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master in de biomedische wetenschappen: klinische moleculaire wetenschappen

Masterproef

Characterization of the anti-UH-RA.21 antibody response and production of a monoclonal cell line

Promotor :
Prof. dr. Veerle SOMERS

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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting klinische moleculaire wetenschappen

Maastricht University

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1 List of Abbreviations

3'UTR	3' untranslated region
Ab	Antibody
ACPA	Anti-citrullinated peptide/protein antibodies
ACR	American College of Rheumatology
ANOVA	Analysis of variance
AUC	Area under the curve
BSA	Bovine serum albumin
CCP	Cyclic citrullinated peptides
cDNA	Complementary deoxyribonucleic acid
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
DAB	3,3'-Diaminobenzidine
DC	Dendritic cell
ECL	Enhanced chemiluminescence
ELISA	Enzyme linked immunosorbent assay
EULAR	European League Against Rheumatism
F	Female
Fc	Fragment crystallizable region
FCS	Fetal calf serum
FDA	Food and Drug Administration
H	Hour
HAT	Hypoxanthine-aminopterin-thymidine
HC	Healthy control
HC.p	Healthy control patient number
hIg	Human immunoglobulin
HLA	Human leukocyte antigen
HP	High performance
HRP	Horse radish peroxidase
HT	Hypoxanthine-thymidine
ICFA	Incomplete Freund's adjuvant
Ig	Immunoglobulin
IL	Interleukin

Isotype Ctrl	Isotype control
KLH	Keyhole Limpet Hemocyanin
M	Male
Max	Maximum
MHC	Major Histocompatibility Complex
Min	Minute
MPBS	Marvel in phosphate-buffered saline
mRNA	Messenger ribonucleic acid
NA	Not available
NEG	Negative
NHS	N-hydroxysuccinimide
OA	Osteoarthritis
OD	Optical density
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline Tween 20
PEG	Polyethylene glycol
POS	Positive
PsA	Psoriatic arthritis
PsA.p	Psoriatic arthritis patient number
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RA.p	Rheumatoid arthritis patient number
RC	Rheumatic control
RF	Rheumatoid Factor
ROC	Receiver Operating Characteristic
RPMI	Roswell Park Memorial Institute
RT	Room temperature
TMB	3, 3’, 5, 5’-tetramethylbenzidine
TNFα	Tumor necrosis factor α
SAS	Serological antigen selection
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SpA	Ankylosing spondylitis
SpA.p	Ankylosing spondylitis patient number

2 Preface

Eight months of practical work, reading scientific articles and writing the thesis have gone by so fast. Therefore, I would like to take the opportunity to thank the people who have guided and supported me throughout this senior internship.

First of all, I would like to thank my promoter, Prof. dr. Veerle Somers, for giving me the opportunity to perform my internship in her research group at the Biomedical Research Institute (BIOMED) of Hasselt University. During these months, her encouragement and guidance has helped me to become a motivated and skilled scientist. A big thank you also goes out to my daily supervisor, Liesbeth De Winter, for sharing me your scientific knowledge and practical tips. For giving me the opportunity to design and implement my own protocols. It gave me more insight in experiments and confidence in the lab. Thank you for carefully reading my thesis and for giving me valuable suggestions. Thanks for everything!

Furthermore, I would like to thank Igna Rutten for her practical advice and for answering my questions.

A word of appreciation also goes out to Prof. dr. Piet Geusens, the team of the ReumaClinic and patients for providing samples that were valuable for my project.

Moreover, thanks to my fellow students and friends for their direct and indirect help needed to successfully complete my thesis.

Last but not least, I would like to thank my parents and brother for supporting me the past five years and for believing in me.

3 Abstract

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disorder of which the origin is still unknown. Characteristic for RA is the inflammation of synovial joints, which generally leads to progressive joint destruction.

Rheumatoid arthritis is mainly diagnosed through clinical manifestations and the presence of serological markers (rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)). However, approximately one third of the established RA patients are seronegative for both RA disease markers and the sensitivity of these markers is proven to be even lower in the early disease phase. Therefore, there is a need for additional RA disease markers in order to diagnose undifferentiated arthritis patients, early RA patients (i.e. symptoms of maximum 1 year) and seronegative RA patients (i.e. RF-negative and anti-cyclic citrullinated peptide (CCP) antibody-negative).

Novel candidate autoantibody markers for early and seronegative RA patients were identified during a previous study by our research group. From these candidate markers, the antibody response directed against UH-RA.21 had the highest sensitivity (29%) and an associated specificity of 95% for RA. UH-RA.21 is a mimotope and contains an epitope that mimics an *in vivo* antigen. Both the epitope and the identity of the *in vivo* antigen were unknown. Therefore, the goal of this study was to characterize the antibody response directed against UH-RA.21 and the corresponding *in vivo* antigen of UH-RA.21.

Polyclonal anti-UH-RA.21 antibodies were purified from blood plasma of RA patients and further analyzed for their specificity, purity and presence of different antibody isotypes. Because of the presence of immunoglobulin G, M and A within purified antibodies, a screening was performed to study the isotype reactivity against UH-RA.21 in healthy controls, RA patients and rheumatic controls. Monoclonal anti-UH-RA.21 antibodies were derived from a cell line generated by hybridoma technology.

The identity of the corresponding *in vivo* antigen of UH-RA.21 was studied using immunoprecipitation followed by western blotting, which resulted in five remaining protein bands. One of these bands possibly contains the corresponding *in vivo* antigen of UH-RA.21 and will be identified by mass spectrometry.

4 Samenvatting

Reumatoïde artritis (RA) is een systemische, chronische aandoening waarvan de etiologie nog onbekend is. Kenmerkend voor RA is de ontsteking van synoviale gewrichten die zonder behandeling kan leiden tot progressieve gewrichtsafbraak. De diagnose van deze aandoening wordt voornamelijk gesteld op basis van klinische manifestaties en de aanwezigheid van serologische merkers (reumafactor (RF) en antilichamen gericht tegen gecitrullineerde proteïnen (ACPA)). Ongeveer 1:3 van de RA patiënten is seronegatief voor de huidige RA ziektemerkers. Verder is aangetoond dat de sensitiviteit van deze merkers lager is in de vroege ziektefasen. Hierdoor is er nood aan extra RA ziektemerkers om ongedifferentieerde artritis patiënten, vroege RA patiënten (symptomen gedurende maximum 1 jaar) en seronegatieve RA patiënten (negatief voor RF en anti-CCP antilichamen) te diagnostiseren.

In een voorgaande studie van onze onderzoeksgroep werden nieuwe kandidaat autoantilichaammerkers geïdentificeerd voor RA. Van deze kandidaatmerkers had de antilichaamreactie gericht tegen UH-RA.21 de hoogste sensitiviteit (29%) en een geassocieerde specificiteit van 95% voor RA. UH-RA.21 is een mimotoop en bevat dus een epitoop dat een *in vivo* antigen nabootst. Zowel het epitoop als de identiteit van het *in vivo* antigen zijn onbekend. Deze studie heeft tot doel om de antilichaamreactie gericht tegen UH-RA.21 en het corresponderende *in vivo* antigen van UH-RA.21 te karakteriseren. Polyklonale antilichamen werden verkregen uit bloedplasma van RA patiënten en vervolgens werden ze geanalyseerd voor hun specificiteit, zuiverheid en aanwezigheid van antilichaamisotypes. Omwille van de aanwezigheid van immunoglobuline G, M en A in de opgezuiverde antilichamen werd er een screening uitgevoerd om de isotype reactiviteit gericht tegen UH-RA.21 te bestuderen in gezonde controles, RA patiënten en reumatische controles. Monoklonale anti-UH-RA.21 antilichamen werden geproduceerd door een cellijn gegenereerd via de hybridoma-technologie. De identiteit van het corresponderende *in vivo* antigen van UH-RA.21 werd bestudeerd door middel van immunoprecipitatie gevolgd door western blotting en resulteerde in vijf overblijvende proteïnebanden. Hiervan bevat één proteïneband mogelijk het corresponderende *in vivo* antigen van UH-RA.21 en deze zal geïdentificeerd worden door middel van massaspectrometrie.

5 Introduction

5.1 Rheumatoid arthritis

Rheumatoid arthritis (RA), the world's most common autoimmune disorder, is mainly characterized by chronic inflammation of diarthrodial joints [1]. In Northern-Europe and Northern-America, RA affects 0.5-1% of the population, while lower prevalence rates (0.1-0.3%) are found in South America, Asia and Africa [2].
The major symptoms of RA are symmetrical joint tenderness, swelling, pain and morning stiffness. Uncontrolled active RA will eventually lead to progressive cartilage and joint destruction and deformity, disability and reduction of quality of life [1, 3-5]. Besides articular symptoms, RA also affects extra-articular tissues, such as skin, heart, blood vessels, muscles and lungs. Therefore, RA is considered a systemic autoimmune disease [5].

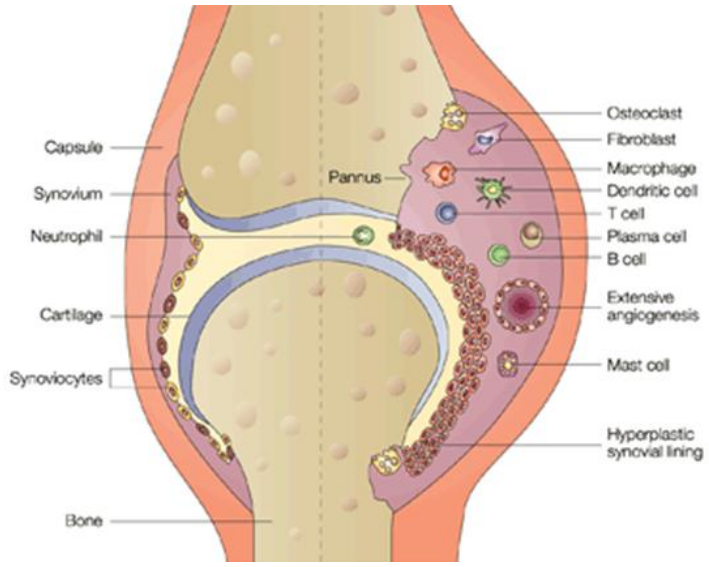
Rheumatoid arthritis is a multifactorial, complex disease in which both genetic (e.g. major histocompatibility complex (MHC) class II genes such as human leukocyte antigen (HLA)-DRB1 01 and 04 variants or polymorphisms in the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) gene) and environmental factors (e.g. smoking, air pollution or bacteria (e.g. *Porphyromonas gingivalis*)) are involved [6-9]. However, the exact pathogenesis is still unknown [1, 3-5, 8, 10].

The autoimmune attack in RA is mainly focused on synovium of joints (Figure 1A). Synovium, a thin highly organised structure between the joint cavity and the fibrous joint capsule, is responsible for the delivery of nutrients to cartilage and the production of lubricants for the joint [11].
Synovium has two separate layers: an intimal lining layer (or synovial lining layer) and a synovial sublining layer (or subsynovium) (Figure 1B). The intimal lining layer mainly consists of intimal macrophages and fibroblast-like synoviocytes and lacks a true basement membrane. Therefore, cells are organised in a continuous network of compacted cells embedded within a specialised extracellular matrix, leading to the formation of a functional

'cellular basement membrane' between the synovial fluid compartment and the synovial sublining. The loosely organised synovial sublining consists of extracellular matrix, scattered fibroblasts, macrophages, mast cells, nerves, blood vessels and lymphatics, resulting in the formation of a microanatomic base for the synovial lining [11, 12].

In RA, the cellular organisation and composition of synovium undergoes striking changes (Figure 1C). At the lining layer, an increase in the number of cells is observed which results in hyperplasia [13, 14]. Macrophage-like synoviocytes often represent the majority of cells, but fibroblast-like synoviocytes also exhibit characteristics of a metabolically active, secretory cellular state. Synovium develops into pannus tissue and migrates into the joint cavity, where it overgrows and invades articular cartilage and subchondral bone, resulting in extensive bone damage and destruction [14]. At the synovial sublining, inflammatory cells such as T-lymphocytes, B-lymphocytes, plasma cells, macrophages, mast cells, natural killer cells and dendritic cells (DC) infiltrate and accumulate [15, 16]. Lymphocytes can be organised into lymphoid follicles resembling germinal centres or they can form diffuse or perivascular infiltrates. Furthermore, small vessels show proliferation and an increase in the number of high endothelial venules is observed [11]. Besides the inflammatory cells, pro-inflammatory cytokines (Tumor necrosis factor α (TNFα), interleukin (IL) 1β, IL-6, IL-15 and IL-17) and tissue-destructive enzymes (matrix metalloproteinases) also contribute to the resulting joint destruction [13].

A



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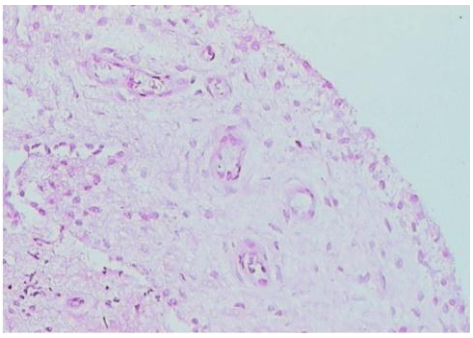

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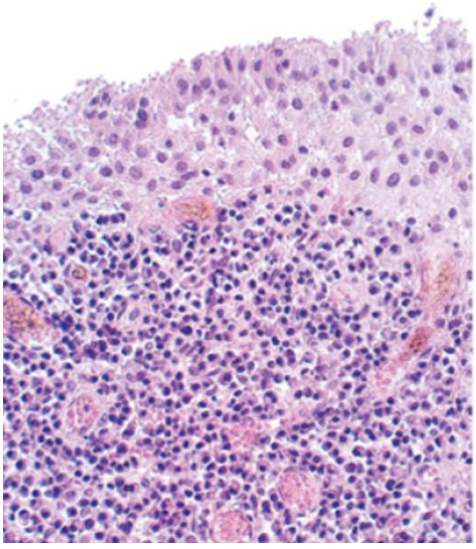

Figure 1: Rheumatoid arthritis joint lesion
A diarthrodial joint, also known as a synovial joint, is composed of two articulating bones and a synovial cavity filled with synovial fluid. The synovial cavity is surrounded by the articular capsule which is composed of synovium and fibrous membrane (a, left). In RA, the structure of the joint undergoes striking changes (A, right) [17]. The cellular organization and composition of normal synovium (B) and RA synovium (C) [18, 19].

5.2 Rheumatoid arthritis and the humoral immune response

Although the etiology of RA remains unknown, numerous studies indicate that the humoral immune response plays an important role in pathological processes of RA [20-22]. This finding is supported by the presence of rheumatoid factor (RF) in a large amount of RA patients [23]. Rheumatoid factor is a circulating immunoglobulin (Ig) M antibody directed against the fragment crystallizable (Fc) region of self-immunoglobulin G. Autoantibodies such as RF can have a pathogenic effect via fixation and activation of the complement system

on target tissues or through their neutralizing action. In addition, Fc receptors on macrophages and DCs can also be activated. The presence and persistence of these autoantibodies show that clones of autoreactive B cells can avoid the process of B cell tolerance and proliferate under continuous stimulation in RA patients [23]. Besides the production of autoantibodies, B cells secrete chemokines and cytokines and function as antigen presenting cells to T lymphocytes in the synovial environment, leading to clonal expansion of T cells [20].

Furthermore, ectopic lymphoid structures are found in RA synovium, supporting the ongoing production of class-switched autoantibodies [24]. Nevertheless, the main evidence for the role of the humoral immune response in RA is provided by the success of B cell-targeted therapies in RA and the Food and Drug Administration (FDA)-approval to use rituximab as a treatment for RA [21, 25, 26]. In addition, rodent models of RA also confirm the role of the humoral immune response in RA, for example, by the pathogenic action of anti-collagen type II antibodies and anti-glucose-6-phosphate isomerase antibodies in collagen induced arthritis (CIA) and serum transfer-induced arthritis models, respectively [27, 28].

5.3 Diagnosis

Immunological and inflammatory processes associated with destructive mechanisms in RA appear at the very beginning of the disease [29]. Therefore, early intervention is crucial to prevent irreversible joint damage and to improve long term disease outcome. Consequently, it is of utmost importance to diagnose RA at a very early phase of disease [5, 10, 30].
At present, the diagnosis of RA is based on fulfillment of classification criteria revised by the American College of Rheumatology (ACR) and European League Against Rheumatism (EULAR) in 2010. These criteria focus on early stage features associated with persistent and/or erosive disease in order to identify patients with undifferentiated inflammatory synovitis. The criteria consist of the presence of synovitis in at least one joint (with absence of a better, alternative diagnosis) and achievement of an individual score of six or more

(of a possible ten) in four domains. The four domains are the number and site of affected joints (score range 0-5), serologic abnormality (RF or anti-citrullinated protein antibodies (ACPA), score range 0-3), elevated acute-phase response (erythrocyte sedimentation rate or C-reactive protein, score range 0-1), and symptom duration (two levels; score range 0-1) [31].

An early diagnosis of RA is very important, but currently, it is still unachievable in many cases [32]. Serological testing for RA disease markers could contribute to an early diagnosis of RA. Current RA disease markers are RF and ACPA and have a specificity of 82-91% and 97%, respectively [4, 10, 31, 33, 34]. However, approximately one third of the established RA patients are negative for both RA disease markers (i.e. seronegative) and the sensitivity of these two RA disease markers is proven to be even lower in the diagnostically important early disease phase [4, 35]. Sensitivity of RF and ACPA is ranging between 40% and 55% in early RA patients and reaches approximately 75% in established RA patients [4, 10, 35, 36]. Therefore, there is a need for additional RA disease markers in order to diagnose undifferentiated arthritis patients, early RA patients (i.e. symptoms of maximum (max) 1 year) and seronegative RA patients (i.e. RF-negative and anti-cyclic citrullinated peptide (CCP) antibody-negative).

5.4 The anti-UH-RA.21 antibody response

In a previous study of our research group, novel candidate autoantibody markers for RA were identified via serological antigen selection (SAS) based on complementary deoxyribonucleic acid (cDNA) phage display [37]. The cDNA phage display library was constructed from RA synovial pannus tissue and was screened for antigen reactivity with autoantibodies present in pooled plasma of early and seronegative RA patients. Fourteen candidate autoantibody markers (protected by patent) were detected in both early and seronegative RA patients. From these fourteen candidate autoantibody markers, the antibody response directed against UH-RA.21, an artificial peptide, was the most promising marker. The UH-RA.21 peptide sequence results from the expression of a normally untranslated messenger ribonucleic acid (mRNA) region (in this case 3'-untranslated region (3'UTR)) and therefore probably comprises a mimotope,

which means that it contains an epitope that structurally mimics an *in vivo* antigen. Although sequence similarity is not excluded, the mimotope and the corresponding *in vivo* antigen are not necessarily identical or similar at amino acid level [37].

Results of the previous study of our research group showed that the antibody response directed against UH-RA.21 had the highest sensitivity (29%) and an associated specificity of 95% for RA, which indicates the biomarker potential of this antibody response (Figure 2A and 2B). Moreover, anti-UH-RA.21 antibodies were detected in synovial fluid of RA patients and one of the three stained RA synovial tissues showed overexpression of the corresponding *in vivo* antigen of UH-RA.21 [37]. Furthermore, preliminary results show that injection of antibodies directed against UH-RA.21 in CIA mouse models exacerbates the symptoms of the disease (Figure 2C), which points toward a potential role of the autoantibody reactivity against UH-RA.21 in the underlying disease processes of RA.

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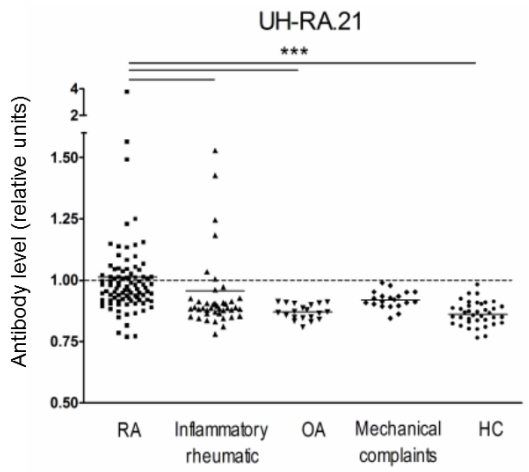

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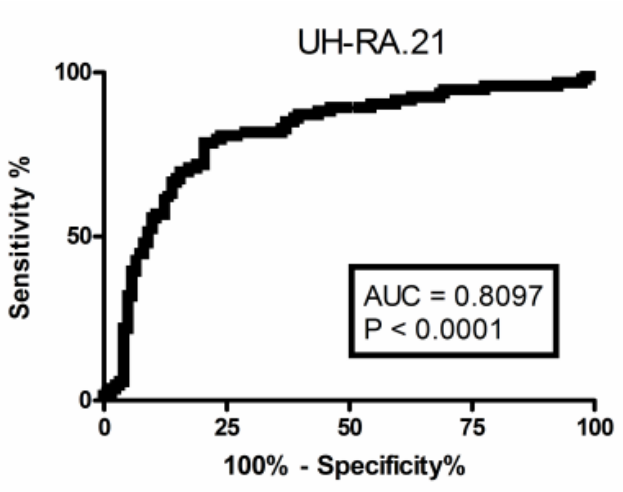

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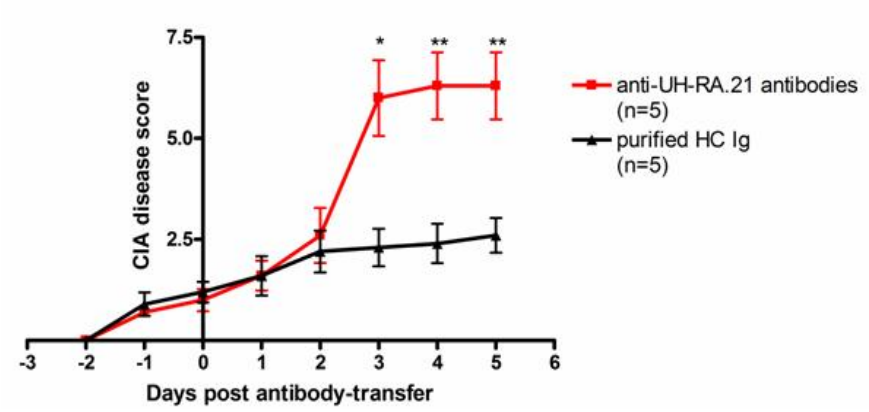


Figure 2: Specificity, sensitivity and pathological role of anti-UH-RA.21 antibodies
(A) Rheumatoid arthritis (RA) patients (n=92), patients with other inflammatory rheumatic diseases (n=43), osteoarthritis (OA) patients (n=20), patients with mechanical joint complaints (n= 20) and healthy controls (HC, n=38) were included in a phage ELISA screening to determine relative amounts of anti-UH-RA.21 antibodies. Results were normalized to a predetermined cut-off score (i.e. three times the standard deviation above the mean optical density (OD) (UH-RA.21 peptide)/OD(empty phage) in the HC group), meaning that patients were considered positive if they had a relative antibody level of >1. Relative anti-UH-RA.21 antibody levels were significantly higher in RA patients compared to the other groups. (B) Receiver Operating Characteristic (ROC)-curve of the anti-UH-RA.21 antibody response. The anti-UH-RA.21 antibody response shows an area under the curve (AUC) value of 0.8097, which represents the capacity to distinct between RA patients and HC (valuable diagnostic test, AUC >0.75). (C) Collagen-induced arthritis (CIA) was induced in DBA/1 mice. At baseline of the disease (CIA disease score of >0 for >1 day), CIA mice were injected intraperitoneally with human anti-UH-RA.21 antibodies (red, n=5) and isotype control antibodies (purified HC Ig, black, n=5). Mice injected with human anti-UH-RA.21 antibodies showed significantly higher disease scores compared to mice injected with human isotype control antibodies. *p<0.05; **p< 0.01; ***p<0.001.

5.5 Aims

UH-RA.21 is a mimotope and contains an epitope that mimics an *in vivo* antigen. Both the epitope and the identity of the *in vivo* antigen were unknown. In this study, the goal was to characterize the antibody response directed against UH-RA.21 and the corresponding *in vivo* antigen of UH-RA.21.

To investigate the identity of the corresponding *in vivo* antigen of UH-RA.21, anti-UH-RA.21 antibodies were required. Polyclonal anti-UH-RA.21 antibodies were derived from blood plasma samples of RA patients. After purification of anti-UH-RA.21 antibodies using affinity chromatography, the specificity and purity of the antibodies was studied and a screening was performed to analyze the isotype reactivity against UH-RA.21 in healthy controls (HC), RA patients and rheumatic controls (RC). Monoclonal anti-UH-RA.21 antibodies were obtained from a cell line generated by hybridoma technology. Next, the identity of the corresponding *in vivo* antigen of UH-RA.21 was studied. Therefore, the reactivity of anti-UH-RA.21 antibodies was analyzed by immunoprecipitation of human synovial tissue lysates followed by western blotting. The identity of the corresponding *in vivo* antigen of UH-RA.21 will provide us more insight in the underlying disease processes of RA.

6 Material and Methods

6.1 Plasma and tissue samples

Polyclonal anti-UH-RA.21 antibodies were purified from blood plasma of RA patients (obtained from rheumatologists in Genk and Hasselt) testing positive for anti-UH-RA.21 antibodies on enzyme linked immunosorbent assay (ELISA). Isotype control antibodies were obtained from blood plasma of randomly selected HC testing negative for anti-UH-RA.21 antibodies on ELISA.

For the IgG and IgA anti-UH-RA.21 antibody screening, plasma samples of 13 HC (Table 1), 42 RA patients and 11 RC were randomly selected. Healthy controls included in the screening had an average age of 32 years and 15% of the subjects were men.

Table 1

Characteristics of HC used for the IgG and IgA anti-UH-RA.21 antibody screening

Patient[a]	Age[b]	Gender[c]
HC.p1	27	F
HC.p2	26	M
HC.p3	33	M
HC.p4	24	F
HC.p5	31	F
HC.p6	24	F
HC.p7	52	F
HC.p8	51	F
HC.p9	26	F
HC.p10	28	F
HC.p11	47	F
HC.p12	25	F
HC.p13	28	F

[a] HC.p, healthy control patient number.
[b] Age of the patient in years.
[c] F, female; M, male.

Of the 42 RA patients that entered the screening, 18 were early RA patients. The average age of the early RA patients was 51 years and 29% of the patients were men. Established RA patients had an average age of 56 years and 42% of the patients were men. Characteristics of RA patients included in the IgG and IgA anti-UH-RA.21 antibody screening were summarized in Table 2.

Table 2

Characteristics of RA patients used for the IgG and IgA anti-UH-RA.21 antibody screening

Patient[a]	RF[b]	Anti-CCP Ab[c]	Disease duration[d]	Age disease onset[e]	Age[f]	Gender[g]
RA.p1	-	+	0,08	29	29	F
RA.p2	+	+	0,08	47	47	M
RA.p3	+	+	0,17	63	63	F
RA.p4	+	+	0,17	35	35	F
RA.p5	+	+	0,17	32	32	F
RA.p6	-	+	0,25	49	49	F
RA.p7	-	-	0,42	57	57	F
RA.p8	+	+	0,5	65	65	F
RA.p9	+++	+	0,5	43	43	F
RA.p10	-	-	0,58	39	39	M
RA.p11	+	+	0,58	65	65	M
RA.p12	+	+++	0,66	45	46	M
RA.p13	-	-	0,75	48	49	F
RA.p14	-	-	0,75	79	80	M
RA.p15	+	+++	0,83	54	55	F
RA.p16	-	-	0,83	53	54	F
RA.p17	+	-	1	53	54	F
RA.p18	-	-	1	58	59	F
RA.p19	+	+++	2	58	60	M
RA.p20	-	-	2	52	54	F
RA.p21	-	+++	2	49	51	M
RA.p22	-	-	2	70	72	F
RA.p23	+	+++	3	36	39	M
RA.p24	-	-	3	58	61	F
RA.p25	+	+++	4	53	57	F
RA.p26	-	-	4	62	66	F
RA.p27	-	-	4	44	48	F
RA.p28	+	+++	5	38	43	F
RA.p29	+	+++	11	44	55	F
RA.p30	+	+++	11	51	62	M
RA.p31	-	-	12	34	46	F
RA.p32	-	-	13	28	41	M
RA.p33	-	-	13	37	50	M
RA.p34	-	-	14	54	68	F
RA.p35	+	+	14	59	73	F
RA.p36	+	+++	14	49	63	M
RA.p37	-	-	16	21	37	M
RA.p38	+	+++	16	57	73	M
RA.p39	-	-	NA[h]	80	NA	M
RA.p40	-	-	14	38	52	F

RA.p41	-	-	20	32	52	F
RA.p42	-	+	NA	67	NA	F

[a] RA.p, rheumatoid arthritis patient number
[b] The RF status was tested with a Serodia-RA Particle Agglutination test, Fujirebio Diagnostics, a cut-off value of 20 IU/ml was used to define a positive test.
[c] The anti-CCP antibody (anti-CCP Ab) status was tested with a Quanta Lite CCP IgG, INOVA Diagnostics, a cut-off value of 20 IU was used to define a positive test.
[d] Disease duration in years.
[e] Age disease onset in years.
[f] Age patient in years.
[g] F, female; M, male.
[h] NA, not available.

To study the specificity of the IgG and IgA anti-UH-RA.21 antibody response, RC were also incorporated in the screening (Table 3). Therefore, 7 ankylosing spondylitis (SpA) patients (average age, 45 years; 50% of the patients were men) and 4 psoriatic arthritis (PsA) patients (average age, 55 years; 50% of the patients were men) were studied.

Table 3

Characteristics of RC patients used for the IgG and IgA anti-UH-RA.21 antibody screening

Patient[a]	RF[b]	Anti-CCP Ab[c]	Disease Duration[d]	Age disease onset[e]	Age[f]	Gender[g]
SpA.p1	NA[h]	NA	NA	NA	NA	NA
SpA.p2	-	NA	4	42	46	F
SpA.p3	-	NA	NA	NA	34	M
SpA.p4	NA	NA	6	24	30	M
SpA.p5	NA	NA	NA	NA	66	M
SpA.p6	-	NA	17	30	47	F
SpA.p7	-	NA	24	20	44	F
PsA.p1	NA	NA	1	52	53	F
PsA.p2	-	NA	6	50	56	M
PsA.p3	NA	-	13	53	66	F
PsA.p4	NA	NA	13	34	47	M

[a] SpA.p, ankylosing spondylitis patient number; PsA.p, psoriatic arthritis patient number.
[b] The RF status was tested with a Serodia-RA Particle Agglutination test, Fujirebio Diagnostics, a cut-off value of 20 IU/ml was used to define a positive test.
[c] The anti-CCP antibody (anti-CCP Ab) status was tested with a Quanta Lite CCP IgG, INOVA Diagnostics, a cut-off value of 20 IU was used to define a positive test.
[d] Disease duration in years.
[e] Age disease onset in years.
[f] Age of the patient at sampling in years.
[g] F, female; M, male.
[h] Not available.

Lysates of knee synovial tissue of an RA patient positive for UH-RA.21 on immunohistochemical stainings and positive for anti-UH-RA.21 antibodies on ELISA were used to carry out immunoprecipitation experiments.

6.2 Immuno-affinity purification of antibodies

Synthetic UH-RA.21 peptides (1 mg; 95% purity; Eurogentec, Seraing, Belgium) were coupled to a HiTrap N-hydroxysuccinimide (NHS)-activated High Performance (HP) column (GE Healthcare, Diegem, Belgium) according to the manufacturer's instructions. Coupling efficiency was determined by spectrophotometry (adsorption at 280 nm, SmartSpec™ Plus, Bio-Rad, Nazareth Eke, Belgium). Plasma of RA patients was filtered (Minisart-Plus filters, pore size 0.45 µm; Sigma-Aldrich, Bornem, Belgium) and incubated with the UH-RA.21 coupled column. Anti-UH-RA.21 antibodies were eluted with IgG elution buffer (pH 2.8; Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) and immediately neutralized with Tris buffer (1M Tris, pH 9). The presence of anti-UH-RA.21 antibodies was confirmed on a peptide ELISA. Anti-UH-RA.21 antibodies were concentrated by use of a Pierce concentrator (cut-off 9K; Thermo Fisher Scientific, Erembodegem-Aalst, Belgium). Antibody concentration was determined using the BCA protein quantification kit (Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) with human IgG as standard.
Isotype control antibodies were purified from blood plasma of HC using a protein A/G column (Thermo Scientific, Erembodegem-Aalst, Belgium). The bound antibodies were eluted with IgG elution buffer (pH 2.8), neutralized with Tris buffer (1M Tris, pH 9) and concentrated. The concentration immunoglobulin was determined by spectrophotometry (adsorption at 280 nm, SmartSpec™ Plus, Bio-Rad, Nazareth Eke, Belgium or NanoDrop ND-1000 spectrophotometry, Isogen Life Science, Temse, Belgium).

6.3 Peptide ELISA

Polystyrene microplates (96 well, Flat bottom, Greiner Bio-one, Wemmel, Belgium) were coated overnight with 100 µl/well 1 µg/ml synthetic UH-RA.21 peptides (85% purity; Eurogentec, Seraing, Belgium) in phosphate buffered

saline (PBS, 1.5 mM KH_2PO_4, 5 mM NA_2HPO_4, 130 mM NaCl) at room temperature (RT). The plates were washed with PBS-0.05% (v/v) Tween 20 (0.05%-PBST) and blocked with 200 µl/well 2% (w/v) Marvel-PBS (MPBS) for 2 hours (h) shaking at 37°C. Following washing, plasma samples diluted 1:100 in 2% MPBS were added in 100 µl/well to the coated ELISA plates for 2 h shaking at RT. After washing, rabbit anti-human-horseradish peroxidase (HRP) antibody (Dako, Heverlee, Belgium) diluted 1:2000 in 2% MPBS was incubated (100 µl/well) for 1 h shaking at RT. The wash steps were repeated and 100 µl/well 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution (Thermo Fisher Scientific, Erembodegem-Aalst, Belgium) was added. After incubation for approximately 6 minutes (min) in the dark, the color reaction was stopped with 50 µl/well 2N H_2SO_4 and absorbance was read at 450 nm (Microplate Reader Infinite M1000 Pro, TECAN, Männedorf, Switzerland).

6.4 SDS-PAGE

To investigate the identity of immunoprecipitates of human synovial tissue lysates and the purity of purified antibodies, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide separating gel, 4% acrylamide stacking gel) was used. Samples were diluted 1:1 in reducing sample buffer (0.5 M Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 1% bromophenol blue) with 0.05% beta-mercaptoethanol and boiled for 4 min. Samples and Precision Plus Protein Standards dual color (Bio-rad, Nazareth Eke, Belgium) ladder were loaded onto the SDS-PAGE gel.

6.5 Dot Blot

To confirm the presence of different antibody isotypes in the purified antibodies, a dot blot analysis was performed. Purified antibodies were both diluted (1:10) and undiluted spotted on a nitrocellulose blotting membrane (Protran BA-85, Whatman, 's-Hertogenbosch, Netherlands). After blocking the membranes with 5% (w/v) MPBS for 30 min shaking, blots were incubated with HRP-labeled rabbit anti-human IgG (Dako, Heverlee, Belgium), HRP-labeled mouse anti-human IgM (BD Biosciences, Erembodegem, Belgium) or HRP-labeled mouse

anti-human IgA (1:100, Invitrogen, Merelbeke, Belgium) in blocking buffer. Next, blots were washed twice 5 min in PBS-0.05% (v/v) Triton X-100. Presence of IgG, IgM or IgA directed against UH-RA.21 was visualized by means of a 3, 3'-Diaminobenzidine (DAB) staining (Sigma, Bornem, Belgium). Dilution series of human IgG (Jackson ImmunoResearch Laboratories, Belgium), IgM (Jackson ImmunoResearch Laboratories, Belgium) and IgA (500 µg/ml to 0 µg/ml in PBS; Jackson ImmunoResearch Laboratories, Belgium) were used as a reference.

6.6 Hybridoma production

To generate a cell line that produces monoclonal anti-UH-RA.21 antibodies, female, 6 to 8-week-old BALB/c mice (Harlan, Belgium) were immunized by intraperitoneal injection of 90 µg UH-RA.21 peptides (85% purity; Eurogentec, Seraing, Belgium) coupled to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) and dissolved in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (ICFA). The immunization protocol is shown in Table 3. Plasma titers of anti-UH-RA.21 antibodies were tested via peptide ELISA with HRP-labeled rabbit anti-mouse Ig (1:2000; Dako, Heverlee, Belgium). Immunized mice could be used to create a monoclonal cell line when a minimum dilution of the plasma (1:1000) corresponded to half of the maximal OD value. When a sufficiently high antibody titer was reached in plasma, spleens of the mice were isolated and homogenized. Spleen cells were fused with Sp2/0-Ag14 mouse myeloma cells at a ratio of 5:1 in 50% polyethylene glycol (PEG) 1500 (Janssen, Beerse, Belgium) for immortal hybridoma production. The hybridoma cells were selected via culture in selective medium. First, cells were cultured in hybridoma culture medium (serum-free Roswell Park Memorial Institute (RPMI, Lonza, Verviers, Belgium) 1640 medium containing 10% fetal calf serum (FCS, Hyclone, Erembodegem, Belgium), 1% (v/v) sodium-pyruvate (Gibco, Merelbeke, Belgium), 1% (v/v) non-essential amino acids (Gibco, Merelbeke, Belgium)) supplemented with hypoxanthine-aminopterin-thymidine (HAT) media supplement (1:50, Invitrogen, Merelbeke, Belgium), IL-6 (1 µg/ml, 1:500) and gentamycine (1:1000, Gibco, Gent, Belgium) and maintained at 37°C in a 5% CO_2 incubator chamber. Because of the presence of aminopterin in

this medium, normal nucleotide synthesis is disrupted. B cells contain a salvage enzyme and can use an alternative nucleotide synthesis so that only B cells fused with Sp2/0-Ag14 mouse myeloma cells will survive. At day 10 after the fusion, selective medium was replaced by hybridoma culture medium containing hypoxanthine-aminopterin (HT) media supplement (1:50, Invitrogen, Merelbeke, Belgium) to restore the normal nucleotide synthesis pathway. Next, cells were cultured in hybridoma culture medium. Cell culture supernatants were screened for the presence of anti-UH-RA.21 antibodies using peptide ELISA with HRP-labeled rabbit anti-mouse Ig (1/2000; Dako, Heverlee, Belgium) to select specific antibody-producing hybridoma clones. The selected hybridomas were cloned until monoclonal anti-UH-RA.21 antibody-producing cell lines were obtained. The isotype of produced anti-UH-RA.21 antibodies was determined using the Rapid ELISA Mouse mAb Isotyping kit (Thermo Fisher Scientific, Erembodegem-Aalst, Belgium). Purity of the produced antibodies was evaluated using SDS-PAGE.

Table 3

Immunization protocol

Day 1	First immunization: 90 µg UH-RA.21 peptides conjugated with KLH[a] in CFA[b]
Day 15	Second immunization: 90 µg UH-RA.21 peptides conjugated with BSA[c] in ICFA[d]
Day 25	Titration
Day 28	Third immunization: 90 µg UH-RA.21 peptides conjugated with KLH in ICFA
Day 38	Titration If the anti-UH-RA.21 antibody plasma titer is sufficiently high (a minimum dilution of the plasma (1:1000) corresponded to half of the maximal OD value), Sp2/0-Ag14 mouse myeloma cells were grown.
Day 42	Spleen isolation and cell fusion

[a] Keyhole Limpet Hemocyanin
[b] Complete Freund's adjuvant
[c] Bovine serum albumin
[d] Incomplete Freund's adjuvant

6.7 Immunoprecipitation and western blotting

Before immunoprecipitation of lysate, protein A/G resin of the Crosslink Immunoprecipitation kit (Thermo Scientific, Erembodegem-Aalst, Belgium) was covalently crosslinked with 50 µg anti-UH-RA.21 antibodies according to the manufacturer's instructions. A protein A/G resin covalently crosslinked with 50 µg isotype control antibodies was used as a negative control. Prior to the immunoprecipitation, 1 mg lysate or protein extract was precleared with

80 µl Control Agarose Resin at 4°C for 1 h to reduce nonspecific protein binding. Precleared extract was incubated overnight with protein A/G resin coupled with anti-UH-RA.21 antibodies and with negative control resin at 4°C and end-over-end mixing. Immunoprecipitates were washed thrice with IP lysis/wash buffer (0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4) and once with conditioning buffer (neutral pH buffer). To elute the absorbed antigens, elution buffer (pH 2.8, contains primary amine) was added to the resin and incubated for 5 min. Resin was centrifuged for 1 min at 1000 x g and flow through was collected. After elution of immunoprecipitates, the resin was re-equilibrated and stored in coupling buffer (0.01 M sodium phosphate, 0.15 M NaCl; pH 7.2) at 4°C.

Immunoprecipitates were separated by a 12% SDS-PAGE gel (as described above) and blotted on a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Overijse, Belgium) for 2 h at 350 mA/100 V. Membranes were blocked for 2 h shaking at RT with 5% (w/v) MPBS supplemented with 0.1% (v/v) Tween 20, 10% rabbit serum and 1:200 rabbit anti-human IgG (Dako, Heverlee, Belgium) or biotinylated rabbit F(ab')2 anti-mouse Ig (Jackson ImmunoResearch Laboratories, Belgium). Following overnight incubation at 4°C with 25 µg anti-UH-RA.21 antibodies or isotype control antibodies in blocking buffer on a shaker, membranes were incubated for 2 h shaking at RT with HRP-conjugated rabbit anti-human IgG (1:250; Dako, Heverlee, Belgium) or HRP-conjugated rabbit anti-mouse Ig (1:250; Dako, Heverlee, Belgium) in blocking buffer. Between incubation steps, washing was performed with 0.1% PBST (2x 1 min, 15 min, 3x 5 min). Immunoreactive bands were visualized by means of DAB staining (Sigma, Bornem, Belgium) or enhanced chemiluminescence (ECL) detection (Thermo Fisher Scientific, Erembodegem-Aalst, Belgium).

6.8 Fractionation of tissue lysate

Knee synovial tissue lysate of an RA patient positive for UH-RA.21 on immunohistochemical stainings and positive for anti-UH-RA.21 antibodies on ELISA was homogenized and separated in protein extracts (cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal protein extract)

by means of the Subcellular Protein Fractionation Kit for tissues (Thermo Scientific, Erembodegem-Aalst, Belgium) according to the manufacturer's instructions.

6.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5. Values of $p<0.05$ were considered significant. Two-Way analysis of variance (ANOVA) followed by a Bonferroni post-test was used to compare the IgG and IgA reactivity against UH-RA.21 in HC, early and established RA patients and RC. Correlations between clinical features of RA patients and the IgG and IgA reactivity against UH-RA.21 were studied using Spearman correlation coefficient.

7 Results

7.1 Polyclonal anti-UH-RA.21 antibodies

To investigate the identity of the corresponding *in vivo* antigen of UH-RA.21, antibodies directed against UH-RA.21 were purified from blood plasma of RA patients testing positive for anti-UH-RA.21 antibodies on ELISA. Isotype control antibodies were obtained from blood plasma of randomly selected, anti-UH-RA.21 antibody negative HC. After purification, the presence and specificity of anti-UH-RA.21 antibodies were confirmed using a peptide ELISA (data not shown). Purified antibodies were evaluated for their purity and presence of different antibody isotypes using a 12% SDS-PAGE gel. Figure 3 shows purified isotype control antibodies, anti-UH-RA.21 antibodies and commercial human IgG, IgM and IgA, respectively. The purified antibodies exhibited a high purity and displayed two or three heavy chain bands. Anti-UH-RA.21 antibodies contained IgG (heavy chain, 50 kD), IgM (heavy chain, 75 kD) and IgA (heavy chain, 55 kD). Isotype control antibodies mainly consisted of IgG with IgA and IgM present in lesser extent.

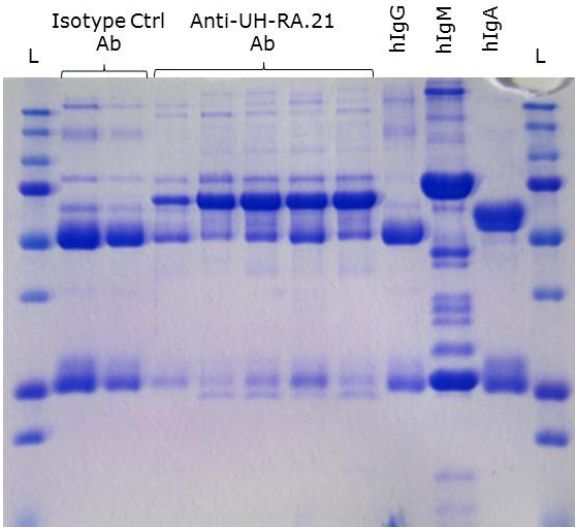


Figure 3: Purity and antibody isotypes in purified antibodies
SDS-PAGE analysis of antibodies purified from randomly selected anti-UH-RA.21 antibody-negative healthy controls and anti-UH-RA.21 antibody-positive rheumatoid arthritis patients. Proteins were visualized with Coomassie Blue. Lane L, Precision Plus Protein standards dual color; Isotype ctrl Ab, Isotype control antibodies; Anti-UH-RA.21 Ab, Anti-UH-RA.21 antibodies; hIgG, monoclonal human IgG; hIgM, polyclonal human IgM; hIgA, monoclonal human IgA.

A dot blot analysis was performed to confirm the presence of different antibody isotypes in purified antibodies. Standard series (500 µg-0 µg) of human IgG (Figure 4A), IgM (Figure 4B) and IgA (Figure 4C) were used as a reference. An anti-UH-RA.21 antibody pool and isotype control antibodies were both undiluted and diluted (1:10) included in the test. The anti-UH-RA.21 antibody pool and undiluted isotype control antibodies contained IgG, IgM and IgA. Diluted isotype control antibodies showed no IgA present.

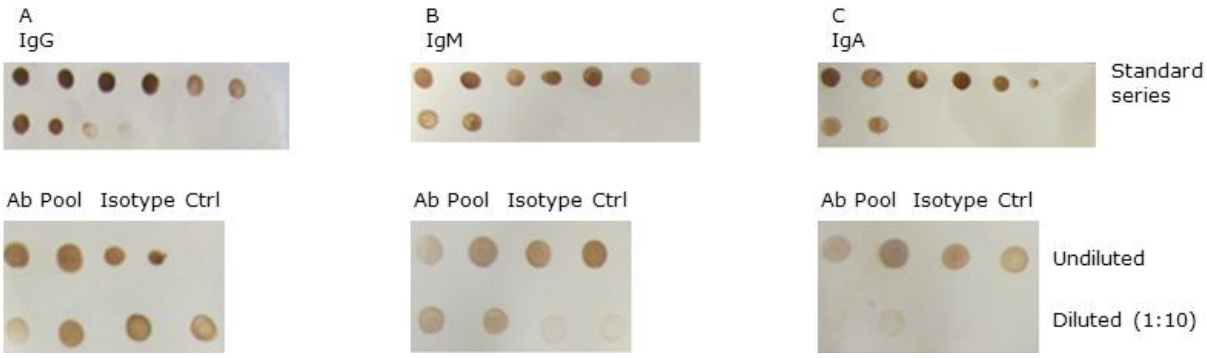


Figure 4: Confirmation of antibody isotypes in purified antibodies
Three distinct nitrocellulose membranes were spotted in duplicate with a standard series of human IgG (A), IgM (B) and IgA (C) (500 µg-0 µg). Furthermore, undiluted and diluted (1:10) anti-UH-RA.21 antibody pool (Ab Pool) and isotype control antibodies (Isotype Ctrl) were spotted in duplicate. Following incubation of the membranes with HRP-labeled rabbit anti-human IgG (A), HRP-labeled mouse anti-human IgM (B) or HRP-labeled mouse anti-human IgA (C), antibody isotypes were detected using a DAB staining.

Because of the presence of IgG, IgM and IgA within purified anti-UH-RA.21 antibodies, isotype reactivity against UH-RA.21 was investigated. The peptide ELISA protocol to detect the IgA reactivity against UH-RA.21 was optimized using a checkerboard (Figure 5A and B). A maximum sensitivity to measure the IgA anti-UH-RA.21 antibody response was obtained by modifying the standard peptide ELISA protocol with coating in a concentration of 5 µg/ml with cyclic UH-RA.21 peptides, a plasma dilution of 1:10 in 2% MPBS, detection with 1:500 HRP-labeled mouse anti-human IgA in 2% MPBS and incubation with TMB for 20 min. After optimization of the peptide ELISA protocol for the IgA reactivity against UH-RA.21, 13 HC, 42 RA patients from which 18 early RA patients (symptoms of max 1 year), 7 SpA patients and 4 PsA patients were included in an IgG and IgA reactivity screening against UH-RA.21. Immunoglobulin G reactivity against UH-RA.21 was measured using the standard

peptide ELISA protocol (as previously described), except for the coating with cyclic UH-RA.21 peptides and control peptides in a coating concentration of 5 µg/ml. Plasma reactivity of isotype antibodies directed against UH-RA.21 was measured and represented by the mean ratio of OD(cyclic UH-RA.21 peptides) to OD(cyclic control peptides). The cut-off for a positive sample (3 times the standard deviation above the mean ratio OD(UH-RA.21 peptides)/OD(cyclic control peptides) of the HC group) was arbitrarily set at 1 relative unite and OD ratios were normalized to it so that plasma samples with a normalized mean OD(cyclic UH-RA.21 peptides)/OD(cyclic control peptides) ratio higher than 1 were considered antibody-positive. The IgG anti-UH-RA.21 antibody response was compared between the five patient groups. A significant difference was found between HC and PsA patients ($p<0.05$), while no difference was observed between HC and early or established RA patients and PsA patients (Figure 5C). Also no difference was found for the IgA anti-UH-RA.21 antibody response between the five patient groups (Figure 5D). The IgG and IgA anti-UH-RA.21 antibody responses were compared separately for each group, but no difference was observed. Of the screened RA patients, 4.8% was positive for IgG and IgA anti-UH-RA.21 antibodies, 21.4% was negative for IgG and positive for IgA anti-UH-RA.21 antibodies, 4.8% was positive for IgG and negative for IgA anti-UH-RA.21 antibodies and 69% was negative for both IgG and IgA anti-UH-RA.21 antibodies. We also looked for correlations between the isotype reactivity against UH-RA.21 and clinical features of the screened RA patients (RF and ACPA status, disease duration, age of disease onset and gender), but no correlations were demonstrated.

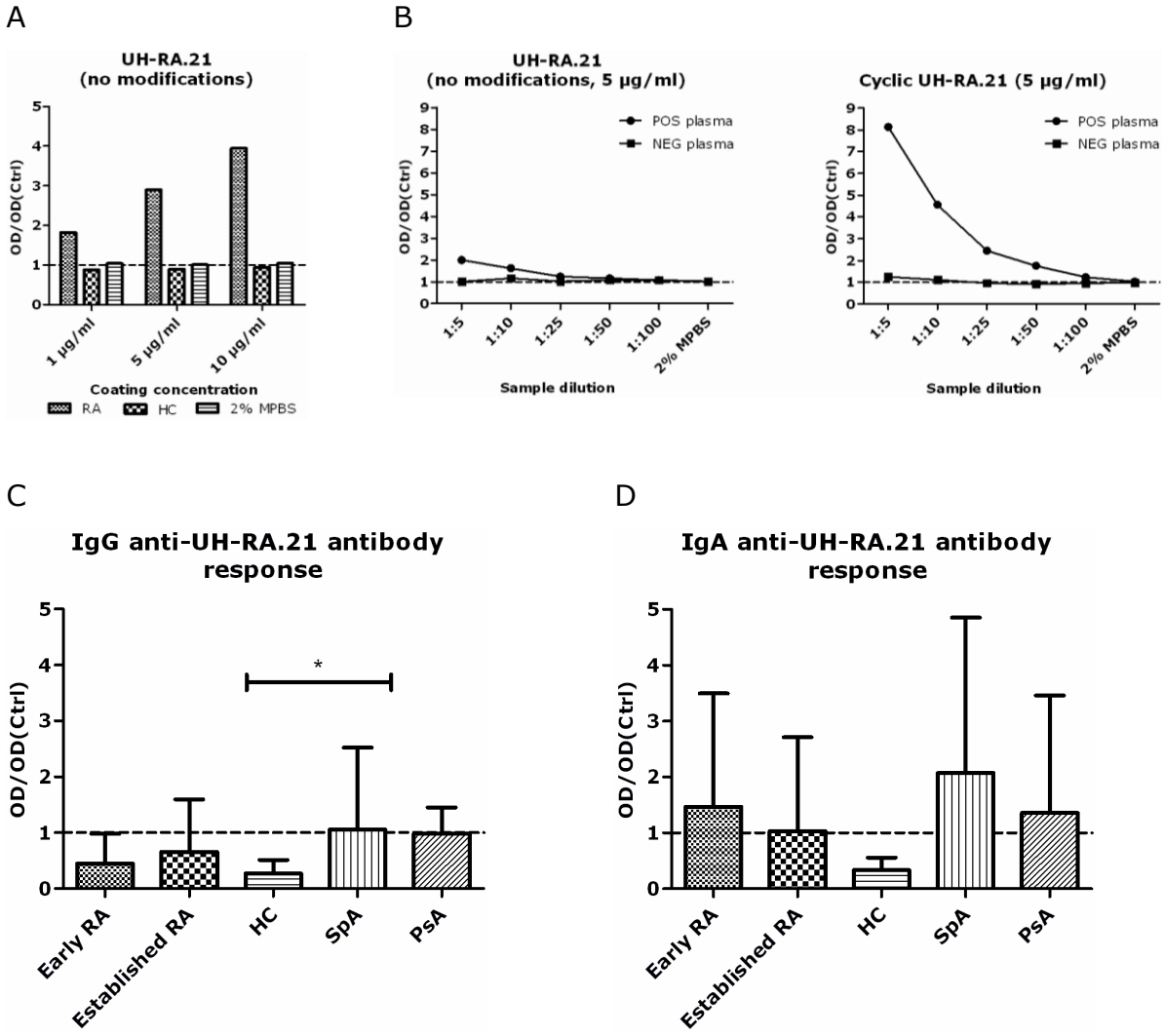


Figure 5: Antibody isotype reactivity against UH-RA.21
(A) Polystyrene microplates were coated with UH-RA.21 peptides (no modifications) and control peptides (WTKTPDGNFQLGGTEP) in a concentration of 1, 5 and 10 µg/ml. Anti-UH-RA.21 antibody-positive RA plasma and anti-UH-RA.21 antibody-negative healthy control plasma was diluted 2:5 in 2% Marvel in phosphate buffered saline (2% MPBS). After incubation with HRP-labeled mouse anti-human IgA (1:500 in 2% MPBS), the IgA reactivity against UH-RA.21 was measured and represented by the mean ratio of optical density (OD) (UH-RA.21 peptides) to OD(control peptides WTKTPDGNFQLGGTEP) (OD/OD(Ctrl)). Plasma with a (mean) ratio higher than 1 was considered positive. (B) Next, coating with 5 µg/ml UH-RA.21 peptides (no modifications) and cyclic UH-RA.21 peptides was compared and the optimal sample dilution was determined. Positive (POS plasma) and negative plasma (NEG plasma) were diluted 1:5 to 1:100. (C and D) IgG and IgA reactivity against UH-RA.21 was measured for 13 healthy controls (HC), 42 rheumatoid arthritis (RA) patients, 7 ankylosing spondylitis (SpA) patients and 4 psoriatic arthritis (PsA) patients using peptide ELISA. Isotype antibody responses directed against UH-RA.21 were represented by the normalized mean ratio of OD/OD(Ctrl). The cut-off score for a positive sample (3 times the standard deviation above the mean ratio OD/OD(Ctrl) of the HC group) was arbitrarily set at 1.0 relative unite and OD ratios were normalized to it so that a plasma sample with a normalized mean OD/OD(Ctrl) ratio higher than 1 was considered positive. Samples were tested in duplicate. (C) Comparison of the IgG anti-UH-RA.21 antibody response in the five patients groups (*$p<0.05$). (D) Comparison of the IgA anti-UH-RA.21 antibody response in the five patient groups.

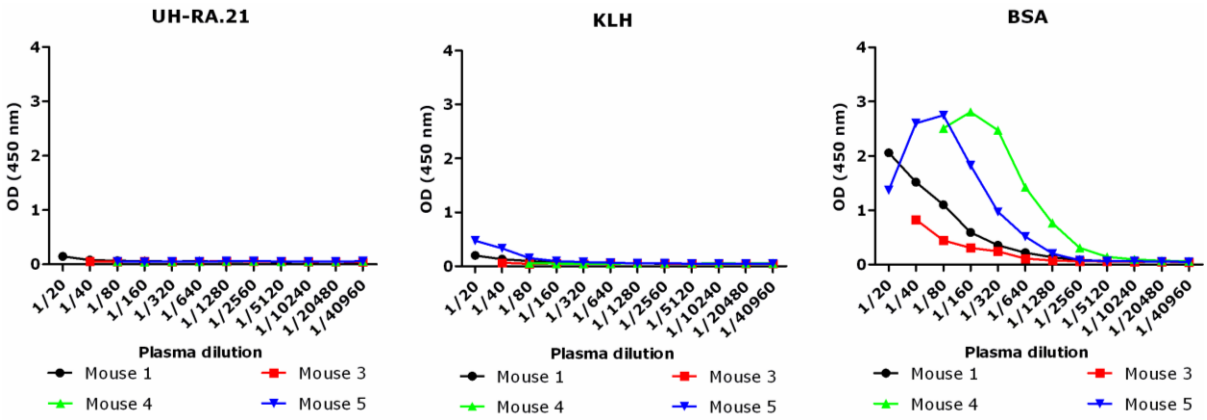
7.2 Monoclonal cell line production

7.2.1 Immunization and titration

For the generation of a cell line that produces monoclonal anti-UH-RA.21 antibodies, female, 6 to 8-week-old BALB/c mice were immunized (Table 3). First, mice were immunized with UH-RA.21 peptides coupled to KLH and dissolved in CFA to enhance the immune response. After 14 days, mice were injected with UH-RA.21 peptides coupled to BSA in ICFA. At day 25, plasma titers of anti-UH-RA.21 antibodies were measured using a peptide ELISA (Figure 6A). Mice could only be used for the production of a monoclonal cell line if anti-UH-RA.21 antibody plasma titers were sufficiently high. Blood plasma was diluted (1:20 – 1:40960) and anti-UH-RA.21 antibody plasma titers were measured. All plasma dilutions showed a very low antibody response directed against UH-RA.21 and KLH and a high anti-BSA antibody response. Therefore, mice received a third immunization with UH-RA.21 peptides coupled to KLH in ICFA. After 10 days, blood plasma titers of anti-UH-RA.21 antibodies were redetermined. As shown in Figure 6B, the antibody response directed against KLH remained the same for all mice, but the anti-BSA antibody response was increased. Mouse 2 had a very low anti-UH-RA.21 antibody response, mouse 1 and 3 exhibited a moderate anti-UH-RA.21 antibody response and mouse 4 and 5 displayed a very high anti-UH-RA.21 antibody response. Because of the high antibody response directed against UH-RA.21, which indicates a sufficient amount of B cells producing specific anti-UH-RA.21 antibodies, mouse 4 and 5 were selected to generate a hybridoma cell line.

A

Day 25*



B

Day 38

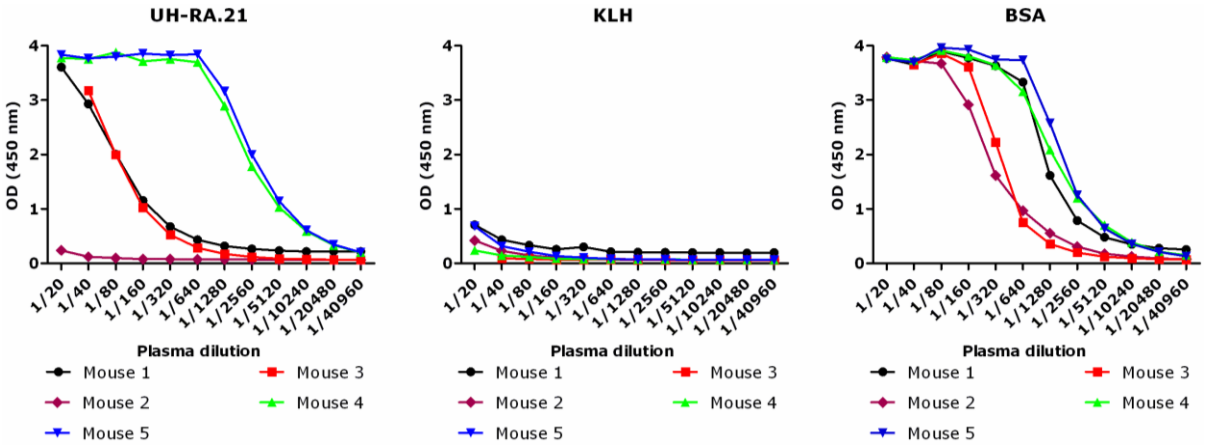


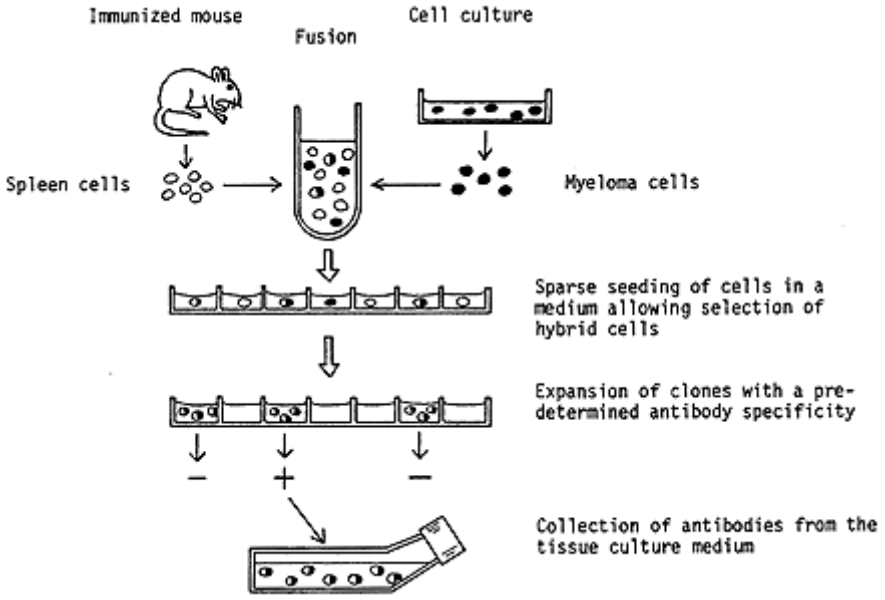
Figure 6: Blood plasma titers of antibodies directed against UH-RA.21, KLH and BSA
The antibody response directed against UH-RA.21, KLH and BSA was studied in dilutional series. At day 25 (A) and 38 (B) after the first immunization, plasma titers of these antibodies were measured using a peptide ELISA. KLH, Keyhole Limpet Hemocyanin; BSA, Bovine serum albumin; OD, optical density. *Data mouse 2 at day 25: not available

7.2.2 Fusion and cloning

Mouse 4 and 5 were sacrificed and spleens were isolated (Figure 7A). Spleen cells were homogenized and fused with Sp2/0-Ag14 mouse myeloma cells using PEG. B cells of the spleen that successfully fused with Sp2/0-Ag14 mouse myeloma cells were selected via culture in selective HAT medium. After a few days, cells were scored for the presence of hybridoma cells (score 0 in absence of hybridoma clones, score 1 in the presence of one group of hybridoma clones (Figure 7B) and score + in the presence of more than one group of hybridoma clones (Figure 7C)). At day 10 after the fusion, cell culture

supernatants were screened for the presence of anti-UH-RA.21 antibodies (Data not shown). Hybridoma cells producing anti-UH-RA.21 antibodies were cloned and screened repeatedly until a monoclonal hybridoma clone was obtained.

A



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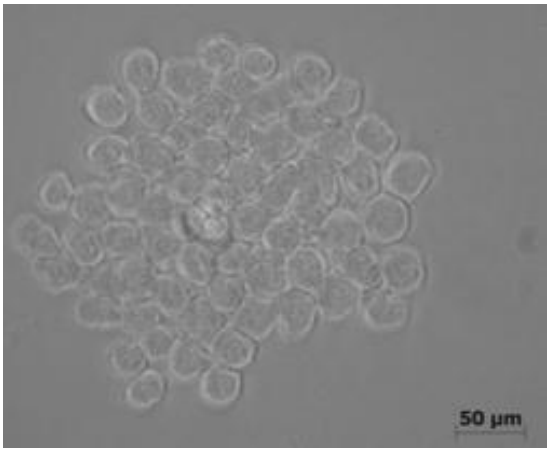

C

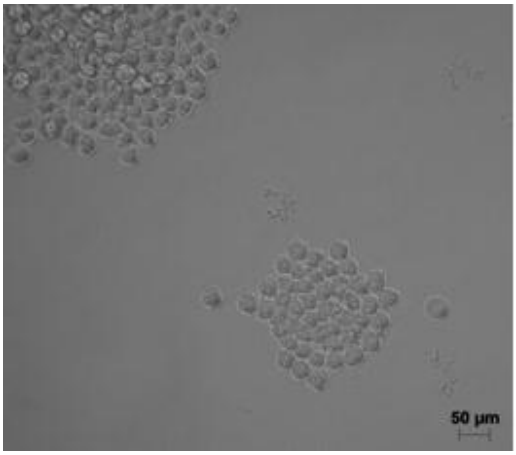

Figure 7: Hybridoma cell line
Principal steps of the hybridoma technology (A). Phase contrast photomicrograph of hybridoma clones derived from the fusion of Sp2/0-Ag14 mouse myeloma cells and B cells of the spleen that produce anti-UH-RA.21 antibodies (one group of hybridoma clones (Score 1, x40)) (B), (several groups of hybridoma clones (Score +, x20)) (C). Photographs were taken with a Zeiss PrimoVert microscope and adapted with AxioVision.

7.2.3 Monoclonal anti-UH-RA.21 antibodies

The isotype of anti-UH-RA.21 antibodies produced by hybridoma cells was determined using the Rapid ELISA Mouse mAb Isotyping kit. As shown in Figure 8A, anti-UH-RA.21 antibodies of tested hybridoma clones showed an IgM antibody isotype with a kappa light chain. SDS-PAGE analysis was performed to confirm the antibody isotype and to study the purity of produced anti-UH-RA.21 antibodies (Figure 8B). Purified anti-UH-RA.21 antibodies exhibited a high purity and displayed a heavy chain band of approximately 73 kD and a light chain band of approximately 25 kD. According to the results, anti-UH-RA.21 antibodies produced by the tested hybridoma clones have an IgM antibody isotype.

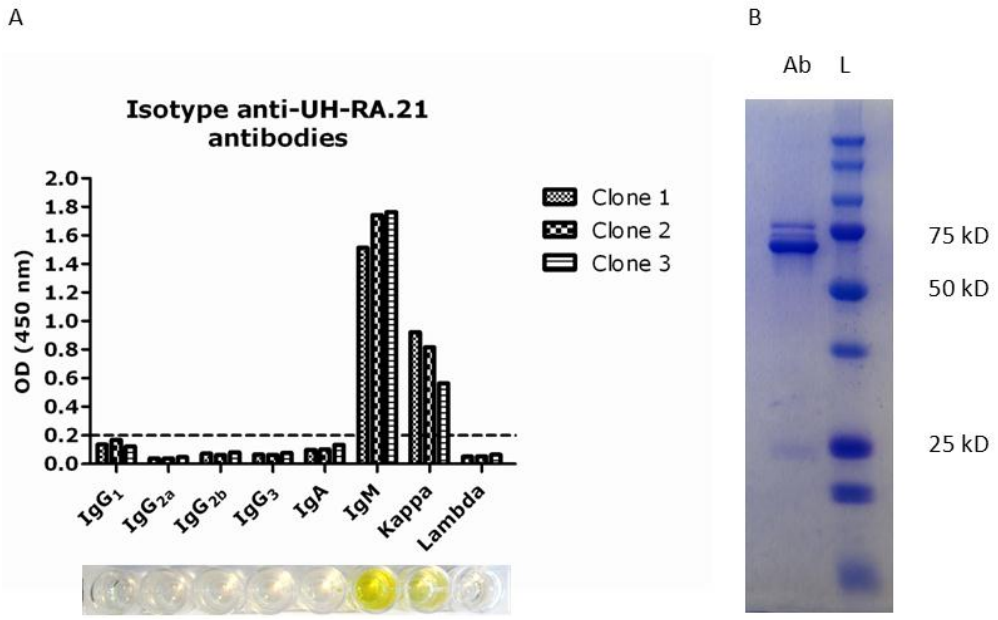

Figure 8: Antibody isotype of the monoclonal anti-UH-RA.21 antibodies
(A) The isotype of produced anti-UH-RA.21 antibodies was determined using the Pierce Rapid ELISA Mouse mAb isotyping Kit. Antibodies of three hybridoma clones were tested and results were considered positive if OD_{450} was ≥0.2. (B) SDS-PAGE analysis was performed to confirm the antibody isotype and to study the purity of produced anti-UH-RA.21 antibodies (Ab). Lane L, Precision Plus Protein standards dual color.

7.3 Immunoprecipitation and western blotting: Optimization

Knee tissue of an RA patient was homogenized and stored in nonyl phenoxypolyethoxylethanol (NP40) or radioimmunoprecipitation assay (RIPA) buffer at -80°C. To verify the quality of the lysates, SDS-PAGE analysis was performed (Figure 9A). Lysates in NP40 and RIPA buffer displayed similar protein band patterns and were well preserved. Western blotting was performed with 5-20 µg knee lysate in NP40 and RIPA buffer. Proteins probed by anti-UH-RA.21 antibodies and isotype control antibodies were visualized using ECL detection (Data not shown). ECL pictures showed a very high background signal and some protein bands, but the lysates were too concentrated to find distinctions between the two blots. Therefore, immunoprecipitation was performed prior to western blotting (Figure 9B). Immunoprecipitates probed by anti-UH-RA.21 antibodies and isotype control antibodies were visualized using ECL detection (Data not shown) and DAB staining (Figure 9C, top). ECL detection again displayed too much background signal. DAB staining revealed protein bands, but no difference was observed between immunoprecipitates probed by anti-UH-RA.21 antibodies or isotype control antibodies. Protein extracts (cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal protein extract) were made of knee lysate in NP40 buffer and western blotting was performed. DAB staining was used to visualize protein bands probed by anti-UH-RA.21 antibodies or isotype control antibodies. Only cytoplasmic and membrane extracts showed a protein band pattern, but without difference in observed antibody reactivity.

New lysates of knee synovial tissue of the same RA patient were obtained and separated into protein extracts (cytoplasmic, membrane, nuclear soluble, chromatin-bound and cytoskeletal protein extract). Immunoprecipitation followed by western blotting was performed and immunoprecipitates were visualized using DAB staining (Figure 9C, bottom). Cytoplasmic and membrane protein extracts showed similar protein band patterns in the first eluate, but the second and third eluate only showed a small protein band of approximately 50 kD. No difference was observed between immunoprecipitates of protein A/G resin coupled with anti-UH-RA.21 antibodies and negative control

resin. We chose to perform further experiments with knee lysates in NP40 buffer. Immunoprecipitation was performed with knee lysate in NP40 buffer, but incubation steps were prolonged. Flow through and immunoprecipitates of protein A/G resin coupled with anti-UH-RA.21 antibodies and negative control resin were used to carry out western blotting. Western blotting was performed in several conditions: probing with human derived polyclonal anti-UH-RA.21 antibodies and isotype control antibodies, mouse derived monoclonal anti-UH-RA.21 antibodies and blocking buffer as a control for aspecific binding of the secondary antibodies. ECL detection showed a broad protein band pattern for blots probed with polyclonal anti-UH-RA.21 antibodies (Figure 9D, right) and isotype control antibodies. Also, aspecific binding of HRP-labeled rabbit anti-human IgG was detected. High background signals and aspecific binding were overcome by using monoclonal anti-UH-RA.21 antibodies. The blot probed with monoclonal anti-UH-RA.21 antibodies showed five protein bands of which four bands were most likely derived from binding of antibodies present in the lysate (Figure 9D, left). The remaining protein band might contain the corresponding *in vivo* antigen of UH-RA.21.

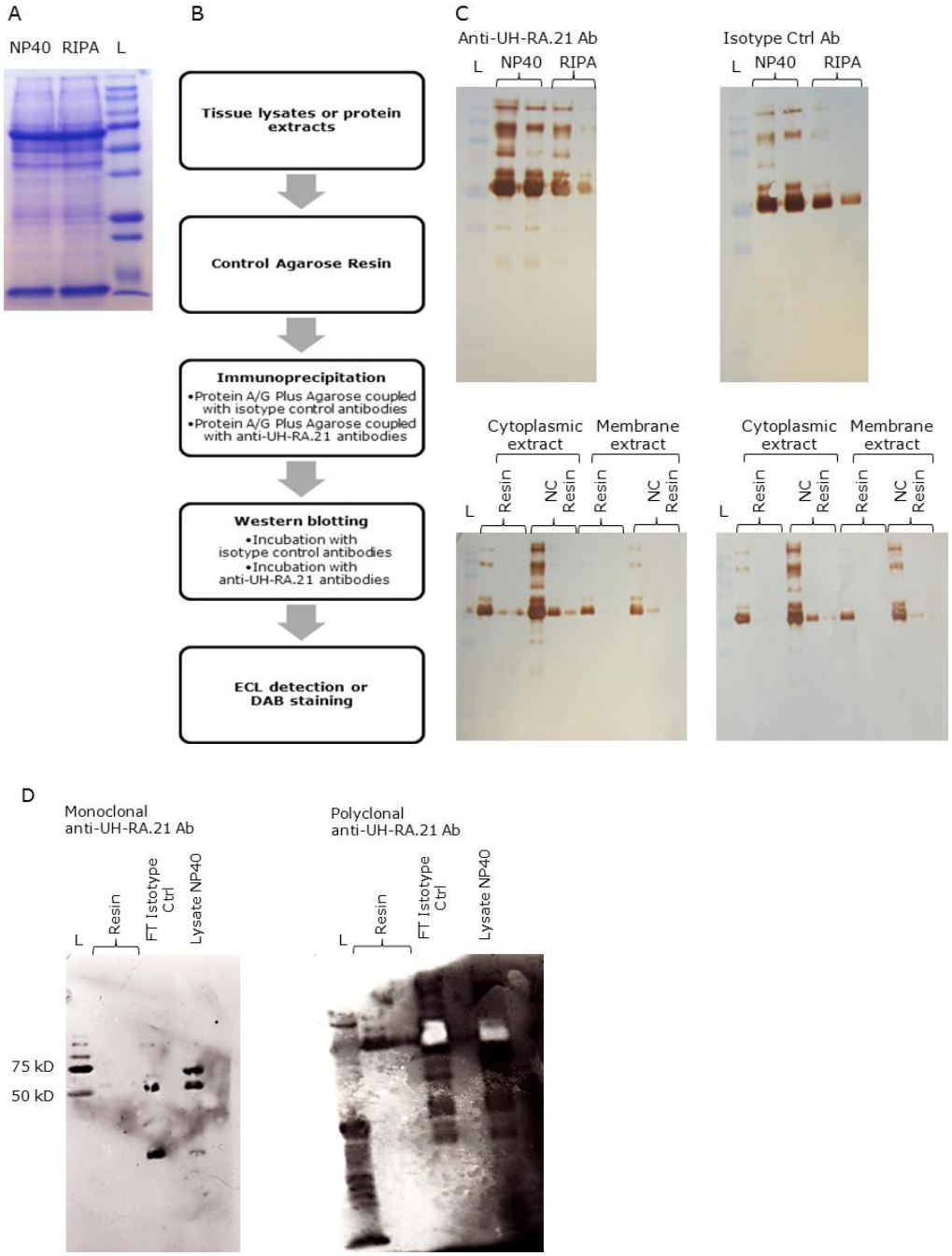

Figure 9: Immunoprecipitation and western blotting (optimization)

(A) Knee lysates in NP40 and RIPA buffer. (B) Immunoprecipitation followed by western blotting, principal steps. (C, top) Immunoprecipitation followed by western blotting performed with knee lysates in NP40 and RIPA buffer. Lysate incubated with protein A/G Plus Agarose coupled with anti-UH-RA.21 antibodies and negative control resin, respectively. (C, bottom) Immunoprecipitation followed by western blotting performed with protein extracts derived from new knee synovial tissue lysates. Probed antigens were visualized using a DAB staining. (D) Immunoprecipitation followed by western blotting performed with knee lysate in NP40 buffer. Proteins probed with monoclonal and polyclonal anti-UH-RA.21 antibodies were visualized using ECL detection. Lane L, Precision Plus Protein standards dual color; Ab, antibody; isotype Ctrl Ab, isotype control antibodies; Resin, protein A/G Plus Agarose coupled with anti-UH-RA.21 antibodies; NC resin, negative control resin, FT Isotype Ctrl, Flowthrough negative control resin.

8 Discussion

In a previous study of our research group, the antibody response directed against UH-RA.21 was selected as the most promising candidate autoantibody RA marker [37]. Its biomarker potential was already indicated by the specificity (95%) and sensitivity (29%) of the marker, but also by presence of anti-UH-RA.21 antibodies in synovial fluid of RA patients and overexpression of the corresponding *in vivo* antigen of UH-RA.21 in RA synovial tissue [37]. Furthermore, preliminary results showed a potential role of autoantibody reactivity against UH-RA.21 in underlying disease processes of RA.

Expression of a normally untranslated mRNA region resulted in the UH-RA.21 peptide sequence. Therefore, the UH-RA.21 peptide sequence probably comprises a mimotope, meaning that it contains an epitope that structurally mimics an *in vivo* antigen. Both the epitope and the identity of the *in vivo* antigen were still unknown. In this study, the goal was to characterize the antibody response directed against UH-RA.21 and the corresponding *in vivo* antigen of UH-RA.21.

To identify the corresponding *in vivo* antigen of UH-RA.21, anti-UH-RA.21 antibodies were required. Polyclonal anti-UH-RA.21 antibodies were obtained from blood plasma of RA patients testing positive for anti-UH-RA.21 antibodies on ELISA. Blood plasma of randomly selected, anti-UH-RA.21 antibody-negative HC was used to purify isotype control antibodies. Purified anti-UH-RA.21 antibodies were investigated for their specificity and purity, but also for the presence of different antibody isotypes. Both anti-UH-RA.21 antibodies and isotype control antibodies showed a high purity. Anti-UH-RA.21 antibodies consisted of IgG, IgM and IgA antibody isotypes. Isotype control antibodies mainly contained IgG with IgA and IgM present in lesser extent. The presence of different antibody isotypes in purified antibodies was confirmed by a dot blot analysis and raised the question whether there was a difference between the IgG and IgA reactivity against UH-RA.21. Therefore, a screening was performed to determine the plasma reactivity of isotype anti-UH-RA.21 antibodies. The screening included 13 HC, 42 RA patients of which 18 were early RA patients and 11 RC patients of which 7 were SpA patients and 4 were PsA patients (randomly selected). According to the results, a significant difference was found

for the IgG anti-UH-RA.21 antibody response between the HC group and the PsA group (Figure 5C, $p<0.05$), while no difference was observed between HC, early or established RA patients and PsA patients. In addition, IgA reactivity against UH-RA.21 did not differ between the five patient groups. Comparing the IgG and IgA anti-UH-RA.21 antibody responses separately for each group, no difference was observed. Of the screened RA patients, 4.8% was positive for IgG and IgA anti-UH-RA.21 antibodies, 4.8% was positive for IgG and negative for IgA anti-UH-RA.21 antibodies, 21.4% was negative for IgG and positive for IgA anti-UH-RA.21 antibodies and 69% was negative for both IgG and IgA anti-UH-RA.21 antibodies. This confirms the previously demonstrated sensitivity of the marker [37]. No correlations were found between the IgG and/or IgA reactivity against UH-RA.21 and clinical features of screened RA patients (RF and anti-CCP antibody status, disease duration, age of disease onset and gender). So far, only a difference was seen for the IgG anti-UH-RA.21 antibody response between HC and PsA patients. To draw definitive conclusions, the screening needs to be repeated and more RA patients and controls must be included. Furthermore, it would also be interesting to analyze the anti-UH-RA.21 antibody response in synovial fluid of RA patients and to search for correlations with clinical features of RA patients. This would contribute to a more complete characterization of the anti-UH-RA.21 antibody response and may give us information on the prognostic value of this marker.

In the past years, many research groups studied the currently used RA disease markers for differences in isotype antibody reactivity and correlations with clinical features of RA patients. Rheumatoid factor was first detected as IgA in pre-RA patients, followed by IgG-RF and IgM-RF. Just before onset of disease, the frequency of IgA-RF and IgM-RF markedly increases [38]. In established RA patients, RF is mainly present in IgM, but serum and synovial fluid also contain IgG-RF and IgA-RF, providing additional diagnostic information. IgM-RF is not specific for RA and studies even suggest that IgA-RF would be a more specific RA marker, compared to IgG-RF and IgM-RF [33, 39]. Furthermore, high titers of IgM-RF and IgA-RF were associated with severe RA forms (such as radiological erosions, more rapid disease progression, worse disease outcome and extra-articular manifestations), implying a considerable prognostic value [39]. However, the most specific serological RA marker is ACPA [33, 36, 40]. Kokkonen H. et al (2010) proved that anti-CCP antibodies were present early in

the disease course and may even precede clinical onset [41]. In pre-RA patients, IgA anti-CCP antibodies were detected in a lower frequency compared to IgG while, IgM anti-CCP antibodies appeared later with a lower frequency [38]. In established RA patients, IgG anti-CCP antibodies were mainly present (74.8%), followed by IgA (52.9%) and IgM (44.5%). Moreover, associations were found between the presence of ACPA and radiographic disease progression (i.e. bone damage), the presence of RA associated HLA-DR alleles or environmental factors such as smoking [40, 42].

Polyclonal anti-UH-RA.21 antibodies derived from blood plasma consist of a heterogeneous mixture of antibodies. Each antibody binds a unique site of the corresponding *in vivo* antigen of UH-RA.21 with a different affinity and represents a different antibody class or subtype. Polyclonal anti-UH-RA.21 antibodies achieve high-affinity interactions with the corresponding *in vivo* antigen of UH-RA.21 via the reactivity with multiple epitopes of this antigen, but the mixture of polyclonal antibodies also contains a lot of irrelevant antibodies. Furthermore, inconsistencies may exist between blood plasma samples and the amount of specific antibodies is also limited. In comparison with polyclonal antibodies, monoclonal antibodies derived from hybridoma clones are highly specific and homogeneous in their binding characteristics and exhibit nanomolar binding affinities. Furthermore, monoclonal antibodies can be selected for their unique epitope binding or functional activities [43]. In order to obtain monoclonal anti-UH-RA.21 antibodies, a cell line was generated by hybridoma technology. After three immunizations with UH-RA.21 peptides, two out of five immunized mice had reached a sufficiently high anti-UH-RA.21 antibody plasma titer (Figure 6), which means that antigen-specific B cells were activated and differentiated into effector cells that actively produce antibodies of different isotypes [44]. Both mice were euthanized and their spleens were isolated for *in vitro* hybridoma cell production. Cell supernatants were screened for the presence of anti-UH-RA.21 antibodies and the isotype of the antibodies was determined. Produced anti-UH-RA.21 antibodies showed a high purity and had an IgM isotype (Figure 8). The isotype of antibodies produced by hybridoma clones is dependent on several factors. At rest, B cells express membrane bound IgG and IgM, which both function as receptors for antigens. After appropriate antigen stimulation, B cells will carry

various types of membrane immunoglobulins and start to proliferate and differentiate into plasma cells. During the differentiation of B cells, heavy chain class switching can occur and the production of IgM isotype antibodies can change into other antibody isotypes [44]. Antibody isotypes not only differ in molecular weight, structure and expression, but they also have a different role in the immune system. Therefore, a specific antibody isotype is necessary for some purposes.
In the future, the mice with a moderate anti-UH-RA.21 antibody response will be boosted with UH-RA.21 peptides to generate a hybridoma cell line that produces IgG or IgA anti-UH-RA.21 antibody isotypes. This will contribute to a more complete characterization of the anti-UH-RA.21 antibody response and its role in underlying disease processes of RA.

After the purification of anti-UH-RA.21 antibodies, the identity of the corresponding *in vivo* antigen of UH-RA.21 could be studied. Therefore, the immunoprecipitation and western blotting protocol had to be optimized. We started with western blotting of knee lysates in NP40 and RIPA buffer. Proteins of the lysate probed by anti-UH-RA.21 antibodies and isotype control antibodies were visualized by ECL detection. ECL results showed a high background signal and demonstrated that the used lysates were too concentrated to find distinctions between the two blots. Therefore, immunoprecipitation was performed prior to western blotting and protein extracts of lysate in NP40 buffer were obtained. But still, a high background signal was present. Consequently, lysates were incubated with negative control resin prior to incubation with protein A/G Plus Agarose coupled with anti-UH-RA.21 antibodies and incubation steps were prolonged. Furthermore, aspecific binding of HRP-labeled rabbit anti-human IgG was detected, which can arise when the same antibodies (or antibodies of the same species) were used to perform both the immunoprecipitation and western blotting. During immunoprecipitation, both antigens and antibodies coupled with the protein A/G Plus Agarose were eluated and size-fractionated by SDS-PAGE, resulting in the binding of light and heavy antibody chains by secondary antibodies [45]. To circumvent this problem, reducing agents from the immunoprecipitation buffers can be excluded, boiling of samples can be avoid so that the tetrameric antibody structure is preserved or antibodies of a different species can be used

to avoid cross-reactivity [45]. Therefore, mouse derived monoclonal anti-UH-RA.21 antibodies were purified and used to perform western blotting, which resulted in five protein bands. Four protein bands were most likely derived from binding with antibodies present within the eluate or lysate. The remaining protein band might contain the corresponding *in vivo* antigen of UH-RA.21. In the future, the remaining protein band will be isolated and identified via mass spectrometry. Western blotting with monoclonal anti-UH-RA.21 antibodies will be performed on immunoprecipitated protein extracts. These experiments will provide us information about the identity and location of the corresponding *in vivo* antigen of UH-RA.21. The expression pattern of the corresponding *in vivo* antigen of UH-RA.21 will also be investigated via immunohistochemistry. First, the corresponding *in vivo* antigen of UH-RA.21 will be localized in human synovial tissue slices. Next, the expression pattern will be studied by comparing tissues of HC and RA patients, tissues of RA patients seropositive and seronegative for the currently used RA disease markers and tissues of early and established RA. We hypothesize that the corresponding *in vivo* antigen of UH-RA.21, an artificial peptide potentially involved in the disease processes of RA, is differentially expressed between HC and RA patients, and between different RA subtypes.

Targets of autoantibodies associated with RA are quite diverse. Waaler E. (1940) first described RF, which was an autoantibody specific for IgG. Other targets were collagen and cartilage proteins, heat shock proteins, enzymes, nuclear proteins and citrullinated proteins such as fibrin or vimentin, which were targeted by ACPA [36, 40].

In conclusion, polyclonal anti-UH-RA.21 antibodies were purified and studied for their specificity, purity and presence of different antibody isotypes. Because of the presence of different antibody isotypes within the purified antibodies, a screening was performed to study the isotype reactivity against UH-RA.21 in HC, RA patients and RC. Monoclonal anti-UH-RA.21 antibodies were obtained from a cell line generated by hybridoma technology.

The identity of the corresponding *in vivo* antigen of UH-RA.21 was studied via immunoprecipitation followed by western blotting with monoclonal anti-UH-RA.21 antibodies, resulting in five remaining protein bands. One of the protein bands might contain the corresponding *in vivo* antigen of UH-RA.21

and will be isolated and identified by mass spectrometry. This will give us more insight into underlying disease processes of RA.

With a prevalence of 1%, RA is the most common severe inflammatory joint disease with an unknown etiology, pathogenesis and radical treatment [36]. Beyond the pain and stiffness, RA affects the social life, physical function and emotional well-being of the patient [46]. Psychologically, the disease has an influence on the self-concept and mood of RA patients. Physically, inflammation of the synovial joints and joint destruction result in disability. Dependent on disease duration and severity, RA patients will become unable to work (2% and 18% for early and late RA patients, respectively) and unemployed [47, 48]. The effect of the disease on socioeconomic consequences is also reflected in the need for help and health care, which is accompanied with high costs for both individual and society. For early and late RA patients, the annual societal direct costs per patient are estimated at € 3055 and € 9946, respectively [48].

The biomarker potential of the anti-UH-RA.21 antibody response was already indicated by the specificity (95%) and sensitivity (29%) of the marker, but also by presence of anti-UH-RA.21 antibodies in synovial fluid of RA patients and overexpression of the corresponding *in vivo* antigen of UH-RA.21 in RA synovial tissue [37]. If undifferentiated arthritis, early and seronegative RA patients can be diagnosed by the anti-UH-RA.21 antibody response in an early phase of disease, treatment can be started early and irreversible joint damage and loss of productivity can be prevented. Therefore, RA patients will be able to continue working and have less need for help and health care, resulting in reduced RA-associated costs. Besides the diagnostic potential of this marker, the prognostic value will be studied by looking for correlations between the anti-UH-RA.21 antibody response and clinical features of RA patients (e.g. RF and ACPA status, age of disease onset, gender, disease activity etc.). Therefore, patients will be better informed about their disease course and treated with an adapted therapy. Furthermore, the characterization of the corresponding *in vivo* antigen of UH-RA.21 will provide novel insights into underlying RA disease processes. In the future, this academic knowledge can contribute to the development of novel and better treatment options for RA patients.

9 References

1. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature. 2003;423(6937):356-61. Epub 2003/05/16.
2. Alamanos Y, Drosos AA. Epidemiology of adult rheumatoid arthritis. Autoimmunity reviews. 2005;4(3):130-6. Epub 2005/04/13.
3. Somers K, Stinissen P, Somers V. Optimization of high-throughput autoantibody profiling for the discovery of novel antigenic targets in rheumatoid arthritis. Ann N Y Acad Sci. 2009;1173:92-102. Epub 2009/09/18.
4. Avouac J, Gossec L, Dougados M. Diagnostic and predictive value of anti-cyclic citrullinated protein antibodies in rheumatoid arthritis: a systematic literature review. Ann Rheum Dis. 2006;65(7):845-51. Epub 2006/04/12.
5. Nell VP, Machold KP, Eberl G, Stamm TA, Uffmann M, Smolen JS. Benefit of very early referral and very early therapy with disease-modifying anti-rheumatic drugs in patients with early rheumatoid arthritis. Rheumatology (Oxford). 2004;43(7):906-14. Epub 2004/04/29.
6. Diratzouian H, Freedman GM, Hanlon AL, Eisenberg DF, Anderson PR. Importance of physical examination in the absence of a mammographic abnormality for the detection of early-stage breast cancer. Clinical breast cancer. 2005;6(4):330-3. Epub 2005/11/10.
7. Eisenberg DP, Adusumilli PS, Hendershott KJ, Yu Z, Mullerad M, Chan MK, et al. 5-fluorouracil and gemcitabine potentiate the efficacy of oncolytic herpes viral gene therapy in the treatment of pancreatic cancer. Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract. 2005;9(8):1068-77; discussion 77-9. Epub 2005/11/05.
8. VanderBorght A, Geusens P, Raus J, Stinissen P. The autoimmune pathogenesis of rheumatoid arthritis: role of autoreactive T cells and new immunotherapies. Seminars in arthritis and rheumatism. 2001;31(3):160-75. Epub 2001/12/12.
9. Klareskog L, Malmstrom V, Lundberg K, Padyukov L, Alfredsson L. Smoking, citrullination and genetic variability in the immunopathogenesis of rheumatoid arthritis. Seminars in immunology. 2011;23(2):92-8. Epub 2011/03/08.
10. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP antibodies: the past, the present and the future. Nat Rev Rheumatol. 2011;7(7):391-8. Epub 2011/06/08.
11. Kiener HP, Karonitsch T. The synovium as a privileged site in rheumatoid arthritis: cadherin-11 as a dominant player in synovial pathology. Best practice & research Clinical rheumatology. 2011;25(6):767-77. Epub 2012/01/24.
12. Henderson B, Pettipher ER. The synovial lining cell: biology and pathobiology. Seminars in arthritis and rheumatism. 1985;15(1):1-32. Epub 1985/08/01.
13. Strand V, Kimberly R, Isaacs JD. Biologic therapies in rheumatology: lessons learned, future directions. Nature reviews Drug discovery. 2007;6(1):75-92. Epub 2006/12/30.
14. Palmer DG. The anatomy of the rheumatoid lesion. British medical bulletin. 1995;51(2):286-95. Epub 1995/04/01.

15. Smeets TJ, Dolhain RJ, Breedveld FC, Tak PP. Analysis of the cellular infiltrates and expression of cytokines in synovial tissue from patients with rheumatoid arthritis and reactive arthritis. The Journal of pathology. 1998;186(1):75-81. Epub 1999/01/06.
16. Yanni G, Whelan A, Feighery C, Bresnihan B. Analysis of cell populations in rheumatoid arthritis synovial tissues. Seminars in arthritis and rheumatism. 1992;21(6):393-9. Epub 1992/06/01.
17. Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. Nature reviews Drug discovery. 2003;2(6):473-88. Epub 2003/05/31.
18. Smith MD. The normal synovium. The open rheumatology journal. 2011;5:100-6. Epub 2012/01/27.
19. Vinay Kumar AKA, Nelson Fausto, RIchard N. Mitchell. Robbins Basic Pathology. 8th Edition ed. Philadelphia: Saunders Elsevier; 2007 2007. 946 p.
20. Nakken B, Munthe LA, Konttinen YT, Sandberg AK, Szekanecz Z, Alex P, et al. B-cells and their targeting in rheumatoid arthritis--current concepts and future perspectives. Autoimmunity reviews. 2011;11(1):28-34. Epub 2011/07/23.
21. Eisenberg R, Looney RJ. The therapeutic potential of anti-CD20 "what do B-cells do?". Clin Immunol. 2005;117(3):207-13. Epub 2005/09/20.
22. Declerck LS. B-Lymphocytes and Humoral Immune-Responses in Rheumatoid-Arthritis. Clinical Rheumatology. 1995;14:14-8.
23. Bugatti S, Codullo V, Caporali R, Montecucco C. B cells in rheumatoid arthritis. Autoimmunity reviews. 2007;7(2):137-42. Epub 2007/11/24.
24. Humby F, Bombardieri M, Manzo A, Kelly S, Blades MC, Kirkham B, et al. Ectopic lymphoid structures support ongoing production of class-switched autoantibodies in rheumatoid synovium. PLoS medicine. 2009;6(1):e1. Epub 2009/01/16.
25. Lim ET, Berger T, Reindl M, Dalton CM, Fernando K, Keir G, et al. Anti-myelin antibodies do not allow earlier diagnosis of multiple sclerosis. Mult Scler. 2005;11(4):492-4. Epub 2005/07/27.
26. Furst DE, Keystone EC, Kirkham B, Kavanaugh A, Fleischmann R, Mease P, et al. Updated consensus statement on biological agents for the treatment of rheumatic diseases, 2008. Ann Rheum Dis. 2008;67 Suppl 3:iii2-25. Epub 2008/12/17.
27. Matsumoto I, Maccioni M, Lee DM, Maurice M, Simmons B, Brenner M, et al. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. Nature immunology. 2002;3(4):360-5. Epub 2002/03/16.
28. Nandakumar KS, Holmdahl R. Antibody-induced arthritis: disease mechanisms and genes involved at the effector phase of arthritis. Arthritis research & therapy. 2006;8(6):223. Epub 2007/01/27.
29. Tak PP, Smeets TJ, Daha MR, Kluin PM, Meijers KA, Brand R, et al. Analysis of the synovial cell infiltrate in early rheumatoid synovial tissue in relation to local disease activity. Arthritis Rheum. 1997;40(2):217-25. Epub 1997/02/01.
30. Tampoia M, Brescia V, Fontana A, Maggiolini P, Zucano A, Pansini N. Proteomic: new advances in the diagnosis of rheumatoid arthritis. Clin Chim Acta. 2005;357(2):219-25. Epub 2005/05/18.

31. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, 3rd, et al. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. Arthritis Rheum. 2010;62(9):2569-81. Epub 2010/09/28.
32. Kumar K, North R, Taylor R, Sculpher M, Van den Abeele C, Gehring M, et al. Spinal Cord Stimulation vs. Conventional Medical Management: A Prospective, Randomized, Controlled, Multicenter Study of Patients with Failed Back Surgery Syndrome (PROCESS Study). Neuromodulation : journal of the International Neuromodulation Society. 2005;8(4):213-8. Epub 2005/10/01.
33. Vallbracht I, Rieber J, Oppermann M, Forger F, Siebert U, Helmke K. Diagnostic and clinical value of anti-cyclic citrullinated peptide antibodies compared with rheumatoid factor isotypes in rheumatoid arthritis. Ann Rheum Dis. 2004;63(9):1079-84. Epub 2004/08/17.
34. Aggarwal R, Liao K, Nair R, Ringold S, Costenbader KH. Anti-citrullinated peptide antibody assays and their role in the diagnosis of rheumatoid arthritis. Arthritis Rheum. 2009;61(11):1472-83. Epub 2009/10/31.
35. van Venrooij WJ, van Beers JJ, Pruijn GJ. Anti-CCP Antibody, a Marker for the Early Detection of Rheumatoid Arthritis. Ann N Y Acad Sci. 2008;1143:268-85. Epub 2008/12/17.
36. G. S. Autoantibodies in rheumatoid arthritis. Autoimmunity. 2006(7):8-10.
37. Somers K, Geusens P, Elewaut D, De Keyser F, Rummens JL, Coenen M, et al. Novel autoantibody markers for early and seronegative rheumatoid arthritis. J Autoimmun. 2011;36(1):33-46. Epub 2010/11/13.
38. Kokkonen H, Mullazehi M, Berglin E, Hallmans G, Wadell G, Ronnelid J, et al. Antibodies of IgG, IgA and IgM isotypes against cyclic citrullinated peptide precede the development of rheumatoid arthritis. Arthritis research & therapy. 2011;13(1):R13. Epub 2011/02/05.
39. Masdottir B, Jonsson T, Manfredsdottir V, Vikingsson A, Brekkan A, Valdimarsson H. Smoking, rheumatoid factor isotypes and severity of rheumatoid arthritis. Rheumatology (Oxford). 2000;39(11):1202-5. Epub 2000/11/21.
40. Harre U, Georgess D, Bang H, Bozec A, Axmann R, Ossipova E, et al. Induction of osteoclastogenesis and bone loss by human autoantibodies against citrullinated vimentin. The Journal of clinical investigation. 2012;122(5):1791-802. Epub 2012/04/17.
41. Kokkonen H, Soderstrom I, Rocklov J, Hallmans G, Lejon K, Rantapaa Dahlqvist S. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. Arthritis Rheum. 2010;62(2):383-91. Epub 2010/01/30.
42. Berglin E, Padyukov L, Sundin U, Hallmans G, Stenlund H, Van Venrooij WJ, et al. A combination of autoantibodies to cyclic citrullinated peptide (CCP) and HLA-DRB1 locus antigens is strongly associated with future onset of rheumatoid arthritis. Arthritis research & therapy. 2004;6(4):R303-8. Epub 2004/07/01.
43. Howard GaKM. Making and Using antibodies, a practical handbook 2007.
44. Napaporn Apiratmateekul PP. A modified hybridoma technique for production of monoclonal antibodies having desired isotypes. Cytotechnology. 2009:7.

45. Lal A, Haynes SR, Gorospe M. Clean Western blot signals from immunoprecipitated samples. Molecular and cellular probes. 2005;19(6):385-8. Epub 2005/09/09.
46. Nagyova I, Stewart RE, Macejova Z, van Dijk JP, van den Heuvel WJ. The impact of pain on psychological well-being in rheumatoid arthritis: the mediating effects of self-esteem and adjustment to disease. Patient Educ Couns. 2005;58(1):55-62. Epub 2005/06/14.
47. Girard F, Guillemin F, Novella JL, Valckenaere I, Krzanowska K, Vitry F, et al. Health-care use by rheumatoid arthritis patients compared with non-arthritic subjects. Rheumatology (Oxford). 2002;41(2):167-75. Epub 2002/03/12.
48. Westhovens R, Boonen A, Verbruggen L, Durez P, De Clerck L, Malaise M, et al. Healthcare consumption and direct costs of rheumatoid arthritis in Belgium. Clin Rheumatol. 2005;24(6):615-9. Epub 2005/05/17.

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Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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