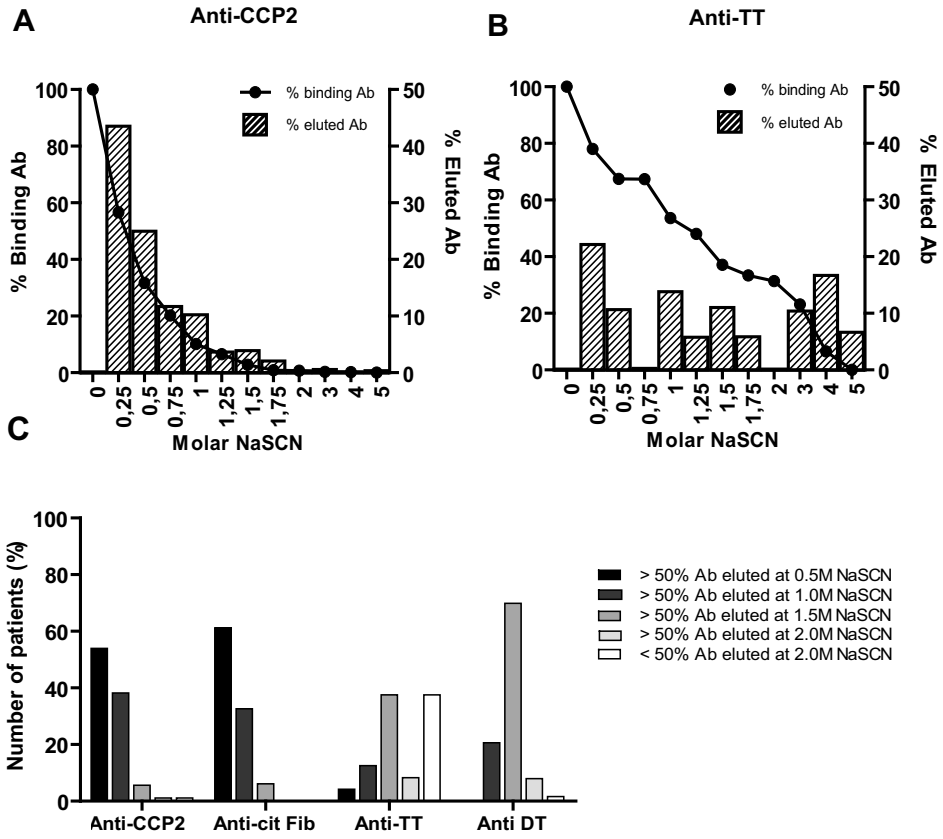


Figure 4: Different elution profiles between ACPA and IgG against recall antigens.
Different patterns of elution between anti-CCP2 IgG (A), and anti-TT IgG (B) following NaSCN elution. Percentage of antibody that was eluted after each consecutive step of NaSCN elution is expressed in bars and the percentage of antibody still bound after NaSCN elution is presented as a linear graph. The elution profiles of anti-CCP2 IgG and anti-TT IgG of one representative patient are shown. (C) The proportion of patients that have different elution profiles for the indicated antibody response.

Dynamics of ACPA IgG avidity

To evaluate whether there is affinity maturation of the ACPA IgG response over time following onset of arthritis, we next determined the avidity of ACPA in a longitudinal fashion, by comparing samples from the same patients at baseline and at 1 and 5 years follow up. We did not observe a correlation between ACPA avidity and baseline clinical characteristic such as age of onset, sex, DAS or CRP (data not shown). We observed no increase in ACPA IgG avidity over time in the patients analyzed (Figure 5A). As expected, the result for avidity measurements of anti-cit Fib antibodies in time confirms this observation (Figure 5B). In addition we did not observe a change in the high avidity (>2M) population of ACPA during follow up of patients (data not shown). These data

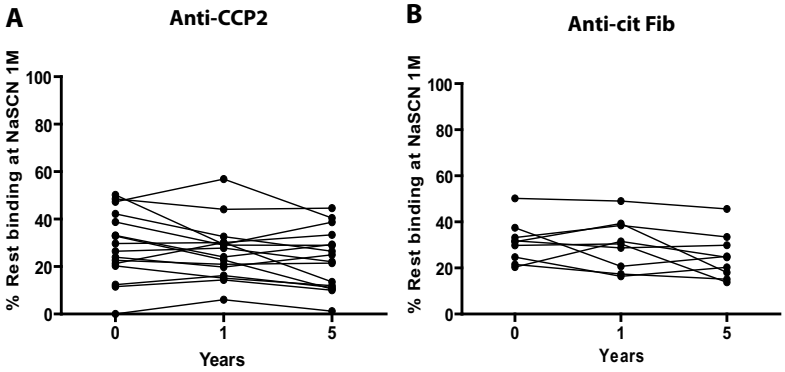
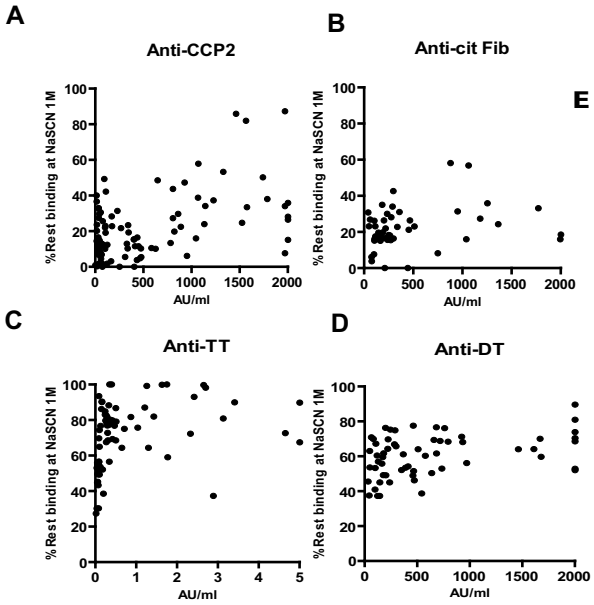


Figure 5: No avidity maturation of ACPA IgG during 5 year follow-up.
The avidity of ACPA in serial samples at baseline, 1 and 5 years follow-up were analyzed. Anti-CCP2 avidity was analyzed in 16 RA patients (A) and anti-cit Fib avidity was analyzed in 9 RA patients (B). The ACPA response did not show extensive avidity maturation.

indicate that during disease progression the ACPA response does not undergo extensive avidity maturation. However, these data do not exclude limited avidity maturation before disease onset.



E Broad ACPA isotype usage in low avidity ACPA

Low	IgG	IgM	IgA	IgG1	IgG2	IgG3	IgG4
I	X	X	X	X	X	X	X
II	X		X	X	X	X	X
III	X		X	X	X	X	X
IV	X			X		X	X
V	X			X	X		X
VI	X	X		X	X		X
VII	X			X			X
VIII	X		X	X	X		X

Figure 6: ACPA avidity maturation, titers and isotypes.
Correlation between the levels and avidity of ACPA IgG; anti-CCP2 (A) and anti-cit Fib (B), and IgG against recall antigens; anti-TT (C) and anti-DT (D). (E) Broad ACPA isotype usage also in patients with low avidity of ACPA IgG. Shown are the 8 patients with the lowest avidity (numbered I – VIII) with their use of isotypes in the ACPA response (X indicates positivity for an isotype).

ACPA avidity maturation, titers and isotypes

We determined the relation between antibody levels and avidity of both ACPA and IgG against recall antigens (Figure 6A-D). The data indicate that the avidity of IgG against recall antigens can be high even if the titer is low, confirming previous results. More importantly, however, patients with a high titer of ACPA IgG still display only a relatively low avidity. This contrasts to antibodies against recall antigens which have a high avidity in case of a high titer.

Likewise, the isotype usage of ACPA was assessed in relation to the avidity of ACPA IgG. Unlike the distribution of the isotype usage of anti-TT antibodies, which is predominated by IgG1 (data not shown)[19]. We analyzed isotype usage in 8 patients with the lowest ACPA avidity and observed that there is broad usage of isotypes even in these patients (Figure 6E). Together these data reveal poor avidity maturation of ACPA IgG, despite high antibody titers and extensive isotype switching.

DISCUSSION

In this study the ACPA response is generally of a much lower avidity than the recall responses and in our limited longitudinal study we did not obtained evidence for avidity maturation during the course of established disease. These data indicate that the regulation of the RA-specific autoimmune response against citrullinated antigens differs from the regulation of recall-responses.

Currently, only limited information is available on the endogenous citrullinated antigens recognized by ACPA. Therefore, we have used three different antigens for the determination of ACPA-avidity; the CCP2 peptide and two citrullinated proteins. The CCP2 ELISA is the most commonly used for the detection of ACPA[22], whereas the proteins cit-Fib and MCV have been shown to be present in the sera, synovial fluid and synovium of RA-patients[23,24] and are recognized by ACPA of most RA-patients[25]. We made similar observations for all three systems analyzed, providing internal confirmation of the results.

To exclude the possibility that the observed low avidity of ACPA detected in serum was a reflection of preferential retention of high avidity antibodies in the inflamed joint we analyzed ACPA avidity during active disease and complete drug free remission, where all clinical activity was absent. These studies indicate that there is no change in the avidity of ACPA detected in serum comparing active disease to remission. As we also did not observe a different in ACPA avidity in paired samples of SF and serum, we consider it unlikely that the preferential retention of high avidity antibodies in the joint due to the presence of citrullinated epitopes explains the low avidity of ACPA, although this possibility can not formally be ruled out.

The difference in the ACPA response as compared to 'conventional' B cell responses studied (i.e. B-cell responses against recall antigens) might be due to the nature of the antigen where citrullinated antigens are likely to be presented constantly in the body at multiple site, where recall antigens are presented for a short amount of time at a localized site. This would lead in a relative abundance of citrullinated antigens in the body and, as a consequence, the absence of competition for antigens by different B-cell clones in the germinal center as to be expected for recall antigens, absence of affinity maturation and hence low-affinity antibodies. Unlike isotype switching, avidity maturation not only critically depends on the presence of antigen, but most likely also on proper amounts of antigen in the germinal centers[26]. This will result in an antibody response that does undergo isotype switching but does not display avidity maturation as we observed both at baseline and at follow-up. We favor this explanation over other possibilities such as, for example, lack of adequate T cell help on a limited amount of time passed after antigen exposure. The ACPA response is characterized by extensive isotype switching, including IgA and IgE[18,21,27]. The generation of these isotypes appears T-helper cell-dependent as patients suffering from hyper IgM syndrome (caused by a gene defect in CD40Ligand) do not develop IgA responses[28,29]. Moreover, the HLA alleles predisposing to RA, only predispose to ACPA-positive RA and not to ACPA-negative disease[30] also indicates the involvement of CD4+ T helper cells in the formation of ACPA. Likewise, avidity maturation of antibody responses against recall antigen takes place within weeks, whereas the disease duration of most RA patients is much longer.

Our data indicate that ACPA producing B cells behave differently as compared to 'conventional' B cells. Recently it was shown in mice that rituximab specifically depletes B cells producing the autoantibodies, while sparing the 'conventional' plasma cells producing the protective antibodies[13]. Therefore therapies targeting the crucial biological mechanisms underlying 'conventional' B cell responses may not work similarly on ACPA producing B cells. In this respect, a possible interaction that could be of relevance is a therapy targeting the survival factors B-cells compete for during the germinal center reaction[31].

In conclusions, our data indicate that the ACPA response is different from antibody responses against recall protein antigens. The ACPA response can be of high titer, can use all isotypes yet is of low avidity that contrasts to antibodies against recall antigens that are of high avidity. These and possibly other differences between ACPA and recall responses are likely reflecting differences in the underlying B cells response. Understanding these differences may be of relevance for the design of novel B cell targeted therapies in RA.

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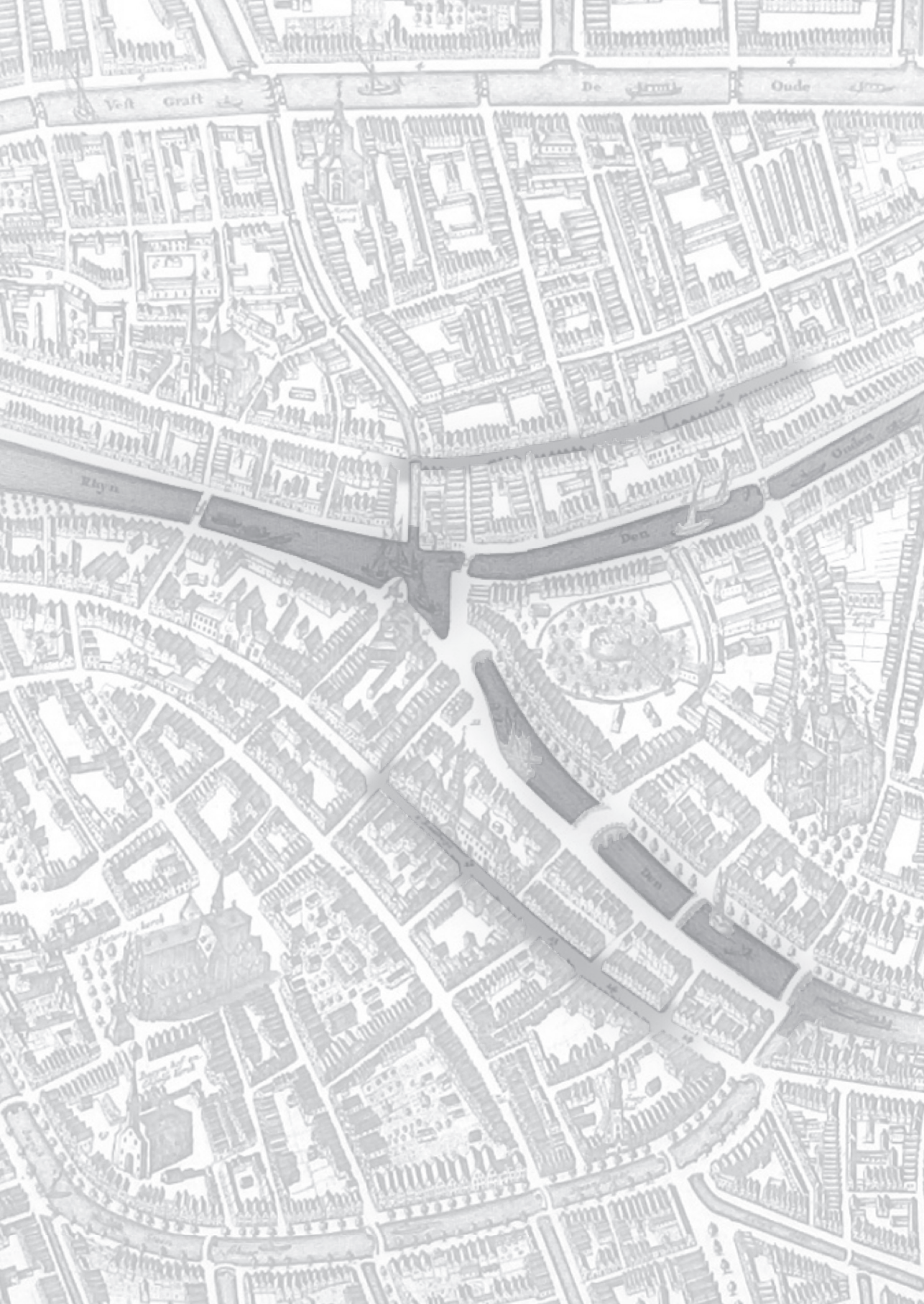
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Chapter 6

Distinct ACPA fine specificities, formed under the influence of HLA shared epitope alleles, have no effect on radiographic joint damage in rheumatoid arthritis.

Scherer HU*, van der Woude D*, Willemze A, Trouw LA, Knevel R, Syversen SW, van der Linden MP, Lie B, Huizinga TW, van der Heijde DM, van der Helm-van Mil AH, Kvien TK, Toes RE.

Ann Rheum Dis. 2011 Aug;70(8):1461-4.

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ABSTRACT

Objectives: HLA shared epitope (SE) alleles are associated with joint destruction, the presence of anti-citrullinated protein antibodies (ACPA) and the ACPA fine-specificity repertoire in rheumatoid arthritis (RA). A large variation in joint destruction is seen within the ACPA-positive patient population, and it is conceivable that certain ACPA reactivities contribute to radiological damage. Here, we investigated whether ACPA fine-specificities, which are formed under the influence of SE-alleles, associate with the extent of radiographic joint damage.

Methods: Antibodies recognizing six citrullinated epitopes were determined in sera of 330 ACPA-positive RA patients genotyped for SE-alleles. The association between SE-alleles, ACPA fine-specificity and radiographic joint damage was assessed using radiographic follow-up data. A second cohort of 154 RA patients with 5- and 10-year radiographic follow-up was used for replication.

Results: SE alleles predisposed to the recognition of certain citrullinated epitopes. However, none of the ACPA fine-specificities studied influenced radiographic joint damage. Importantly, although SE-alleles associated with radiographic damage in the total RA population, this association was no longer detectable after stratification for the presence of ACPA.

Conclusions: SE-alleles are instrumental in shaping the ACPA repertoire. However, ACPA fine-specificities formed under the influence of SE-alleles do not seem to affect joint destruction.

INTRODUCTION

HLA shared epitope (SE) encoding genes are the most prominent genetic risk factor for rheumatoid arthritis (RA). SE-alleles exert their risk effect by predisposing for anti-citrullinated protein antibody (ACPA)-positive RA[1]. This and other findings have led to the concept that RA represents at least two distinct disease entities (ACPA-positive and –negative RA) with different pathogenetic mechanisms[2,3].

The strength of the SE effect on susceptibility to ACPA-positive disease depends on the number of SE-alleles present, with two alleles conferring a higher risk of disease than one allele. The fact that ACPA are also found in SE-negative patients, albeit in lower frequency, indicates that SE alleles are not absolutely required for the development of ACPA[1]. It has been postulated that SE-alleles primarily facilitate priming and activation of T helper cells, which can then provide help to ACPA-producing B cells. Such help is required for isotype-switching and generation of a potent and long-lasting antibody response.

It is conceivable that certain citrullinated antigens are more potent than others in activating T cells in the context of SE-alleles. Only very few T-cell epitopes have so far been found, and no skewing of T-cell responses has been described. However, SE-alleles were found to associate with antibodies targeting peptides from citrullinated vimentin, but not with the presence of antibodies recognizing citrullinated fibrinogen[4]. This differential modulation of the ACPA response by SE-alleles, and the fact that disease phenotypes vary greatly among ACPA-positive patients, has raised the question whether certain ACPA fine-specificities might associate with a more severe disease phenotype. If so, designing assays that test for these specificities would be of prognostic value and could influence treatment decisions in the clinic.

In the present study, we analysed the relation between SE-alleles, different ACPA fine-specificities and their independent effects on disease outcome.

METHODS

Patients and Radiographs

ACPA fine-specificity was determined on baseline serum samples of RA patients participating in the Leiden Early Arthritis Clinic (EAC)[5]. The present study included patients who presented between March 1993 and November 2006 and who fulfilled the 1987 revised ACR criteria for RA within the first year of follow-up. Annual radiographs of hands and feet were assessed as previously described[6].

Replication of the association between SE-alleles and radiographic progression in relation to ACPA status was performed using data of Norwegian RA patients with a

maximum disease duration of 4 years included in 1992-1993 in the European Research on Incapacitating Disease and Social Support (EURIDISS) project[7-9]. Radiographs of the hands were available for 154 patients at baseline and assessed according to the Sharp-van der Heijde method [10] by one experienced reader with known time order.

Anti-CCP2 assays

Anti-CCP2 antibody levels were measured by ELISA (EAC: Immunoscan RA Mark 2; Eurodiagnostica, Arnhem, The Netherlands; EURIDISS: INOVA Diagnostics, San Diego, CA, USA).

ACPA fine-specificity assays

Antibodies against the citrullinated (Cit) and the arginine-containing form of two peptides derived from vimentin (Vim1-16; Vim59-74), two peptides derived from fibrinogen (Fibα 27-43; Fibβ 36-52), one peptide derived from alpha-enolase (Eno 5-20) and against citrullinated myelin basic protein (MBP) were determined by in-house ELISA as previously described[4, 11].

Cut-off values were defined as the mean plus three times the standard deviation of the values of 30 control subjects (anti-CCP-negative EAC patients diagnosed with gout). Recognition was deemed to be citrulline-specific when the following requirements were met: 1) OD value citrullinated peptide > cut-off, and 2) OD difference (=OD citrullinated peptide – OD arginine-containing peptide) ≥ 0.1. The number of patients recognizing both the citrullinated and the arginine-variant above cut-off levels was small (~ 3%).

Statistical analysis

Association between fine-specificity recognition and SE-alleles was assessed using chi-square tests. Association of fine-specificities with the rate of joint destruction was assessed using a repeated measurement analysis on log-transformed radiological data. Adjustments were made for age, gender and treatment strategy used at the time of inclusion as previously described[5, 6]. No association between inclusion period and recognition of separate ACPA fine specificities was found (data not shown). For the EURIDISS cohort, non-parametric Mann-Whitney U-test was used because of non-normal distribution of the data despite log-transformation. For comparison, Mann-Whitney U-test was also applied to the Leiden EAC where indicated.

RESULTS

HLA SE-alleles associate with several ACPA fine-specificities

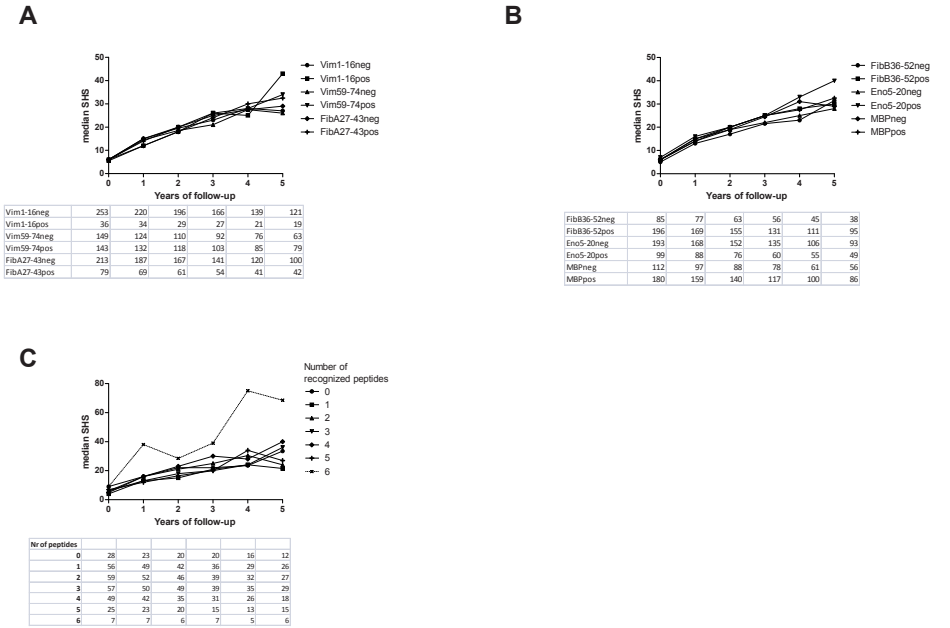
SE-alleles associate with the presence of certain ACPA fine-specificities[4]. We first extended these findings by analyzing reactivity to more citrullinated epitopes, by increasing the number of patients studied, and by analyzing the effect of the SE gene dose on ACPA fine-specificity. Three out of six citrullinated epitopes studied associated with the presence of SE-alleles (table 1). Reactivity to citrullinated vimentin 59-74, citrullinated alpha-enolase 5-20 and citrullinated MBP was found in significantly increased frequency in SE-positive patients, whereas no such effect was observed for the other three antigens. The effect was independent of the number of SE-alleles, as patients with one or two SE-alleles displayed a comparable profile of ACPA epitope recognition. These data indicate that one SE allele is sufficient to facilitate the development of certain ACPA fine-specificities, and that presence of a second SE allele does not further skew the ACPA profile towards more frequent recognition of a specific epitope.

Table 1: Association of HLA SE-alleles with ACPA fine-specificity.
Displayed is the number of patients within the ACPA-positive patient population who harbor ACPA recognizing defined citrullinated antigens. Chi-squared test over all 3 allelic groups (Vim 1-16: $p = 0.88$; Vim 59-74: $p < 0.001$; Fib-α: $p = 0.35$; Fib-β: $p = 0.92$; α-enolase: $p = 0.022$; MBP: $p = 0.006$).

	Number of HLA SE-alleles			
	0 n=65	1 n=192	2 n=73	Total n=330
Recognition of citrullinated:				
Vimentin 1-16	8/63 (13%)	25/188 (13%)	8/73 (11%)	41/324 (13%)
Vimentin 59-74	16/64 (25%)	100/190 (53%)	46/73 (63%)	162/327 (50%)
Fibrinogen-α 27-43	20/64 (31%)	51/190 (27%)	15/73 (21%)	86/327 (26%)
Fibrinogen-β 36-52	43/63 (68%)	128/181 (71%)	52/73 (71%)	223/317 (70%)
α-enolase 5-20	12/64 (19%)	70/190 (37%)	27/73 (37%)	109/327 (33%)
MBP	30/64 (47%)	128/190 (67%)	51/73 (64%)	209/327 (64%)

The ACPA fine-specificity repertoire does not predict future radiographic progression

ACPA-positive patients suffer from more severe disease than ACPA-negative patients. However, it is unknown whether distinct ACPA fine-specificities are pathogenetically driving the inflammatory process and thus lead to a more severe disease outcome. Considering radiographic joint damage as the most objective sign of disease severity in RA, we analyzed the association between baseline recognition of specific citrullinated antigens and progression of radiographic joint damage in ACPA-positive patients over the course of 5 years (figure 1, panels A and B). The ACPA fine-specificity repertoire in individual patients did not change during this time period[12]. We did not detect a



Vim1-16neg	253	220	196	166	139	121
Vim1-16pos	36	34	29	27	21	19
Vim59-74neg	149	124	110	92	76	63
Vim59-74pos	143	132	118	103	85	79
FibA27-43neg	213	187	167	141	120	100
FibA27-43pos	79	69	61	54	41	42

FibB36-52neg	85	77	63	56	45	38
FibB36-52pos	196	169	155	131	111	95
Eno5-20neg	193	168	152	135	106	93
Eno5-20pos	99	88	76	60	55	49
MBPneg	112	97	88	78	61	56
MBPpos	180	159	140	117	100	86

Nr of peptides						
0	28	23	20	20	16	12
1	56	49	42	36	29	26
2	59	52	46	39	32	27
3	57	50	49	39	35	29
4	49	42	35	31	26	18
5	25	23	20	15	13	15
6	7	7	6	7	5	6

Figure 1: Association between different ACPA fine-specificities and radiographic progression.
(A,B) Median Sharp-van der Heijde scores (SHS) during 5 year follow-up of ACPA positive patients from the Leiden EAC testing positive or negative for six different ACPA fine-specificities at baseline. (C) Median SHS in ACPA-positive patients grouped by the number of ACPA fine-specificities recognized. All statistical comparisons (repeated measurement analysis) were non-significant ($p>0.05$). Numbers of patients in each group are given in the table below each figure. The line representing the group of patients recognizing six fine-specificities diverts from the rest due to the low number of patients in this group. In order to highlight this, this group is depicted with a dotted line. Radiographic data was available for n=266 ACPA-positive patients at baseline and n=132 at year 5.

difference in radiographic outcome in patients who harbour ACPA of one of the fine-specificities tested. Also, patients with ACPA that recognized more citrullinated peptides did not suffer from a higher degree of progression of joint damage than patients with a limited ACPA repertoire (figure 1, panel C). These data indicate that harbouring ACPA of any of the specificities tested has no direct influence on the progression of radiographic joint damage.

Stratification for ACPA status abolishes the effect of HLA SE-alleles on radiographic joint damage

None of the fine-specificities tested was found to associate with radiographic progression. A drawback of this analysis is that lack of association does not exclude that the investigation of other citrullinated peptides would have revealed a positive association with long-term joint damage. As this limitation can persist in case more (non-associating)

reactivities would be analyzed, we reasoned that an additional way to investigate the relationship between the ACPA recognition profile and radiographic outcome is to study the effect of SE-alleles on joint damage in ACPA-positive disease only. Since SE-alleles affect the recognition of certain citrullinated epitopes (table 1), SE-alleles can be interpreted as a surrogate marker for the constitution of the ACPA response.

While SE-alleles associated with the degree of radiographic joint damage in the total RA population (figure 2A), they no longer contributed to radiographic joint damage in ACPA-positive disease (figure 2B). We sought replication of this observation in the Norwegian EURIDISS cohort. The findings in this cohort with 5 and 10-year radiographic follow-up confirmed our results: there was no association between the presence of SE-alleles and radiographic damage in ACPA-positive disease (figure 2C and D).

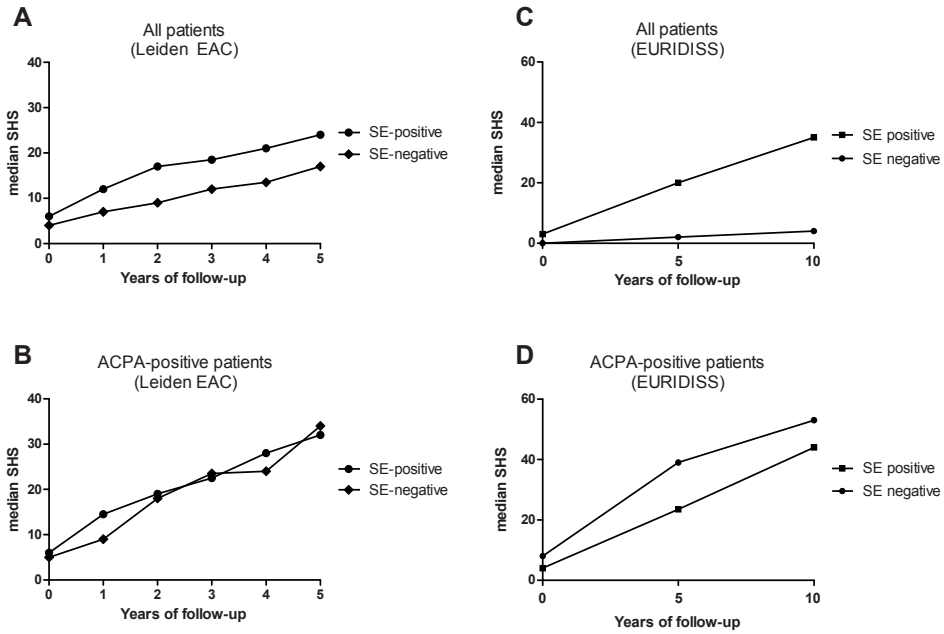


Figure 2: Shared epitope (SE) alleles exert no effect on radiographic progression after stratification for ACPA.

Median Sharp-van der Heijde scores (SHS) in relation to SE positivity in the entire RA population and after stratification for ACPA-positive disease in patients of the Leiden Early Arthritis Clinic (A/B) and of the EURIDISS cohort (C/D). For the Leiden EAC: radiographic data was available for n=481 patients at baseline and n=214 at year 5, of which n=266 were ACPA-positive (data for n=132 available at year 5). (A) Repeated measurement analysis: $p = 0.013$; Mann-Whitney U test $p < 0.001$ at year 1, $p < 0.001$ at year 2, $p = 0.007$ at year 3, $p = 0.005$ at year 4, $p = 0.012$ at year 5; (B) repeated measurement analysis: $p > 0.05$; all Mann-Whitney U p-values > 0.05. For the EURIDISS cohort: radiographic data was available for n=154 patients at baseline and n=142 at year 10, of which n=94 were ACPA-positive (data for n=83 available at year 10). (C) Mann-Whitney U test $p = 0.029$ at baseline, $p < 0.001$ at year 5, $p < 0.001$ at year 10; (D) Mann-Whitney U test $p = 0.36$ at baseline, $p = 0.93$ at year 5, $p = 0.97$ at year 10.

DISCUSSION

Recent findings on ACPA fine-specificity and its association with SE alleles have fuelled a hypothesis in which (a) ACPA are pathogenetically driving the disease and in which (b) certain ACPA fine-specificities (developing preferentially under the influence of SE-alleles) could be more pathogenic than others, leading to more severe joint destruction over time[4,13]. Further insights into which ACPA fine-specificities might be associated with disease severity could therefore have prognostic value and contribute to our understanding of disease pathogenesis.

Here, we could not detect an association between ACPA fine-specificities and radiographic joint damage. An anti-citrulline immune response to 3 out of 6 of the epitopes studied developed preferentially in patients harboring SE-alleles, but this did not translate into more severe radiographic outcome. Also, the number of citrullinated epitopes recognized by an individual patient did not influence the degree of joint destruction. Although we accounted for baseline treatment strategy in this analysis, and although no association between year of inclusion and recognition of the separate ACPA fine-specificities was found, we cannot fully exclude that treatment effects later in the disease course could have influenced our results. The number of citrullinated epitopes also limits our study, and it cannot be excluded that other epitopes would have been more useful for this purpose. We addressed this issue by using SE-alleles as a surrogate marker for those ACPA fine-specificities that develop under the influence of SE-alleles. After stratification for ACPA, SE-alleles no longer contributed to joint damage. Based on this finding, we consider it unlikely that a SE-associated ACPA fine-specificity can be identified that predicts disease course in RA. If such a predictive recognition profile exists, antibodies recognizing this epitope are likely to be generated independent of SE-alleles.

Our findings are relevant for strategies aimed at identifying patients that are at risk for rapidly progressive disease and provide evidence that the recognition profile of the ACPA response is unlikely to have a relevant impact on radiographic progression.

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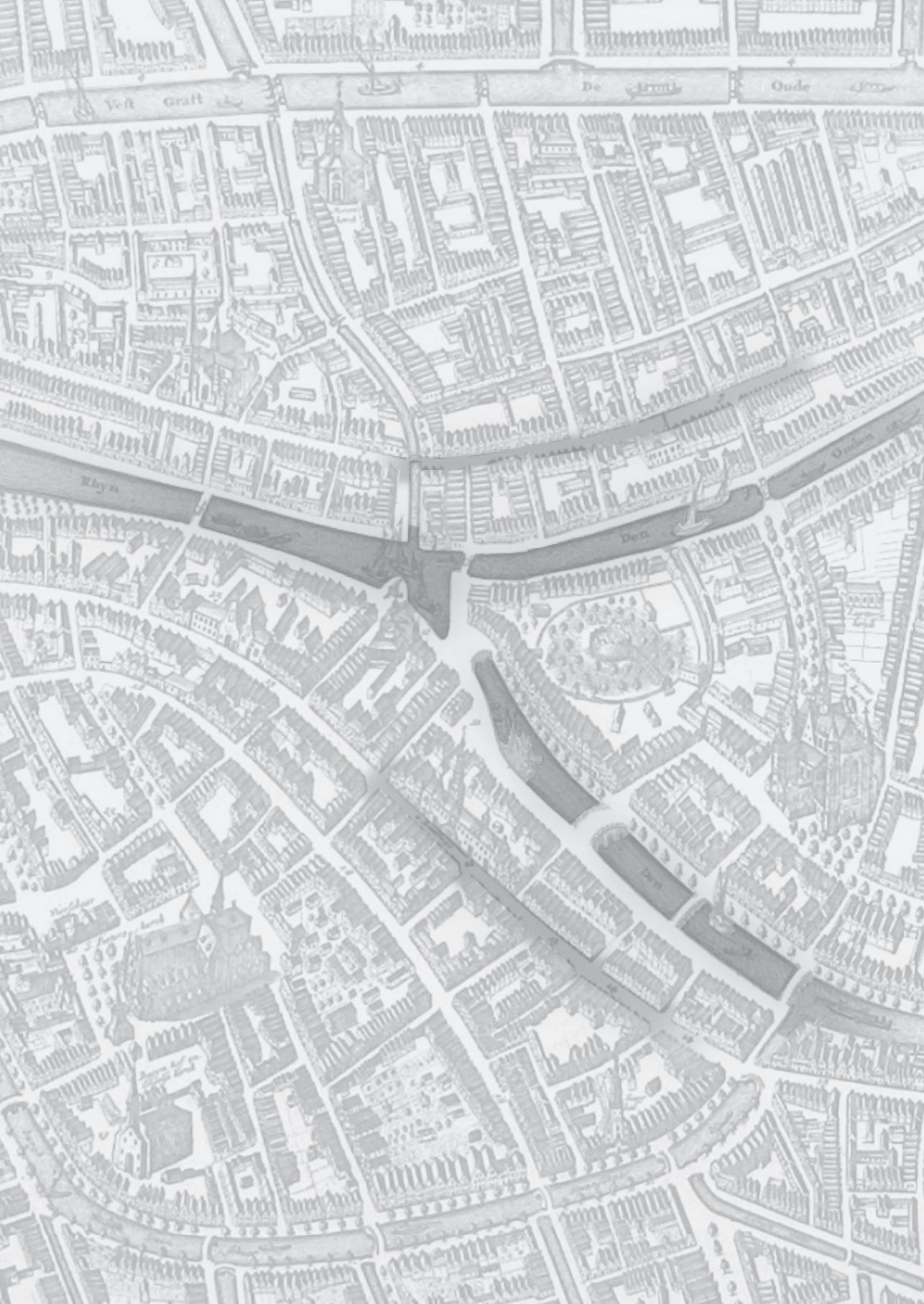
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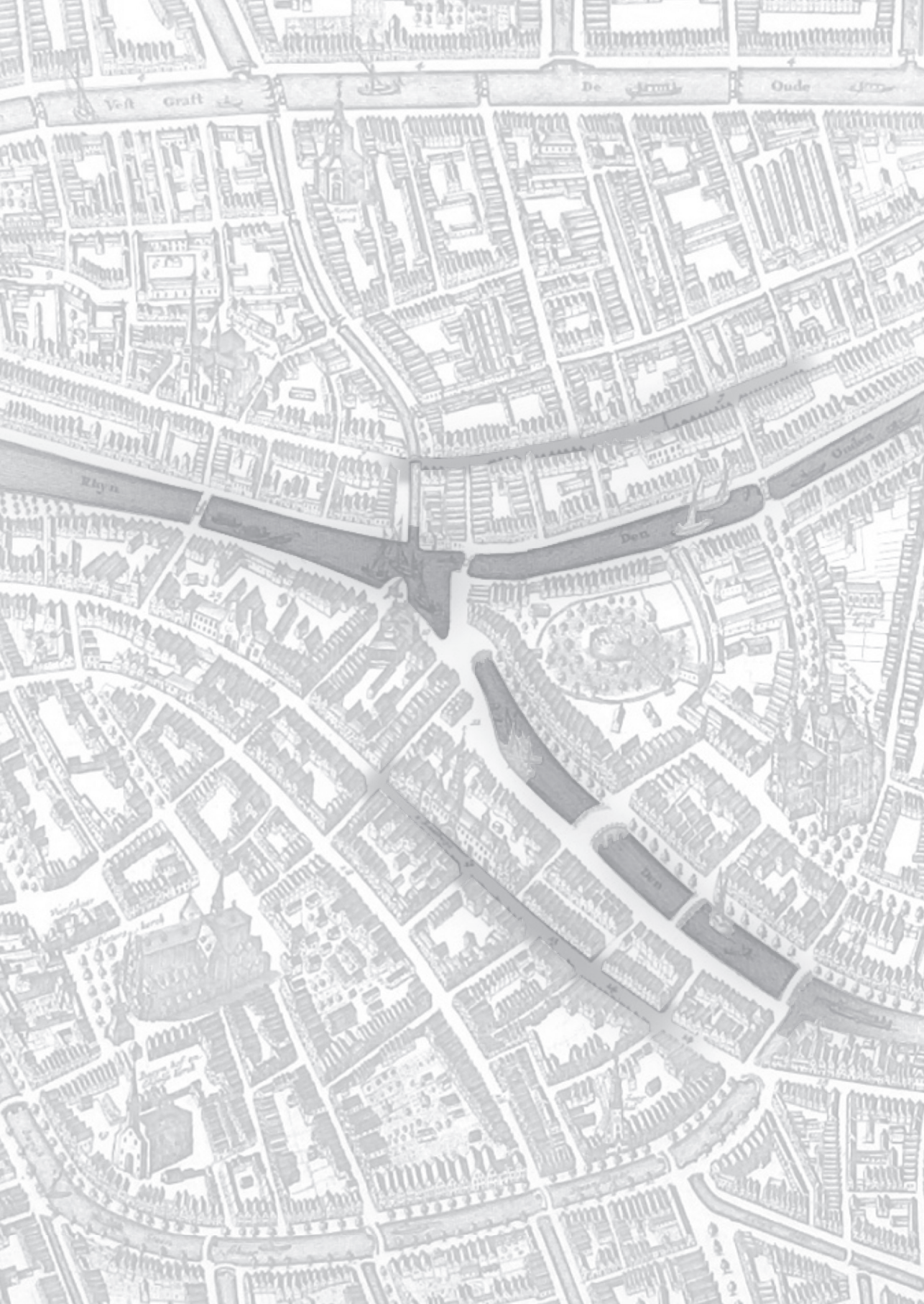
Part III

Genetic contribution to joint destruction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Chapter 7

Association of the 6q23 region with the rate of joint destruction in rheumatoid arthritis.

Scherer HU, van der Linden MP, Kurreeman FA, Stoeken-Rijsbergen G, Cessie S, Huizinga TW, van der Helm-van Mil AH, Toes RE.

Ann Rheum Dis. 2010 Mar;69(3):567-70.

ABSTRACT

Objective: Two novel genetic polymorphisms on chromosome 6q23 are associated with susceptibility to rheumatoid arthritis (RA). Both polymorphisms (rs6920220 and rs10499194) reside in a region close to the gene encoding tumor necrosis factor α-induced protein 3 (TNFAIP3). TNFAIP3 is a negative regulator of NFκB and as such involved in inhibiting TNF-Receptor mediated signalling effects. Interestingly, the initial associations were detected in patients with long-standing RA. However, no association was found for rs10499194 in a Swedish early arthritis cohort. As this could be caused by overrepresentation of patients with severe disease in cohorts with long-standing RA, we analyzed the effect of the 6q23 region on the rate of joint destruction.

Methods: Five single nucleotide polymorphisms (SNPs) in 6q23 were genotyped in 324 Dutch patients with early RA. Genotypes were correlated to progression of radiographic joint damage for a follow-up time of 5 years.

Results: Two polymorphisms (rs675520 and rs9376293) associated with severity of radiographic joint damage in ACPA+ patients. Importantly, the effects were present after correction for confounding factors such as secular trends in treatment.

Conclusions: Our data associate the 6q23 region with the rate of joint destruction in ACPA+ RA.

INTRODUCTION

Recent whole genome association scans have revealed novel genetic polymorphisms associated with susceptibility to ACPA+ RA[1,2]. Among those, two single nucleotide polymorphisms (SNPs), rs6920220 (A allele) and rs10499194 (C allele), were found to independently associate with ACPA+ disease. Both SNPs map to a single linkage disequilibrium block spanning ~60 kb in a region on chromosome 6q23 that lacks known genes or transcripts. The closest genes are oligodendrocyte lineage transcription factor 3 (*OLIG3*) and tumor necrosis factor α-induced protein 3 (*TNFAIP3*). The latter is of potential importance to RA pathogenesis, as the protein TNFAIP3 acts as a negative regulator of NF-κB[3]. So far, however, functional relevance of the reported polymorphisms is unknown.

Rs6920220 was initially identified in ACPA+ RA patients (minor allele OR 1.38) originating from the United Kingdom (UK)[1]. It was further replicated in an extended UK-based case-control study[4]. Rs10499194 was initially identified in North American ACPA+ patients (the Brigham Rheumatoid Arthritis Sequential Study, BRASS; minor allele OR 0.67)[2]. Replication was successful in two additional US cohorts selected from the North American Rheumatoid Arthritis Consortium (NARAC). Replication failed, however, in ACPA+ patients of a Swedish population-based inception cohort (the Epidemiological Investigation of Rheumatoid Arthritis cohort, EIRA)[2]. This latter finding is of interest, as both BRASS and NARAC are cohorts of patients with long-standing RA (mean disease duration BRASS: 15.4 ± 12.8 years[5]; NARAC: 14.3 ± 11.1 years[6]). The EIRA study, however, was designed to identify incident cases of RA as soon as possible after disease onset, resulting in an estimated mean disease duration at inclusion of only 10 months[7].

Association of a genetic polymorphism in cohorts of patients with longstanding disease but absence of this association in an early arthritis cohort led us to hypothesize that the 6q23 region would associate with disease severity in ACPA+ patients. Very little information is currently available on the effects of genetic variation on outcome measures in RA[8]. Therefore, we genotyped five SNPs in a Dutch early arthritis cohort (the Leiden Early Arthritis Clinic, EAC) and correlated genotyping data to progression of radiographic joint damage for a maximum follow up of 5 years.

PATIENTS AND METHODS

Patients

The Leiden EAC is a population-based inception cohort that includes patients with self-reported symptom duration of ≤ 2 years[9]. DNA samples of 324 patients consecutively

included between 1993 and 2003 were used for analysis. For further details see supplementary file 1.

SNP selection and genotyping

Five SNPs (rs1878658, rs675520, rs9376293, rs10499194 and rs6920220) were selected based on a haplotype analysis across the 6q23 locus published previously.[2] All SNPs are in imperfect linkage disequilibrium to one another (supplementary table 1). Genotyping was performed using pre-designed Taqman allelic discrimination probes (Applied Biosystems). Each 384 well plate contained 10 ng sample DNA per well and at least 8 negative and 6 positive controls. Genotype calls and clusters were manually checked for discrepancies and doubtful calls were rejected. No SNP deviated from Hardy-Weinberg equilibrium. Genotyping call rates were 96.5% (rs1878658), 98% (rs675520), 95% (rs9376293), 94% (rs10499194), and 98.1% (rs6920220).

Serology and Radiographs

Serum samples were tested for citrulline-specific IgG antibodies using a commercially available ELISA kit (Immunoscan Mark2, Eurodiagnostica, The Netherlands). Radiographs were scored according to the Sharp van der Heijde method [10] with known time order by one blinded, independent trained reader (supplementary file 1).

Statistical analysis

Association between genotypes and radiographic scoring data was analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL). P-values < 0.05 were considered significant. All p-values reported are two-sided.

Two approaches were chosen for statistical analysis. First, the average increase in Sharp van der Heijde scores during the follow-up period was estimated per person by regression analysis. Subsequently, the average increase (slope) of scores per genotype was compared non-parametrically using the Mann-Whitney rank-sum test.

We observed an influence of the time of inclusion (1993-2003) on the progression of radiographic joint damage reflecting most likely an improvement of treatment intensity during this 10 year time period. In order to account for this effect, we performed, as a second approach, a mixed model analysis described in detail in supplementary file 1.

RESULTS

Radiographic scores of 324 Dutch RA patients (181 ACPA+, 143 ACPA-) were available for analysis. At least five radiographic follow-up observations were available in 57% of patients. A dominant model was chosen for analysis, as the frequency of patients homo-

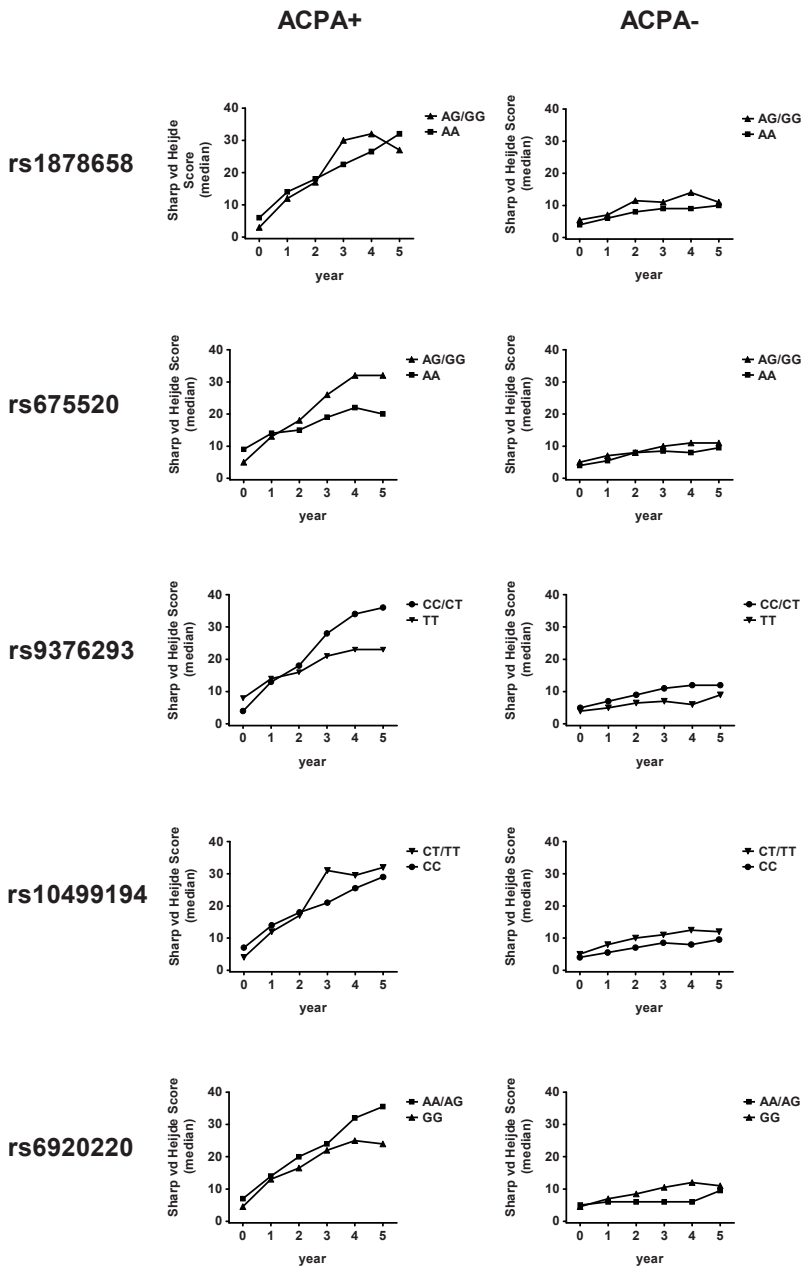

Figure 1: Development of median Sharp van der Heijde scores plotted according to genotype/allele in ACPA+ (left column) and ACPA- (right column) RA patients. Year 0 equals baseline-values. Regression analysis was performed in order to estimate the average increase (slope) in Sharp van der Heijde scores over time. Slopes were subsequently compared using the non-parametric Mann-Whitney test (for the ACPA+ subgroup: p = 0.37 (rs1878658); p = 0.007 (rs675520); p = 0.021 (rs9376293); p = 0.05 (rs10499194); p = 0.76 (rs6920220)).

zygous for the minor allele of rs1878658 (G), rs10499194 (T) and rs6920220 (A) was ≤ 5%. Figure 1 depicts the influence of genotypes on radiographic joint damage. ACPA+ and ACPA- subgroups were analyzed separately. Median scores and interquartile ranges (IQR) are provided for ACPA+ patients in table 1 (for ACPA- patients see supplementary table 2).

No influence of genotypes on radiographic joint damage was observed in ACPA- patients (figure 1). In ACPA+ patients, however, two polymorphisms showed reproducible association with disease progression over time. Presence of the G allele of rs675520 was found to associate with increased Sharp van der Heijde scores, as a significant difference was observed when the average increase (slope) in radiographic scores over time was compared with G as the dominant allele (median slope AG/GG = 4.6, AA = 2.3; Mann-Whitney p = 0.007). In order to account for an effect of improving treatment strategies on radiographic progression during the 10 year period in which patients were included into the study, we next performed a mixed model analysis. This analysis identified the year of inclusion as a significant variable influencing the extent of radiographic joint damage (p = 0.005). After correcting for the year of inclusion, however, we still observed a significant influence of the G allele of rs675520 (AG/GG vs. AA, p = 0.026).

Similar to the G allele of rs675520, we noted an influence of the C allele of rs9376293 on progression of radiographic joint damage (figure 1). The average increase (slope) in Sharp van der Heijde scores over time was significantly higher for C allele carriers as compared to T homozygotes (median slope CC/CT = 4.5, median slope TT = 3.0, Mann-Whitney p = 0.021). After correcting for the year of inclusion as described above a trend effect of the C allele remained (p = 0.097).

For rs1878658, rs10499194 and rs6920220, no significant influence of individual genotypes on radiographic joint damage was noted.

DISCUSSION

The 6q23 region has recently been associated with disease susceptibility in RA. This region contains no known transcripts. The closest genes with known function are *OLIG3* and *TNFAIP3*. *TNFAIP3* encodes protein A20, a TNF-α induced negative regulator of NF-κB[3,11]. Decreased levels of A20 lead to uncontrolled NFκB-activity, resulting in increased inflammation. This observation makes *TNFAIP3*/A20 and the 6q23 region interesting candidates that could modulate inflammation also in RA.

We were intrigued by recent differential findings for rs10499194, a SNP on chromosome 6q23 close to *TNFAIP3*, in cohorts with differing disease duration. The major allele (C) was found to associate with disease susceptibility in ACPA+ RA patients in three cohorts with long-standing disease, but not in an early arthritis cohort[2]. This indicated

Table 1: Median Sharp van der Heijde scores (M) and interquartile ranges (IQR; 25 - 75% percentiles) per genotype for ACPA+ RA patients (# = number of patients). Genotypes were combined for rs1878658 (G), rs10499194 (T) and rs6920220 (A), as the frequency of patients homozygous for the respective minor allele was ≤5% for these SNPs.

	year																	
	0			1			2			3			4			5		
ACPA+	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**
rs1878658																		
AA	6	2 - 13	126	14	6 - 24	111	18	10 - 32	113	22.5	11 - 42	98	26.5	14 - 51.5	86	32	14 - 51.5	89
AG/GG	3	1 - 7	40	12	5.5 - 20.5	40	17	6 - 32	39	30	11.5 - 56.5	34	32	16 - 69	31	27	17 - 74	31
rs675520																		
AA	9	4.5 - 13	33	14	6.5 - 21	32	15	8 - 27.5	33	19	9 - 34	27	22	13.5 - 39.5	24	20	12.5 - 47.5	21
AG	5	1 - 10	83	12	5 - 21	73	18	9.5 - 32.5	78	25	11.5 - 54	68	32.5	14 - 61	58	33	17 - 62.5	66
GG	5	2 - 11	51	14	7 - 27	48	18	8 - 37	43	26	14 - 48	39	28	16 - 63	37	32	20 - 64	35
AG/GG	5	2 - 10	134	13	6 - 24.5	121	18	9.5 - 33	121	26	12 - 53	107	32	15 - 61	95	32	18.5 - 61	101
rs9376293																		
CC	6.5	3 - 13.5	28	15	9 - 28	27	27	12 - 49.5	25	33	15.5 - 58	24	35	16.5 - 62	24	35	16.5 - 56	21
CT	4	1 - 9	84	12	5 - 21	79	17	7 - 32.5	78	21	10.5 - 54	66	32	14 - 68	57	36	20 - 68	61
TT	8	3 - 13	54	14	6 - 21	46	16	10.5 - 25.5	50	21	12 - 30.5	44	23	13.5 - 38	38	23	13 - 47	40
CC/CT	4	2 - 10	112	13	6 - 26.5	106	18	8 - 36	103	28	11 - 54.5	90	34	15 - 63	81	36	19 - 67.5	82
rs10499194																		
CC	7	2 - 12.5	90	14	6 - 21.5	77	18	9.5 - 28	82	21	12 - 35	71	25.5	14 - 45	62	29	14 - 48	62
CT/TT	4	2 - 7.5	65	12	6 - 24.5	64	17	7 - 37	61	31	11 - 55	55	29.5	14.5 - 67	50	32	15 - 68	51
rs6920220																		
AA/AG	7	2 - 12	58	14	6 - 21	53	20	10 - 32.5	57	24	12 - 48	47	32	18 - 56	35	35.5	18 - 65	42
GG	4.5	2 - 10.5	108	13	6 - 25	99	16.5	8 - 30.5	96	22	11 - 48	86	25	14 - 61	84	24	14 - 51	79

a potential impact of the 6q23 region on disease severity. In order to test for such an impact, five SNPs were genotyped in a cohort of Dutch patients with early RA. These SNPs had previously been shown to identify common haplotypes in 6q23 [2]. We identified two SNPs for which presence of alleles was associated with increased joint destruction in ACPA+ patients. Carriers of the G allele of rs675520 developed increased Sharp van der Heijde scores over time. A similar effect, although weaker, was found for the C allele of rs9376293. Interestingly, no association was found for any of the SNPs in ACPA- individuals. Although this does not exclude a contribution of the 6q23-region to disease severity in ACPA- disease, the latter observation is in line with recent reports detecting an association of the 6q23 region with disease susceptibility in ACPA+ patients only [4]. No effect on disease severity was observed for rs10499194 and rs6920220. Based on our data we cannot rule out the possibility that either SNP exerts a weak effect that requires larger sample numbers for detection or that cannot be observed during the first years of disease. Interestingly, we observed nominally higher scores for the risk-conferring A allele of rs6920220 without reaching statistical significance. The discrepancy between SNPs associating with susceptibility and radiographic progression also indicates that the causal variant at this locus has not yet been identified. Given the large area of linkage disequilibrium surrounding these SNPs, further fine-mapping and functional characterization will have to be performed.

Data linking newly identified genetic polymorphisms to disease outcome in RA are only beginning to emerge. Our data are unique, as they cover a long period of radiographic follow-up and have been scrutinized for artefacts such as secular trends in treatment intensity. Albeit based on relatively low patient numbers, our data indicate a contribution of the 6q23 region to the rate of joint destruction in ACPA+ RA, thereby further refining our understanding of the effects exerted by this locus. Replication of our findings in other cohorts is needed. Nonetheless, this is the first study demonstrating such an effect for genetic polymorphisms located outside the HLA-region in ACPA+ RA patients.

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SUPPLEMENTARY INFORMATION ON PATIENTS AND METHODS

Patients

All patients met the American College of Rheumatology 1987 revised classification criteria for RA and were of Caucasian origin based on self-reported ethnicity. Written informed consent was obtained from all participants, and the study was approved by the local institutional review board.

The Leiden Early Arthritis Clinic (EAC) is a population-based inception cohort that includes patients with self-reported symptom duration of ≤ 2 years. Follow-up visits are performed and radiographs of hands and feet are taken on a yearly basis. DNA samples of 324 patients (67.6% female; mean age 56.3 ± 15.4 years) consecutively included between 1993 and 2003 for who radiographic scoring data and ACPA status were available were used for analysis. 2003 as the latest year of inclusion was chosen in order to allow a five year follow-up period for all patients.

Radiographic scoring

The number of patients with available radiographs varied per time-point (for ACPA+ patients: n = 168 at baseline and n = 153, 154, 134, 119, and 122 at year 1 to 5, respectively; for ACPA- patients: n = 135 at baseline and n = 121, 109, 93, 81, and 65 at year 1 to 5, respectively). In total, radiographs of 324 patients (181 ACPA+, 143 ACPA-) were used for analysis. All radiographs were scored by one experienced reader who was blinded with respect to the patient's autoantibody status, treatment, clinical outcome and genotyping results. Scoring was performed with known time order, which is more sensitive to change compared to scoring with unknown time sequence[1]. For quality control, radiographs of 60 randomly selected RA-patients were rescored by the same reader. This selection comprised 499 radiographs, consisting of 149 baseline radiographs and 350 radiographs during follow-up. Reliability of radiographic scoring was calculated. Intraclass-observer correlation coefficients (ICC) were 0.91 for all scored radiographs, 0.84 for baseline radiographs and 0.97 for the radiographic progression rate.

Statistical analysis

Four different treatment strategies were applied to patients included in the EAC depending on the year of inclusion. Patients included between 1993 and 1995 were treated initially with analgesics and subsequently with chloroquine or sulphasalazine if they had persistent active disease (delayed treatment)[2]. From 1996 to 1998 patients were promptly treated with either chloroquine or sulphasalazine (early treatment)[2,3]. From 1998 to 2002 patients were promptly treated with either sulphasalazine or methotrexate (early treatment) and patients included in 2002 or later were promptly treated with either

sulphasalazine or methotrexate combined with treatment adjustments based on disease activity (early and disease activity based treatment).

To take advantage of the prospective character of the EAC, consisting of repeated measurements, and to avoid multiple testing by performing statistical tests for each time point, a linear mixed model for longitudinal data was used, with the log transformed sharp score as response variable, to compare the radiological progression between genotype groups. We explored different correlation structures between the repeated measurements, and based on the Akaike's information criterion, an autoregressive correlation structure with heterogeneous variances was chosen. This 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. The year of inclusion into the study was entered into the model to correct for possible confounding effects. 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 [2]. The interaction between treatment strategy (i.e. inclusion year) and time was significant in all five analyses of the present study ($p<0.05$).

SUPPLEMENTARY TABLE 1:

Table 1: Comparison of the LD-parameters obtained from HapMap (CEU population in rel 24 Phase II Nov 08) and the Leiden dataset.

		HapMap CEU		Leiden Dataset	
SNP1	**SNP2**	**D'**	**r^2**	**D'**	**r^2**
rs1878658	rs675520	1.0	0.112	1.0	0.155
rs1878658	rs9376293	1.0	0.274	1.0	0.263
rs1878658	rs10499194	1.0	0.623	0.98	0.5
rs1878658	rs6920220	1.0	0.028	0.929	0.04
rs675520	rs9376293	0.931	0.356	0.893	0.482
rs675520	rs10499194	1.0	0.191	0.98	0.289
rs675520	rs6920220	1.0	0.209	0.987	0.289
rs9376293	rs10499194	1.0	0.441	0.982	0.478
rs9376293	rs6920220	1.0	0.102	0.985	0.172
rs10499194	rs6920220	1.0	0.045	0.988	0.087

SUPPLEMENTARY TABLE 2:

Table 2: Median Sharp van der Heijde scores (M) and interquartile ranges (IQR; 25 - 75% percentiles) per genotype for ACPA- RA patients (# = number of patients). Genotypes were combined for rs1878658 (G), rs10499194 (T) and rs6920220 (A), as the frequency of patients homozygous for the respective minor allele was ≤5% for these SNPs.

	year																
	0			1			2			3			4			5		
ACPA-	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**	**M**	**IQR**	**#**
rs1878658																		
AA	4	2 - 10	103	6	3 - 12	95	8	3 - 18	79	9	5 - 17.5	69	9	3 - 19.5	57	10	5 - 22	47
AG/GG	5.5	1 - 12	30	7	4 - 15	25	11.5	4 - 21.5	28	11	4 - 21	23	14	4 - 24	23	11	4 - 27	18
rs675520																		
AA	4	2 - 7	28	5.5	1.5 - 16	26	8	5 - 21	21	8.5	4 - 14	20	8	2.5 - 19	16	9.5	5.5 - 19.5	16
AG	5	1 - 10	71	6	3 - 12	64	7.5	3 - 14.5	56	8	5 - 19	49	10	3 - 20	42	10	4.5 - 20.5	33
GG	5.5	1 - 11	36	8	2 - 21	31	9.5	4 - 21.5	32	12	7 - 22.5	24	14	5 - 26	23	14.5	6 - 31.5	16
AG/GG	5	1 - 10	107	7	3 - 12	95	8	3 - 16.5	88	10	5 - 20.5	73	11	4.5 - 23.5	65	11	5 - 27	49
rs9376293																		
CC	4.5	0.5 - 11	22	5	1 - 10	19	9	1 - 12	19	12	5 - 19.5	17	12	2.5 - 20	13	9	3.5 - 44.5	8
CT	5	2 - 11	69	7	4 - 17.5	61	8	4 - 20.5	57	11	5 - 21.5	44	12	5 - 25	43	12.5	6 - 27	34
TT	4	2 - 7	43	5	2 - 10	41	6.5	3 - 18.5	32	7	3 - 11	31	6	3 - 10.5	24	9	4 - 14	23
CC/CT	5	1 - 11	91	7	4 - 15	80	9	3.5 - 17.5	76	11	5 - 21.5	61	12	5 - 23.5	56	12	5 - 27	42
rs10499194																		
CC	4	1.5 - 7	76	5.5	2.5 - 11.5	70	7	3 - 16	56	8.5	5 - 16.5	52	8	3 - 17.5	44	9.5	5.5 - 20.5	36
CT/TT	5	1 - 12	53	8	3 - 17.5	46	10	3.5 - 21.5	49	11	4.5 - 22	38	12.5	4.5 - 24.5	36	12	4 - 27	28
rs6920220																		
AA/AG	5	2 - 8.5	45	6	3 - 12	39	6	3 - 21	31	6	3 - 14	29	6	3 - 12	23	9.5	2.5 - 17.5	18
GG	4.5	1 - 10	88	7	2.5 - 13.5	81	8.5	4 - 18	76	10.5	6 - 20.5	62	12	5 - 22.5	57	11	5 - 27	46

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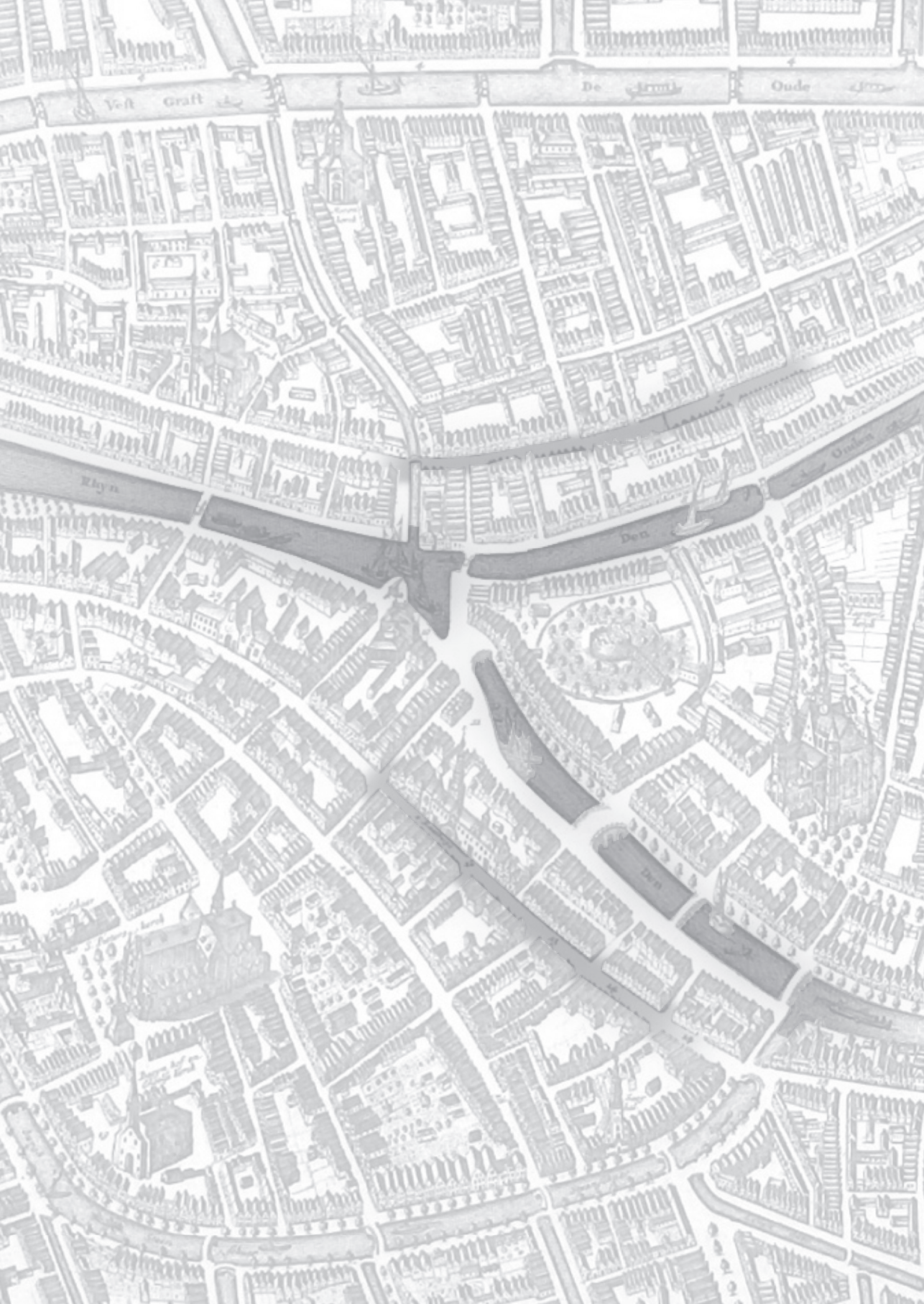
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Chapter 8

Summary and Discussion

PART I: T CELL CONTROL OF INFLAMMATION

Regulatory T (Treg) cells are an attractive tool for the immunotherapy of autoimmune diseases. This is based on the notion that Treg cell deficiency or dysfunction is part of the pathogenetic mechanism underlying these conditions, but also on the important immunosuppressive properties of these cells [1-3]. Crucial to this belief is the observation that severe multiorgan autoimmunity develops in the complete absence of Treg cells, for which examples exist in both mice and men [4,5]. Isolating T cells from peripheral blood of a diseased patient, generating or expanding a regulatory T cell subset *in vitro*, and re-infusing functional Treg cells in high quantity as cellular components that suppress inflammation comes close to the perception of an ideal, patient tailored therapeutic concept. Enticing is the use of the patient's own cells, which excludes graft rejection. Prerequisite for such an approach, however, is detailed knowledge on how Treg cells suppress inflammation, which subsets are responsible for the suppressive effects, and which role Treg cells play in the development and chronicity of the respective disease.

Collagen induced arthritis (CIA) is a mouse model that shares many clinical, serological and histological characteristics with human RA [6]. Amongst other features, the development of antibodies to collagen type II (CII) and to self-antigens is considered crucial to its pathogenesis [7,8]. These antibodies develop upon immunization of susceptible mice with heterologous CII in combination with an adjuvant [7]. T cells in this model are thought to mainly provide help to B cells during their development into antibody producing plasma cells. Indeed, depletion of Treg cells before disease induction leads to increased titers of collagen type II specific antibodies and to enhanced disease severity [9]. This is in line with the direct suppressive effects of Treg cells on effector T cells described in *in vitro* studies [10]. Conversely, one would expect that effective inhibition of effector T cell function would lead to a reduction in antibody titers, as less B cells receive the T cell help needed to differentiate into plasma cells. Earlier work has demonstrated that a single adoptive transfer of Treg cells in established CIA can indeed effectively inhibit arthritis [11]. Of interest, however, this effect was not paralleled by a reduction of the CII-specific antibody response. This intriguing observation indicates that Treg cells can dampen inflammation by mechanisms other than the inhibition of effector T cells. As a proof of principle, we injected mice with complete Freund's adjuvant to induce a T cell independent acute phase response, as evidenced by the production of serum amyloid P. Also here, adoptive transfer of Treg cells could inhibit inflammation, underlining the ability of Treg cells to inhibit not only effector T cells, but also T cell independent immune responses (unpublished observation). **Chapter 2** describes experiments dedicated at elucidating the mechanism underlying this observation.

We hypothesized that the inhibition of T cell independent immune responses is likely to require a Treg cell derived soluble factor able to interfere with acute phase reactants.

Injection of adjuvants induces high levels of TNF-α, making soluble TNF receptors likely candidates for the effects observed [12]. In addition, Treg cells had previously been shown to express high levels of TNFRII [13]. Our studies indeed revealed that Treg cells, but not effector T cells, shed TNFRII from the surface upon stimulation, and that this soluble TNFRII is able to interfere with TNF-mediated effects both *in vitro* and *in vivo*. Importantly, the ability to shed TNFRII was also found for human Treg cells. Although CD25+FoxP3- human effector T cells constitutively expressed TNFRII, only the regulatory population expressing high levels of FoxP3 was capable of shedding the receptor upon stimulation. Additional analysis of the human Treg cell population showed that the HLA-DR expressing subgroup, which is known for its strong suppressive effects on effector T cells [14], also possessed the strongest TNFRII shedding capacity.

Several aspects of this observation have implication for Treg cells in RA. First, due to the important role of TNF-α in RA pathogenesis, TNFR shedding might be an important feature that could be exploited for optimizing Treg cell based immunotherapy. In fact, Etanercept, a soluble TNFRII fused to a human Fc tail, is a highly effective therapeutic in active RA [15]. Enrichment for the TNFR shedding population could therefore yield Treg cells with enhanced anti-inflammatory capacity for therapeutic use.

Second, our finding contributes to a better understanding of the interplay between TNF-α and TNFR expression with regard to Treg cell function in RA. In this context, two concepts are being debated that try to explain why Treg cells fail to control inflammation in this disease: impaired Treg cell function versus increased resistance of effector T cells to Treg mediated suppression [16]. Data on this issue are conflicting, as are data concerning the number and functionality of Treg cells in RA. Supporting the first concept, several studies have demonstrated inhibiting effects of TNF-α on Treg cells mediated by TNFRII, and found an expansion of Treg cells in RA patients upon treatment with TNF inhibitors [17-19]. These data are in contrast to more recent studies showing that TNF-α can promote, by signaling via TNFRII, expansion, proliferation and survival of Treg cells [20]. In fact, TNF-α even induced expression of TNFRII and, as in our study, constitutive TNFRII expression was found highest on those Treg cells that also most strongly expressed FoxP3. In line with this, TNFRII positive Treg cells possessed strong suppressive capacity [21], indicating that TNFRII expression is closely linked to suppressive function. Based on our study, it is likely that part of the anti-inflammatory effects exerted by these cells is mediated by shedding of soluble TNFRII. In line with these stimulatory effects of TNF-α for Treg cell function, and in keeping with the second concept, TNF-α was also found to induce resistance of effector T cells to suppression by Treg cells [22]. This concept fits with the finding of elevated TNFRII expression by synovial fluid Treg cells in RA, which also showed increased suppressive function *in vitro* [23-25]. Treatment with TNF-α inhibitors could, therefore, lead to sensitization of effector T cells for Treg mediated suppressive effects, partly explaining the beneficial effects of this treatment in RA. However, as the

network of molecules involved in these interactions is actually much larger, including the TNF superfamily member lymphotoxin (TNFβ) that can bind TNFRII, further study is warranted to fully understand the effects.

In summary of **chapter 2**, we demonstrated a novel mechanism by which Treg cells can inhibit T cell independent inflammation. This functional feature could be exploited for Treg cell based immunotherapy, while it contributes to the understanding of Treg cell function in RA.

PART II: CHARACTERISTICS OF THE IMMUNE RESPONSE TO CITRULLINATED ANTIGENS

RA develops on the basis of a heterogeneous, complex interplay of different factors that eventually determine its clinical presentation. Among the available biomarkers that help in diagnosis and risk assessment, anti citrullinated protein antibodies (ACPA) have proven most useful[26]. This is mainly due to their unique specificity and their prognostic value, both for disease development in the pre-RA period and for disease progression in established RA. Several aspects of the ACPA immune response remain puzzling, however, and raise the question as to what extent ACPA are truly involved in RA pathogenesis. Most striking is the observation that ACPA titers do not reflect disease activity, at least in established disease[27,28]. In fact, patients can undergo complete, drug free remission despite high ACPA serum titers. Also pre-disease, ACPA can be present for many years without causing apparent pathology. This indicates that ACPA could themselves be non-pathogenic but surrogate markers of a yet unknown, underlying immune response. Next to data from murine studies, however, several recent lines of evidence show a role of human ACPA in complement activation, mast cell degranulation and osteoclast activation[29-31]. These observations make it likely that ACPA do in fact contribute to the disease process. However, ACPA might require one or several additional components to acquire pathogenic effector functions, or could possess specific, variable features that determine pathogenicity. Part II of the thesis focuses on this latter aspect, with emphasis on features of the ACPA Fc tail and of the interaction between ACPA and its antigen.

Characteristics of the ACPA Fc tail

The Fc tail of human antibodies can activate complement and bind to specific cell surface receptors. In addition, the Fc tail of IgG can itself serve as antigen for rheumatoid factors (RF). The degree to which the Fc tail interacts with the immune system depends, in part, on the isotype. IgM and IgG_3 are potent activators of complement, while IgG_1 more strongly binds to activating Fc receptors on, for example, macrophages[32]. In addition, the Fc tail of IgG carries two highly variable glycan moieties, which can modulate its

inflammatory potential. Based on this, we hypothesized that the degree of ACPA pathogenicity could, in part, depend on its Fc specific glycosylation profile. This notion was fuelled by early studies that showed aberrant glycosylation of IgG molecules in RA[33].

An obstacle in the ACPA specific analysis of Fc linked glycans was the lack of methods to isolate ACPA to high purity. Therefore, we first developed a technique that allowed purification of ACPA from small quantities of serum samples in a high throughput manner **(chapter 3)**. This was achieved by purifying ACPA based on affinity to plate bound citrullinated antigen using commercially available ELISA plates. Eluting ACPA from these plates yielded sufficient amounts of IgG for Fc glycosylation analysis, while no non-specific IgG was found in eluates from plates incubated with ACPA negative serum. Subsequent to elution, IgG ACPA were digested with trypsin to peptide fragments of known mass, which allowed for the analysis of peptides carrying Fc-linked glycans by mass spectrometry. We noted, however, that not all ACPA were eluted from the plate under the conditions employed. As this could bias the analysis towards ACPA of low avidity, we additionally digested ACPA on plate while still bound to the antigen, and analyzed the resulting Fc glycopeptides in the same manner. Importantly, no difference was noted in the Fc glycosylation profile between eluted ACPA and those digested in-plate, indicating that the eluted fraction was representative of the overall ACPA glycan profile. In later studies on ACPA avidity (see below), we additionally analyzed Fc glycosylation profiles of high and low avidity ACPA separately, without observing specific differences (unpublished observation).

As a second step, we employed this technique to a set of serum and synovial fluid samples of patients with ACPA positive arthritis **(chapter 4)**. ACPA specific Fc glycosylation profiles and Fc glycosylation of total, non-specific IgG from individual patients were studied in parallel. This analysis revealed that ACPA indeed display a specific, pro-inflammatory glycan profile, characterized by a lack of sialic acid and galactose residues. This feature was most prominent in synovial fluid, i.e. the site of inflammation. This latter finding is of interest, as it shows qualitative differences between ACPA in different compartments. Importantly, the Fc glycosylation profile of the non-specific IgG fraction in serum and synovial fluid was comparable, making it unlikely that the lack of sialic acid and galactose residues was due to enzymatic modification of the antibodies post-secretion. It also indicates that ACPA specific B cells possess distinct features, which are not, or to a lesser extent, present in "conventional" B cells. Similar conclusions are supported by the observation that ACPA are primarily of low affinity **(chapter 6)**, which could point to a distinct developmental pathway of these B cells. We also noted that the agalactosylated G0 form of ACPA was particularly frequent in patients that harbored rheumatoid factor (RF). This is intriguing, as it suggests an interaction between ACPA and RF. Such an interaction is also suggested by observations from cohort studies, which report increased risk for RA development in individuals positive for both autoantibodies

as compared to patients positive for only ACPA[34]. Most RF bind to the CH2 domain of human IgG, which also harbors the Fc-glycosylation site[35]. Therefore, it is not unlikely that Fc-linked glycans can modulate RF binding. In fact, several studies have shown that RF bind preferentially to Fc domains with G0 glycans[36-39]. IgG molecules isolated from synovial fluid derived immune complexes also showed a high G0 content[40]. However, while these considerations could point to a facilitated formation of immune complexes containing ACPA and RF, they do not explain why individuals with G0-ACPA were found to be more frequently RF positive. To answer this question, it will be intriguing to analyze whether lack of galactoses on the ACPA Fc tail exposes immunogenic neoantigens that could trigger the development of RF.

In summary, in **chapters 3 and 4**, we describe characteristic features of ACPA, which are relevant to their inflammatory potential. As Fc glycosylation of human IgG can be modulated ex vivo[41], this offers interesting options for therapeutic intervention.

Characteristics related to antigen recognition

ACPA specific for a mutated form of citrullinated vimentin have recently been shown to bind to osteoclasts, thereby inducing osteoclast activation[31]. In line with this, human anti citrulinated vimentin antibodies, but not control IgG, enhanced bone degradation in the mouse. Although it was not examined if ACPA of other specificities would have similar effects, this finding raises the question as to whether certain ACPA fine specificities might be more pathogenic than others. In such a scenario, the composition of the ACPA fine specificity repertoire could variably change, while overall ACPA serum levels remain stable. In fact, the number of epitopes recognized by ACPA was found to broaden prior to disease onset and showed association with early disease progression[42,43]. Patients with undifferentiated arthritis that progressed to RA within one year recognized more citrullinated epitopes, including vimentin-derived peptides, than patients that remained in the undifferentiated state in the same period of time[43]. However, once RA had developed, no further broadening of the epitope recognition profile was noted. Also, no specific pattern or sequence of antigen recognition could be discerned that differentiated progressing from non-progressing patients[42,43]. This finding suggests that epitope spreading, without preference for a certain fine specificity, reflects merely a general progression of the ACPA specific immune response in the early stage of disease. Still, it was not possible to exclude increased pathogenicity of individual ACPA reactivity's based on these data. To address this question, we studied whether recognition of specific citrullinated antigens would relate to increased radiographic joint destruction over time **(chapter 6)**. Analyzing the ACPA recognition profile at the time of diagnosis was considered feasible, as no further broadening of the fine specificity repertoire at later stages had previously been noted[43]. Of interest, our analysis revealed that none of the fine specificities tested showed preferential association with the degree of joint destruction during 5-year follow-up.

Also the number of epitopes recognized did not relate to an increase in joint damage. As the number of epitopes analyzed was limited in this study, we additionally analyzed whether SE-alleles, which can serve as a surrogate marker for the composition of the ACPA fine specificity repertoire, would themselves favor a profile with increased risk for joint destruction. Also here, however, no effect of the SE-allele was noted within the ACPA positive patient group. Therefore, based on these data, we consider it unlikely that ACPA exert differential pathogenic effects due to recognition of specific epitopes.

Little is known on the origin of ACPA producing B cells, on their development and on the stage at which tolerance to citrullinated antigens is broken. Similar to the epitope recognition profile, also the number of ACPA isotypes present in RA sera is increased at the time of disease onset as compared to the pre-disease state[34,44]. Also here, no further spreading of the isotype profile was observed in established disease[45]. The continuous presence of ACPA IgM in some patients, however, points to a continuously regenerating immune response[44]. While these data underline the notion that mainly the magnitude of the ACPA response contributes to disease development, they also indicate that ACPA producing B cells undergo extensive isotype switching. Isotype switching is part of the B cell maturation process, requires T cell help, and occurs in parallel to affinity maturation in germinal centers[46]. Affinity maturation requires B cells to compete for antigen presented by follicular dendritic cells, and only those B cells that express high affinity B cell receptors receive sufficient survival signals to develop into antibody secreting plasma cells or memory cells[47]. As a result, classical germinal center reactions generate high affinity, class-switched B cells. While it is generally accepted that this applies to B cells that develop upon encounter of foreign antigens, e.g. upon vaccination or infection, it is uncertain whether the same holds true for autoreactive B cells. An important difference could be the abundant and continuous presence of self-antigen in autoimmune diseases, which could impair affinity maturation due to a lack of competition for antigen between B cells. To test this hypothesis in the context of the ACPA response, and in order to gain insight into the development of ACPA specific B cells, we studied the avidity of polyclonal ACPA, and compared it to the avidity of antibodies against recall antigens in the same patients **(chapter 5)**. Of interest, ACPA avidity was significantly lower than that of tetanus or diphtheria toxoid specific antibodies, with no difference between ACPA isolated from serum or synovial fluid. Moreover, ACPA avidity was low irrespective of ACPA titer, disease activity and the number of isotypes used. Also, no avidity maturation could be detected over time, and based on an additional limited number of citrullinated antigens studied no difference was noted with regard to antibodies of differing specificity. These data indicate that ACPA specific B cells undergo a different developmental process than “conventional” B cells, in that affinity maturation and isotype switching are uncoupled.

Taken together, based on the considerations above, we understand the ACPA response as a continuous, polyclonal reaction to self-antigens that broadens before disease onset and generates low avidity antibodies of all isotypes. Of those, IgG ACPA harbor Fc tails with a distinct, pro-inflammatory glycan profile, which might facilitate their interaction with RF. Once established, ACPA isotype usage, fine specificity repertoire and avidity remain stable. In this context, neither the number of antigens recognized, nor ACPA specific for a particular antigen known so far, seem to specifically drive the disease process.

PART III: GENETIC CONTRIBUTION TO JOINT DESTRUCTION

Genetic variation, in part, contributes to RA susceptibility. Although more than 30 genetic polymorphisms have so far been identified that show this association in patients of European descent [48], little is known on the underlying mechanisms by which genetic risk translates to biologic effects. Analysis is often difficult, as some of these polymorphisms are part of an inherited haplotype and do not necessarily represent the causal genetic variant. Other polymorphisms reside in intergenic regions with unknown function, rendering it difficult to predict their potential effects. Some indications, however, can come from careful analysis of the cohorts in which a polymorphism is found. We were intrigued by the observation that a single nucleotide polymorphism (SNP) that had been reported as a risk factor for ACPA positive RA, showed significant association with disease susceptibility in a cohort of patients with established RA but failed to associate with early RA [49-51]. We hypothesized that this could indicate a genetic contribution of the 6q23 region to disease severity. To this end, we studied association of this region with the degree of joint destruction in patients of the Leiden Early Arthritis Clinic **(chapter 7)**. The SNP initially identified resides in an intergenic region on chromosome 6q23, close to the gene coding for TNFAIP3 (A20). A20 is a protein involved in negative regulation of NFκB [52]. Variation in A20 gene expression or protein function could, therefore, directly impact on inflammation. In fact, mice deficient for A20 expresssion in myeloid cells spontaneously develop severe destructive polyarthritis with high levels of inflammatory cytokines in serum [53]. In analogy, overexpression of A20 by viral transfer of A20 cDNA to mice with collagen-induced arthritis via intra-articular injection induced sustained reduction in disease severity, also in non-injected joints [54].

When analyzing five SNPs that identify common haplotypes in the 6q23 region, two (rs675520 and rs9376293) significantly associated with joint damage in our study. Carriers of the G allele of rs675520 and of the C allele of rs9376293 showed increased joint destruction over time (5 years). Of interest, the SNPs associating with disease severity were not identical to the SNPs associating with disease susceptibility. This could partly be due to small sample size of our study, but also suggests that the causal variant in

this region has yet to be defined. Similarly, different SNPs in this region were found to associate with disease susceptibility in different ethnic groups (rs6920220 in Europeans and rs10499194 in Asians), pointing in the same direction [55].

Taken together, our study linked genetic variation in the 6q23 region to disease outcome in RA. While the causal variant is yet unknown, accumulating evidence on the protein level underlines that A20, encoded by *TNFAIP3*, the gene closest to the polymorphisms described, could indeed be involved in regulating inflammation in RA. Of interest, association of the 6q23 region with the degree of joint destruction has recently been replicated in another study, confirming our results [56].

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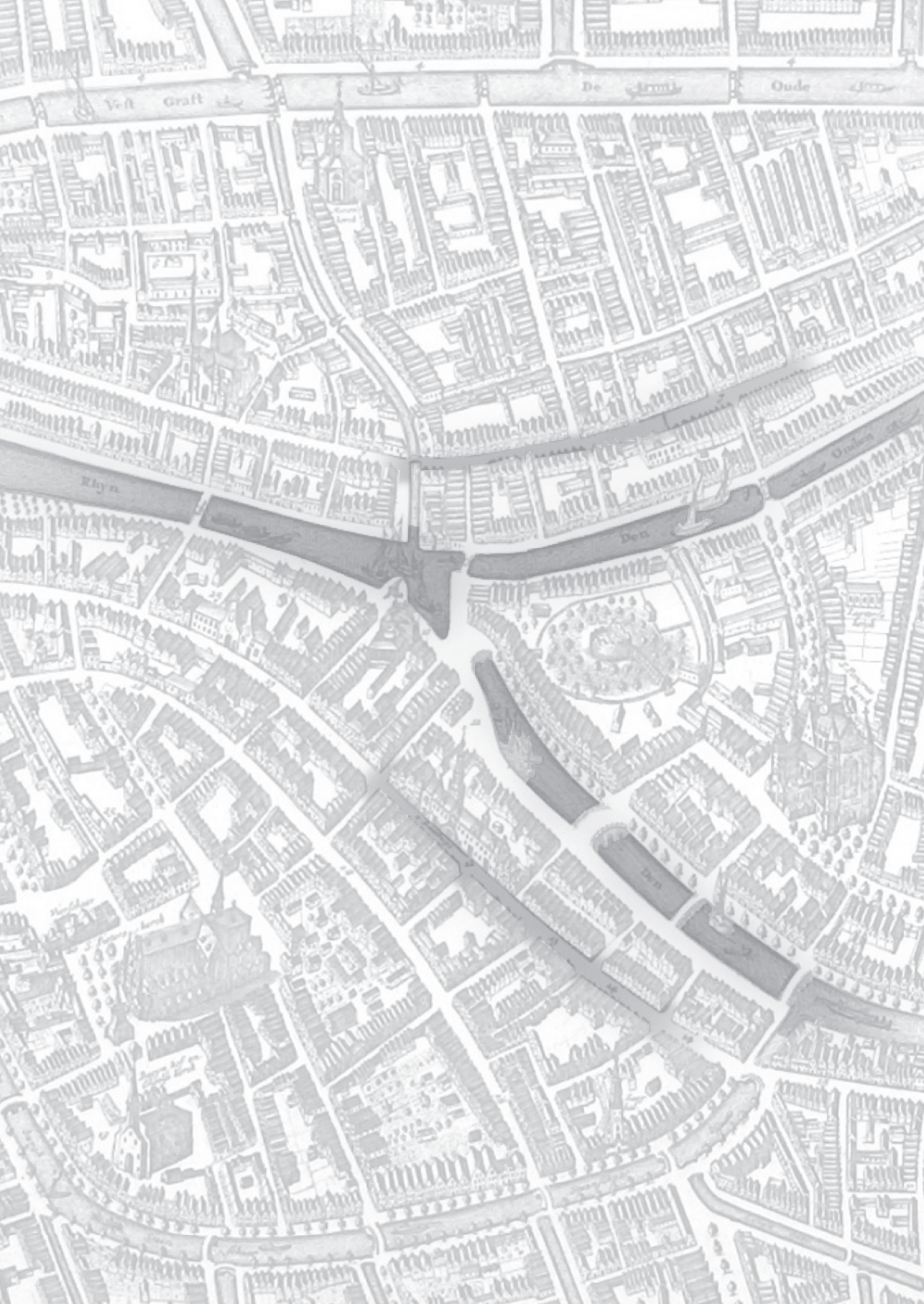
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Chapter 9

Nederlandse Samenvatting

NEDERLANDSE SAMENVATTING

Het ziektebeeld reumatoïde artritis (RA) ontstaat door een complex samenspel van verschillende factoren. Genetische aanleg vormt een belangrijke basis. Vervolgens zijn er effecten van omgevingsfactoren waarna autoantistoffen, afweercellen, hormonen en lokale factoren uit bot en kraakbeen leiden tot een karakteristieke vorm van gewrichtsontsteking. Hoewel het klinisch beeld van RA bij patiënten met lang bestaande ziekte typisch en onmiskenbaar is, is de presentatie van de patiënt in een vroeg stadium heel variabel. In deze fase is het beloop van het ziektebeeld vaak moeilijk in te schatten, omdat zowel rustige, zich sluipend verslechterende vormen als vormen met een agressief, snel tot gewrichtsschade leidend beloop voorkomen. Recent onderzoek heeft laten zien dat, op basis van de aanwezigheid van autoantistoffen tegen gecitrullineerde eiwitten (anti-citrullinated proteïne antistoffen, ACPA), het ziektebeeld valt in te delen in ten minste twee vormen: ACPA-negatieve en ACPA-positieve RA. ACPA zijn vaak al jaren voor de eerste klachten in het serum van patiënten aanwezig, ze voorspellen het ontstaan van RA en zijn markers voor een ernstiger beloop met veel gewrichtsschade. Ook ontwikkelen ACPA zich bij voorkeur bij patiënten met een specifieke genetische achtergrond. Vooral de aanwezigheid van bepaalde bloedgroepen op witte bloedcellen, de HLA allelen die als "shared epitope" allelen bekend staan, spelen een belangrijke rol bij RA. Aanvankelijk dacht men dat deze bloedgroep een risicofactor was voor het ontstaan van RA, maar het is duidelijk dat deze erfelijke factor alleen een risico vormt voor het ontstaan van ACPA-positieve ziekte. Gezien deze associaties is er grote belangstelling voor een beter begrip van de door ACPA veroorzaakte biologische effecten. Ook is het van belang kennis te hebben van de bijdrage die andere genetische risicofactoren of bepaalde afweercellen zoals T-cellen leveren aan het ontstaan van het ontstekingsproces. Het in dit proefschrift gepresenteerde promotieonderzoek omvat deze aspecten en is daarom onderverdeeld in drie delen. Deel I beschrijft het onderzoek naar een mechanisme waardoor de activiteit van ontsteking door zogenaamde regulatoire T-cellen kan worden geremd. Deel II beschrijft het onderzoek naar de eigenschappen van ACPA die deze ziekteactiviteit zouden kunnen beïnvloeden. Ten slotte beschrijft deel III het effect dat een recent gevonden genetisch variant kan hebben op radiologische gewrichtsschade ten gevolge van RA.

DEEL I: CONTROLE VAN ONTSTEKING DOOR REGULATOIRE T-CELLEN

Regulatoire T (Treg)- cellen hebben als voornaamste eigenschap dat zij de functie van effector T-cellen kunnen remmen. Hierdoor kunnen Treg-cellen het ontstaan van auto-immuniteit voorkomen. Dit wordt duidelijk in situaties waarbij door een genetisch defect geen Treg-cellen aanwezig zijn. In de muis, maar ook in de mens leidt een gebrek aan

Treg-cellen tot ernstige auto-immuun ziekte waarbij van bijna alle organen, inclusief de gewrichten, zijn betrokken. Gebaseerd op deze observatie is de gedachte geuit dat Treg-cellen bij patiënten met RA ontbreken of onvoldoende werkzaam zijn, dan wel dat effector T-cellen ongevoelig zijn geworden voor de remmende effecten van Treg-cellen.

In een proefdiermodel voor artritis spelen effector T-cellen een belangrijke rol bij het ontwikkelen van de ziekte doordat ze hulp verlenen aan B-cellen. Hierdoor kunnen deze B-cellen de juiste autoantistoffen aanmaken. Deze autoantistoffen zijn pathogeen en kunnen de ziekte overdragen aan een gezonde muis. De therapeutische toediening van Treg-cellen in dit model remt het beloop van artritis. Hierbij valt echter op dat de Treg-cellen geen invloed hebben op de hoeveelheid autoantilichamen. Deze bevinding gaf aanleiding tot de hypothese dat Treg-cellen ontsteking ook op een andere, effector T-cel onafhankelijke, wijze kunnen remmen. In **hoofdstuk 2** wordt een nieuw, hierbij passend, mechanisme beschreven. Geactiveerde Treg-cellen kunnen een oplosbare factor produceren die de effecten van TNF-α, een ontsteking veroorzakend eiwit, remt. TNF-α is een van de meest belangrijke mediatoren van ontsteking bij patiënten met RA, en therapeutische remming van TNF-α hoort bij de standaard behandelingen van deze ziekte. Treg-cellen dragen een receptor voor TNF-α, TNFRII, op hun oppervlak. Deze receptor wordt bij activering afgesplitst van de celwand. Dit geldt niet alleen voor Treg-cellen van de muis, maar werd ook voor humane Treg-cellen gevonden. Door de secretie van TNFRII wordt TNF-α weggevangen waardoor de ontsteking wordt geremd. Hoewel deze bevinding een verklaring kan zijn voor de positieve effecten van Treg-cellen op het beloop van artritis in de muis blijft het onduidelijk waarom dit mechanisme niet voldoende is om activiteit van RA in de mens te remmen. Uit verder onderzoek zal moeten blijken waarom Treg-cellen in patiënten met RA de ziekte niet kunnen voorkomen of onder controle kunnen brengen.

DEEL II: EIGENSCHAPPEN VAN DE AFWEERREACTIE TEGEN GECITRULLINEERDE EIWITTEN

Zoals beschreven hebben ACPA een grote diagnostische maar ook voorspellende waarde in het kader van RA. Recente data laten zien dat ACPA directe ziektebevorderende eigenschappen hebben waardoor ontsteking mede veroorzaakt of versterkt kan worden. ACPA kunnen echter ook in hoge concentratie aanwezig zijn zonder dat er sprake is van actieve gewrichtsontsteking. ACPA zijn in het bloed van gezonde mensen gevonden jaren voordat deze mensen klachten ontwikkelden. Deze bevindingen suggereren dat naast de aanwezigheid van ACPA nog een cofactor nodig is om ontsteking te veroorzaken, of dat ACPA variabele eigenschappen bezitten die betrokken zijn bij (het ontstaan van) de gewrichtsontsteking. Hierbij kan ondermeer gedacht worden aan een structurele eigenschap aanwezig op deze antistoffen.

Antilichamen zijn eiwitten die suikerstructuren dragen. Het Fc gedeelte van IgG moleculen bevat twee suikers die in vorm en samenstelling variëren. Afhankelijk van deze samenstelling varieert de halfwaardetijd van het antilichaam en kan IgG in verschillende mate met cellen van het afweersysteem in contact treden, deze activeren of remmen en het complement systeem activeren. Er is al langer bekend dat IgG moleculen in serum van RA-patiënten minder galactose dragen dan IgG moleculen van gezonde donoren. Gebaseerd op deze bevinding gaan de hoofdstukken 3 en 4 in op de vraag in hoeverre de suikerstructuren op het Fc gedeelte van ACPA ontsteking kunnen bevorderen. **Hoofdstuk 3** beschrijft de ontwikkeling van een techniek waardoor het mogelijk werd ACPA uit een kleine hoeveelheid serum op te zuiveren en aansluitend het suikerprofiel te meten. **Hoofdstuk 4** maakt gebruik van deze methode voor het bepalen van ACPA Fc suikerprofielen van een groter aantal patiënten met ACPA positieve artritis. Interessant genoeg werd gevonden dat ACPA inderdaad een ontsteking bevorderend suikerprofiel dragen omdat ACPA veelal de suikers siaalzuur en galactose missen. Het ontbreken van deze suikers maakt de communicatie met afweercellen via activerende Fc receptoren makkelijker. Opvallend was de bevinding dat ACPA in synoviaal vocht het meest inflammatoire profiel hadden. Deze eigenschap is specifiek voor ACPA omdat dit niet werd gevonden voor het "niet-specifieke" IgG in het synoviale vocht. Deze observatie suggereert dat ACPA producerende B-cellen antilichamen maken met een specifieke suikerstructuur die mogelijk extra kunnen bijdragen aan het ziekteproces. Inderdaad werd een sterke correlatie gevonden tussen activiteit van de ziekte en gebrek aan galactose. Nader onderzoek moet nu laten zien of de verandering van de suikerstructuur aan ACPA voorafgaat aan de stijging van ziekteactiviteit. Dit zou kunnen worden verwacht indien er een causale relatie aanwezig zou zijn.

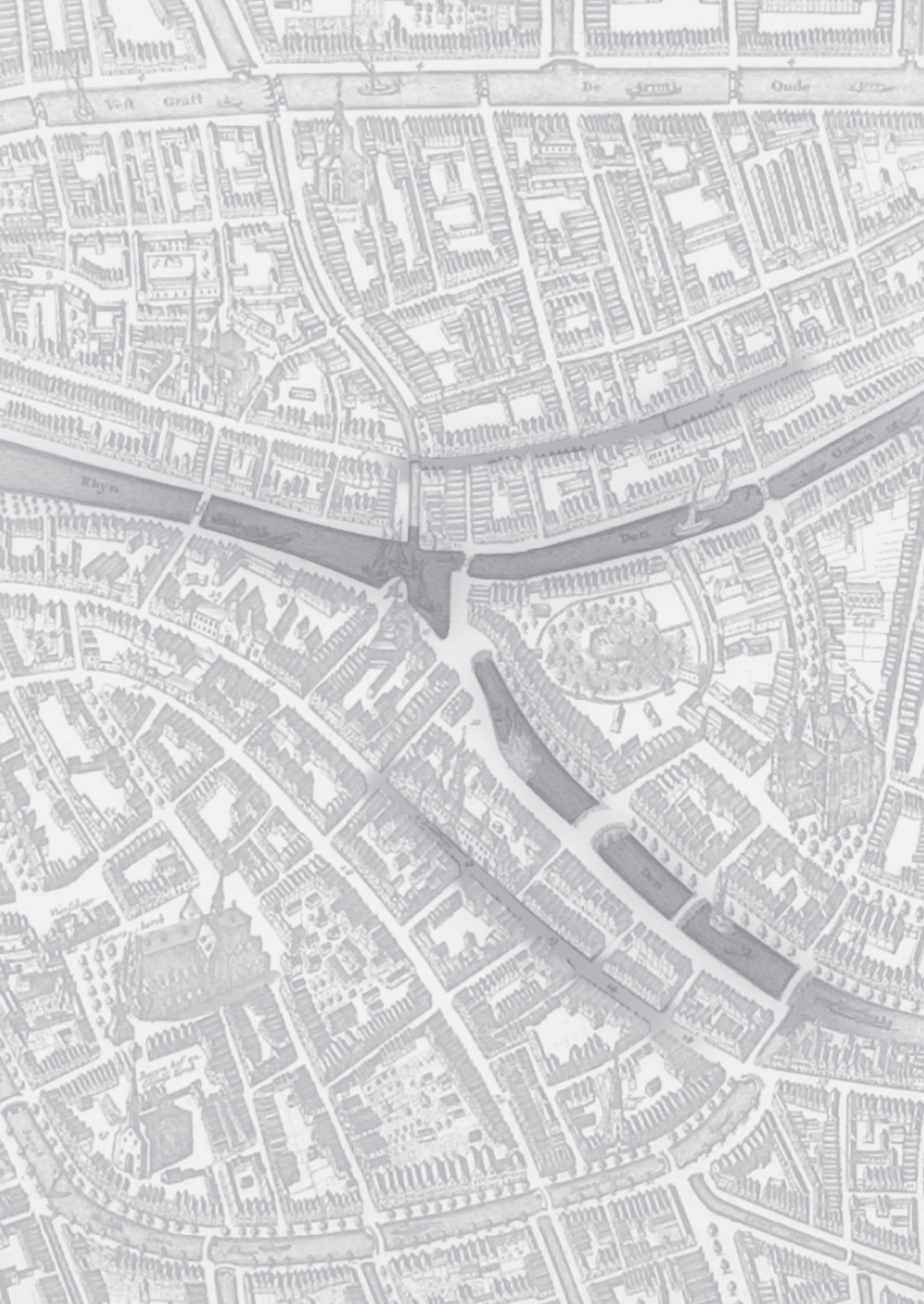
Deze data geven ook aanleiding tot de vraag of ACPA producerende B-cellen mogelijk een aparte ontwikkeling ondergaan. ACPA bestaan in alle isotypes. Dit geeft aan dat ACPA zeer waarschijnlijk de klassieke stappen van ontwikkeling in het germinal center doorlopen. Hier gaan B-cellen o.a. over van de productie van het IgM isotype naar de productie van het IgG, A of E isotype. Ook ondergaat de B-cel in een germinal center verdere uitrijping waardoor de geproduceerde antistoffen antigenen beter, met een hogere affiniteit, kunnen herkennen. Deze rijping vindt plaats indien na competitie tussen B-cellen voor het antigeen dat ze herkennen. De B-cel met de hoogste affiniteit zal in dit proces overleven. Daar autoantigenen zoals gecitrullineerde eiwitten overal aanwezig zijn, is het de vraag of er competitie tussen B-cellen optreedt nodig voor affiniteitrijping van antistoffen. Inderdaad vonden wij, zoals beschreven in **hoofdstuk 5**, dat dit niet het geval blijkt te zijn. ACPA bleken, op groepsniveau, vooral een lage aviditeit te hebben. De aviditeit veranderde niet in de loop van de tijd en verschilde significant van de aviditeit die werd gevonden voor anti-tetanus of anti-diphteria toxine antilichamen. Alles overwegende impliceert dit dat ACPA producerende cellen een proces van ontwikke-

ling doorlopen dat verschilt van dat van conventionele B-cellen. Dit zou in de toekomst aanknopingspunten kunnen geven voor een gerichte interventie in dit proces.

Tevens staat in deel II beschreven in hoeverre het herkenningsprofiel van ACPA, gebruikmakend van verschillende gecitrullineerde antigenen, correleert met verschillende stadia van het ziekteproces. De ACPA-reactie is polyclonaal. Dit betekent dat ACPA verschillende gecitrullineerde antigenen herkennen. Dit maakt de aanname mogelijk dat bepaalde antigenen "belangrijker" zijn voor de ziekte dan andere, waardoor variatie in het herkenningsprofiel van ACPA zou kunnen correleren met stadia van ziekte. Gebaseerd op deze overweging is in **hoofdstuk 6** beschreven of de fijn-specificiteit van ACPA relateert met het ontstaan van radiologische schade. Analyse van vijf verschillende fijn-specificiteiten liet ook na stratificatie van de onderliggende genetische variatie geen aanwijzing zien dat het herkenningsprofiel geassocieerd is met de mate van radiologische gewrichtsdestructie. Dit betekent, dat het onwaarschijnlijk is dat herkenning van de aanwezigheid van een bepaalde specificiteit binnen de groep van ACPA bijdraagt aan het ziekteproces.

DEEL III: BIJDRAGE VAN GENETISCHE VARIANTEN AAN RADIOLOGISCH GEWRICHTSDESTRUCTIE

Er zijn inmiddels meer dan 30 genetische polymorfismen geïdentificeerd die het risico verhogen om RA te ontwikkelen. Het blijkt tot nu toe echter uiterst moeilijk het onderliggende mechanisme voor elk factor te bepalen. Een recent ontdekte risicofactoor ligt in een regio tussen twee genen op chromosoom 6q23, waarbij associatie met het gen dat codeert voor TNF-AIP3 (A20) het meest waarschijnlijk lijkt. A20 is een eiwit dat zorgt voor de negatieve regulatie van NFκB, een transcriptiefactor die o.a. de expressie van TNF-α bevordert. In het kader van RA is A20 interessant, omdat bij muizen de afwezigheid van A20 tot ernstige artritis leidt, terwijl de overexpressie van A20 artritis kan remmen. Opvallend was dat het risico bevorderende genetische polymorfisme alleen gevonden werd in een cohort van patiënten met lang bestaande ziekte, maar niet in cohorten met kort bestaande RA. Dit gaf aanleiding tot de aanname dat deze variant mogelijk invloed heeft op ernst van die ziekte. In **hoofdstuk 7** is beschreven of in totaal 5 genetische polymorfismen in de 6q23 regio de kans verhogen op het ontstaan van gewrichtsschade. Interessant genoeg werd gevonden dat twee polymorfismen inderdaad significant geassocieerd zijn met een ernstig beloop van RA. In combinatie met de data op eiwitniveau dragen deze bevindingen bij aan de hypothese dat de 6q23 regio direct invloed heeft op het ziekteproces dat leidt tot schade bij RA.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Curriculum Vitae

List of publications

Dankwoord

CURRICULUM VITAE

Hans Ulrich Scherer werd in 1975 geboren in München. Vanaf 1986 woonde hij in Berlijn. In 1991/1992 bezocht hij voor een jaar Northfield Mount Hermon School in Massachusetts, Verenigde Staten in het kader van het programma ASSIST (American Secondary Schools for International Students and Teachers). Hij slaagde in 1994 voor het eindexamen gymnasium in Berlijn. Vervolgens voltooide hij gedurende 17 maanden de vervangende dienstplicht in Avignon, Frankrijk in het kader van het vrijwilligers programma ICE (Initiatief Christenen voor Europa). In 1995 begon hij aan zijn studie geneeskunde aan de Vrije Universiteit Berlijn, die hij vervolgens voortzette aan de Eberhard Karls Universiteit Tübingen en de Humboldt Universiteit Berlijn. Zijn eerste contact met basaal biomedisch wetenschap kwam tot stand tijdens een stage aan de International Agency for Research on Cancer in Lyon, Frankrijk. Vervolgens deed hij twee jaar lang onderzoek in het immunologisch laboratorium van de afdeling cellulaire biologie van de Universiteit Tübingen. In deze tijd ontving hij een studiebeurs en was hij lid van het Graduiertenkolleg "Zellbiologie in der Medizin" van de Duitse Organisatie voor Wetenschappelijk Onderzoek (DFG). Met de resultaten van het onderzoek verricht in Tübingen behaalde hij zijn Duitse doctorstitel met het predicaat "magna cum laude". Hij studeerde af in 2002.

In 2003 begon hij aan zijn opleiding tot specialist interne geneeskunde / reumatologie aan het Charité Universitair Medisch Centrum te Berlijn. Als ontvanger van een Articulum Fellowship heeft hij in 2006/2007 14 maanden onderzoek verricht bij de afdeling reumatologie van het Leids Universitair Medisch Centrum (LUMC). Na het afronden van zijn opleiding tot internist in Berlijn keerde hij in 2010 terug naar Leiden voor het voortzetten van wetenschappelijk onderzoek. Daarnaast volgt hij zijn opleiding tot reumatoloog. Voor zijn wetenschappelijk onderzoek won hij diverse reisbeurzen evenals de Abstract Award en de Young Investigator Award van de European League Against Rheumatism (EULAR). Hij werd uitgenodigd deel te nemen aan het ACR/EULAR Exchange Programma in 2012.

Hij woont samen met Ute Braig-Scherer en zijn twee dochters Anouk en Milou in Leiden.

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* : included in this thesis
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DANKWOORD

A frequently quoted statement at the Leiden Department of Rheumatology is accredited to former head of department professor Ferry Breedveld and claims: "science is a social affair". Looking back on how this thesis came about, no delineation would fit better.

2005, EULAR congress in Vienna: Dear René (professor Toes), had you not suddenly been standing in front of my poster at this meeting, six years after we had met in the lab of professor Hans-Georg Rammensee in Tübingen and later lost sight of each other, neither would I live in Leiden today nor would this thesis have ever been written. Since then, you have been invaluable mentor, ever-optimistic motivator, unreserved promoter, and, at the same time, good friend – a combination I cannot esteem high enough and for which I sincerely thank you! Professor Huizinga, dear Tom, you granted me the opportunity to become part of your vivid department, you gave me the chance to experience its stimulating, enthusiastic atmosphere, to learn and grow within its shell and to profit from its clinical and scientific potential. This confers pride and challenge every day and I am deeply grateful for your outright confidence and commitment, for the perspectives you offer, and for your willingness to help wherever you can.

I am deeply indebted to many colleagues, basic researchers and clinicians, who have helped and supported me with much patience and comprehension. Not all can I mention by name, and I apologize to those I might miss. Leendert, Andreea and Fina, you are and have been tremendously important, offering help on many scientific issues, providing knowledge, suggestions and criticism. I take much motivation and inspiration from working with you! Ellen, it is a particular privilege that I may rely on your experience and advice. Your contribution to this thesis has been invaluable! Gerrie, your help with genotyping (chapter 7) needs acknowledgment, thank you! Others from my "early" days at D3: Wang, Wanda, Geertje, Anouk, Onno and those who are still there: Aleida, Annemarie, Nivine, Marjolijn, Joris. All of you generate an atmosphere, which makes it worth taking the effort to squeeze basic research in between clinical duties.

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