

Journal of Clinical & Cellular Immunology

Open Access

# **Research Article**

# Anti-Citrullinated Protein Antibodies as Novel Therapeutic Drugs in Rheumatoid Arthritis

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#### Abstract

We have developed a novel antibody-based treatment for rheumatoid arthritis. In a collagen antibody-induced arthritis mouse model, anti-citrullinated protein antibodies (ACPAs) prevent the onset and/or exacerbation of inflammation, and prevent or strongly reduce joint damage. For the development of this novel therapy, we focussed on rheumatoid arthritis-specific citrullinated peptide epitopes. A growing number of studies indicate that modifications of arginines into citrulline residues are responsible for the initial triggering of autoimmunity and the breaking of tolerance. We identified a subset of human recombinant ACPAs that prevent the onset of inflammation in both collagen-induced arthritis and collagen antibody-induced arthritis mouse models for rheumatoid arthritis. Therapeutic administration of these antibodies resulted in the arrest of the inflammation and prevented a further increase of the inflammatory response. Prophylactic administration significantly prevented the onset of inflammation. Histological analysis of inflamed joints from ACPA treated mice revealed a significant decrease in joint damage, as compared to control animals. To identify the differentiating therapeutic epitope recognized by the therapeutic ACPA (tACPA), we performed comparative immunoprecipitations with therapeutic and non-therapeutic ACPAs using human PAD4deiminated HEK-293 cell lysates, followed by mass spectrometry analysis. The differentiating peptide epitope that is recognized by tACPAs only, is a citrullinated domain of Histone-2A. A second generation of antibodies was selected against this domain by means of phage display and by hybridoma generation. The second generation of tACPAs was comprised of even more potent inhibitors of the inflammatory response. Here, we describe the identification of a series of antibodies directed against a citrullinated epitope present in murine and human histone-2A. In mouse models for rheumatoid arthritis we demonstrate that these tACPAs exhibit a strong anti-inflammatory activity and prevent the occurrence of swelling and joint damage. We propose anti-histone-2A tACPA as a novel therapeutic treatment for rheumatoid arthritis patients.

**Keywords:** Anti-citrullinated protein antibody (ACPA) therapy; Collagen antibody-induced arthritis (CAIA) mouse model; Rheumatoid arthritis; Inflammation; Citrullination; Histone; Peptidyl arginine deiminase (PAD)

**Abbreviations:** ACPA: Anti-Citrullinated Protein Antibody; tACPA: therapeutic Anti-Citrullinated Protein Antibody; BSA: Bovine Serum Albumin; CAIA: Collagen Antibody-Induced Arthritis; CIA: Collagen-Induced Arthritis; CNS: Central Nervous System; DMARD: Disease Modifying Anti-Rheumatic Drug; emPAI: exponentially modified Protein Abundance Index; HE: Haematoxylin and Eosin; IP: ImmunoPrecipitation; i.p.: intraperitoneal; MmAb: Mouse monoclonal Antibody; NETs: Neutrophil Extracellular Traps; PAD: PeptidylArginine Deiminase; NSAID: Non-Steroidal Anti-Inflammatory Drug; PBS: Phosphate Buffered Saline; PBS-T: Phosphate Buffered Saline-Tween20; PG: ProteoGlycan; RA: Rheumatoid Arthritis; RhmAb: Recombinant human monoclonal Antibody; RT: Room Temperature; scFv: single chain variable Fragment; SLE: Systemic Lupus Erythematosus; SO: Safranin O

#### Introduction

Citrullination is a post-translational modification of arginine residues, in which peptidylarginine is converted into peptidylcitrulline. Citrullination is catalyzed by peptidylarginine deiminase (PAD) enzymes in a calcium-dependent manner [1], and has important roles in the normal functioning of the immune system, skin keratinization, insulation of neurons, gene regulation, and in the plasticity of the central nervous system [2].

Recently, citrullination has become an area of increased interest because of its involvement in various inflammatory pathological conditions, such as Alzheimer's disease and other neurodegenerative diseases, rheumatoid arthritis (RA), multiple sclerosis, psoriasis, chronic obstructive pulmonary disease, and even in cancer [3-6]. Still, it is unclear whether citrullination is the cause or the consequence of these pathological conditions. For an extensive discussion of citrullination in physiological and pathological conditions we refer the reader to recently published excellent reviews by Baka et al. [2] and Mohanan et al. [7].

In the process of citrullination, positively charged arginine residues are replaced by neutral citrulline residues. This change in net charge may induce conformational changes and thus the formation of new neo-epitopes. These conformational changes may possibly alter the function, activity, and/or half-life of the modified protein. Five PAD isoforms are distinguished in human and mouse, each of which has a different tissue distribution. PAD1 is expressed in the epidermis and uterus, while PAD2 is widely expressed in muscle tissue, the central nervous system (CNS) [1], and macrophages. PAD3 is localized in hair follicles and in the skin, whereas the less intensively studied PAD6, the only PAD enzyme for which enzymatic activity has not been detected, is found in early embryos and ovaries [1]. PAD4 (also

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Received November 30, 2012; Accepted January 24, 2013; Published January 30, 2013

**Citation:** Chirivi RGS, Jenniskens GJ, Raats JMH (2013) Anti-Citrullinated Protein Antibodies as Novel Therapeutic Drugs in Rheumatoid Arthritis. J Clin Cell Immunol S6: 006. doi:10.4172/2155-9899.S6-006

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designated PAD5) is mainly expressed by cells of the hematopoietic lineage [1], which is consistent with its role in modulating the immune response. Biochemical and genetic evidence strongly suggests that PAD4 contributes to the onset and progression of RA. The cellular localization of PAD is noteworthy, since expression levels are correlated with inflammation (e.g. cell infiltration, hypervascularization, and synovial lining hyperplasia), whereas intracellular and extracellular proteins show elevated levels of citrullination at sites of inflammation. In addition, PAD2 and PAD4 are present in RA synovial fluid [8,9].

The mechanisms by which PAD enzymes influence various physiological processes are increasingly being clarified. Citrullination of histones by PAD4 may affect gene regulation, is implicated in tumorigenesis [10], and is required for the release of neutrophil extracellular traps (NETs) [11]. NETs have been linked to several human autoimmune disorders, including systemic lupus erythematosus (SLE) [5].

The pathophysiological role of protein citrullination in RA is unclear, since it is still unknown whether citrullination reflects ongoing inflammation or whether it plays a crucial role in the onset of pathogenesis in RA.

In an effort to unravel the role of citrullines in RA, we engineered recombinant anti-citrullinated protein antibodies (ACPAs). A subset of these ACPAs exhibits a strong anti-inflammatory activity and prevents joint damage in collagen antibody-induced arthritis (CAIA) and collagen-induced arthritis (CIA) mouse models for RA. We identified the main differentiating therapeutic epitope to be the N-terminal sequence of histone-2A, which comprises a citrullinated arginine at position 3. Here, we propose the masking of this citrullinated epitope from the immune system as the mechanism of action of therapeutic ACPA (tACPA). We propose that specific tACPA can be used as a novel treatment method in RA patients.

# **Materials and Methods**

## Phage display antibody selection

First generation ACPAs were selected by means of phage display, as described [12]. Briefly, the (auto) antibody repertoires of RA-patients were isolated from their B-cell repertoire, and used to generate single chain variable fragment (scFv) libraries. These libraries were subjected to four rounds of affinity selection against cyclic citrullinated peptide-1 (CFC1). Antibody clones were selected based on their reactivity with CFC1 and lack of reactivity with the non-citrullinated cyclic peptide CFC0, resulting in the following ACPA: RhmAb2.101, RhmAb2.102, RhmAb2.104.

Second generation ACPAs were selected in a similar way as described for first generation ACPA [12]. Briefly, human-derived scFv phage display libraries were affinity selected against human PAD2 (hPAD2)- or hPAD4-deiminated forms of human histone-2A and histone-4, antigenic peptide 1 from histone-2A (Table 3), and CFC1 peptide. Antibody clones were picked based on their citrulline-dependent reactivity with antigenic peptide 1, PAD2-, and PAD4-deiminated human histone-2A and -4 (and derived peptides), and lack of reactivity with the non-deiminated histones and peptides.

Only the antibody clones that showed reactivity with the antigenic peptide 1 from histone-2A were cloned into our proprietary eukaryotic expression vector set and produced in transiently transfected human embryonic kidney (HEK) 293 or stably transfected CHO-K1 cells. Resulting in the following ACPA: RhmAb2.108, RhmAb2.109, Page 2 of 13

RhmAb2.110, RhmAb2.111, and RhmAb2.112.

#### Hybridoma-derived antibodies

Antibodies against antigenic peptide 1 from histone-2A (Table 3) were raised in DBA/J1 mice. At day 125 of the immunization procedure, serum samples were analyzed for citrulline-dependent reactivity. Spleens of responder mice were dissected following a final booster. Splenocytes were harvested and fused with a mouse myeloma cell-line (NS-1) according to internal proprietary procedures. Supernatants of the resulting hybridoma cell lines were analyzed for citrulline dependent reactivity with peptide 1 from histone-2A.

Resulting ACPA MmAb22.101 and MmAb22.102 were purified from the culture medium of the corresponding hybridoma cell lines.

#### Cloning and production of recombinant antibodies

Antibody variable domain-coding sequences of all selected ACPAs were synthesized according to Stemmer et al. [13], and cloned into mammalian expression vectors encoding full size human or mouse antibodies. Human antibodies were of the isotype IgG1 (kappa or lambda; in accordance with the isotype of the variable domains); mouse hybridoma derived antibodies were of the IgG1/kappa isotype. Subsequently, these constructs were used to generate transiently transfected HEK293 and stably transfected CHO-K1 ACPA-producing cell lines. Therapeutic and non-therapeutic ACPA were isolated from culture supernatants of transfectants and hybridoma cell lines, using Mab Select affinity columns on an Akta-FPLC system (GE Healthcare Life Sciences, Diegem, Belgium) and buffer exchanged to phosphate-buffered saline (PBS).

#### Miscellaneous antibodies

Unrelated isotype-matched control antibody against human U1-70k protein (RhmAb2.201) and anti-citrullinated fibrinogen antibodies (MmAb13.101, MmAb13.102, and MmAb13.103) were obtained from Modiquest Research BV, (cat. no.: MQR2.201, MQ13.101, MQ13.102, and MQ13.103, respectively).

#### Collagen antibody-induced arthritis mouse model

The commercially available collagen antibody induced arthritis (CAIA) mouse model from ModiQuest Research B.V. (cat. no.: MQ18.101) was used according to manufacturer's specifications to induce arthritis in mice. On day 0, male DBA/J1 mice (5-6 mice/group) of the age of 8 weeks, were injected intra-peritoneally (i.p.) with a mix of 8 anti-collagen antibodies (3.2 mg). On day 3, mice received another i.p. injection containing 25 µg LPS, which triggers and synchronizes the inflammation among mice. To differentiate between the prophylactic and therapeutic potential we injected ACPA i.p. (1mg/mouse) on day 3, when inflammation was still absent, or on day 7 when inflammation was already present. In a subset of experiments mice were treated on day 7 with tACPA i.p. (1mg/mouse) together with dexamethasone on days 7, 8, and 9 (1mg/kg/day) until inflammation disappeared. Mice were scored daily for signs of inflammation in their paws with a maximum of 35 days. Scoring was performed according to the method outlined in Table 1, which results in a maximum arthritis score per animal of 8.

The decision to administer ACPAs on day 3 after anti-collagen antibody injection was based on data of an experiment which showed that citrullinated epitopes appear in the paws of mice with experimentally-induced arthritis on day 4 (data not shown).

Macroscopical signs of inflammation	Score
1-2 Swollen toes	0.25
3-4 Swollen toes	0.50
Slightly swollen footpad or ankle	0.50-0.75
Swollen footpad or ankle	1.00
Swollen toes+slightly swollen footpad	1.25
Swollen toes+swollen footpad	1.5
Swollen footpad+swollen ankle	2.00

Mice with collagen antibody induced arthritis (CAIA) or collagen induced arthritis (CIA) were scored daily for signs of inflammation in their paws with a maximum of 35 days. Scoring was performed according to the method outlined in the table, resulting in a maximum arthritis score of 8 per animal.

Table 1: Inflammation score in CAIA and CIA mouse models for RA.

#### Collagen-induced arthritis mouse model

Chicken type II collagen was diluted with 0.05 M acetic acid to a concentration of 2 mg/ml and emulsified in an equal volume of complete freunds adjuvant. DBA/J1 mice (9 mice/group) of the age of 8 weeks, were immunized intradermally at the tail base with 100  $\mu$ g/100  $\mu$ l of emulsion. On day 21, the animals were given i.p. booster injections with 100  $\mu$ g chicken type II collagen diluted in PBS. On day 22 mice received another i.p. injection containing 25  $\mu$ g LPS, which triggers and synchronizes the inflammation among mice. The experimental group received i.p. injections with 6 mg RhmAb2.102 on day 22 and 30. Mice were scored daily for signs of inflammation in their paws until day 35. Scoring was performed according to the method outlined in Table 1, which results in a maximum arthritis score of 8 per animal.

Mice were kept at the central animal facility of the Radboud University Nijmegen in a standard room with a day/night rhythm of 06:00/18:00 h at a temperature of 21°C and a humidity of 50–60%. Mice were housed in Macrolon cages and fed ad libitum. All procedures involving animals were approved by the animal care committee of the Radboud University Nijmegen Medical Centre, The Netherlands, and conformed to the guidelines of the Dutch Council for Animal Care and the NIH.

#### Histology

Right hind paws were used for double blind histological analysis. Tissue was fixed for 4 days in 4% formaldehyde, decalcified in 5% formic acid, and subsequently dehydrated and embedded in paraffin. Standard frontal sections of 7 µm were mounted on SuperFrost slides (Menzel-Gläser, Braunschweig, Germany). Haematoxylin and eosin (HE) staining was performed to study joint inflammation (inflammatory cell influx; Figure 3D). The severity of inflammation in the joints was scored on a scale of 0-3 (0=no cells, 1=mild cellularity, 2=moderate cellularity, and 3=maximal cellularity). Figure 3A shows the macroscopical inflammation on day 35. To study proteoglycan (PG) depletion from the cartilage matrix (Figure 3E), sections were stained with Safranin O (SO), followed by counterstaining with fast green. Depletion of PG was determined using an arbitrary scale of 0-3 (0=normal, fully intact cartilage; 3=PG-depleted cartilage). Chondrocyte death (Figure 3F) was scored on a scale of 0-3, ranging from no loss of chondrocyte nuclei (0) to complete empty cartilage surface (3). Cartilage and bone erosion (Figures 3B and 3C) were graded on a scale 0-3, ranging from no damage (0) to complete loss of the cartilage or bone structure (3). Histopathological changes in the joint were scored on five semi-serial sections of joint spaced 70 µm apart. Scoring was performed doubleblind, without previous knowledge of the experimental conditions.

Human tissue arrays with various sections of organ tissue from

healthy adult donors (AMS Biotechnology Limited, Oxon, UK) were removed from the -80°C freezer and dried in air for 30 minutes. Tissue arrays were fixed in 4% paraformaldehyde for 15 minutes, and incubated an additional 15 minutes with 1% H<sub>2</sub>O<sub>2</sub> in methanol. Subsequently, sections were stained with MQ22.101 (0.125 µg/ml) for one hour in PBS+5% normal rabbit serum. Sections were washed in PBS and incubated with 13 µg/ml rabbit-anti-mouse IgG-HRP antibody (Dako, Glostrup, Denmark) for one hour in PBS+5% normal rabbit serum. After washing in PBS, peroxidase was developed with diaminobenzidine substrate for 10 minutes. Finally, sections were counterstained with hematoxylin for 1 minute, dehydrated, and closed in Permount mounting medium (Fisher Scientific, Waltham, MA, USA). Human tissue arrays contained the following tissues: adrenal gland, breast, cerebellum, cerebrum, cervix, heart, kidney, liver, lung, ovary, pancreas, placenta, salivary gland, skeletal muscle, skin, small intestine, spleen, stomach, thyroid, uterus.

#### Immunoprecipitation of antigens

HEK293 cells were harvested, washed once with PBS, spun down, and cells were resuspended in 15  $\mu$ l ice-cold lysis buffer (20 mM Tris pH7.4, 10 mM  $\beta$ -mercaptoethanol, 100 mM NaCl, 10% glycerol, and protease inhibitors).

The cell samples were sonicated 4 times for 15 seconds on ice and lysates centrifuged at 3000 rpm for 5 minutes. Supernatant was transferred to a clean tube and deiminated for 2 hours at  $37^{\circ}$ C by adding 10 mM CaCl<sub>2</sub>, 5 mM DTT, and 1 U human PAD4 per 2 mg of protein (ModiQuest Research B.V.; cat no: MQ16.203).

Immunoprecipitations (IPs) were performed on deiminated HEK293 lysates with antibodies RhmAb2.101 and RhmAb2.102, or with non-antibody-conjugated Protein A beads. Briefly, 30 µl Protein A Sepharose Fast Flow was washed 5 times with 1ml IPP500 (10 mM Tris-HCl, pH8.0, 500 mM NaCl, 0,1% NP40, and 0,1% Tween-20), and coupled to 20 µg RhmAb2.101 or RhmAb2.102, under constant rotation at room temperature (RT), for 1 hour. Beads were subjected to three washes with 1 ml IPP500, one wash with 1 ml IPP150 (10 mM Tris-HCl, pH8,0, 150 mM NaCl, 0,1% NP40, and 0,1% Tween-20), and subsequently incubated with 300 µl deiminated HEK293 cell lysate under constant rotation at RT, for 2 hours. Beads were washed three times with 1 ml of IPP150, after which a small part was used for SDS-PAGE to determine if the IP procedure with the HEK293 cells was successful. Immunoprecipitated proteins on RhmAb2.101, RhmAb2.102, and control beads were eluted with 50  $\mu l$  elution buffer (100 mM sodium citrate, pH3.0), neutralized with 10 µl 1M Tris-HCl, pH9.0, and stored at -20°C (until SDS-page electrophoresis and mass spectrometry).

#### SDS-polyacrylamide gel-electrophoresis

10x sample buffer (0.25 M Tris, pH6.8, 8% SDS, 35% glycerol, 2.5%  $\beta$ -mercaptoethanol, and 0.2% bromophenol blue) was added to the IP samples and boiled for 5 minutes. Samples were loaded on an SDS–PAGE gel (15%) and separated using Bio-Rad mini-Protean-II equipment (Bio-Rad, Veenendaal, The Netherlands). Gels were stained with Coomassie Brilliant Blue.

#### Mass-spectrometry analysis

RhmAb2.101- and RhmAb2.102-immunoprecipitates of huPAD4deiminated HEK293 proteins were loaded onto a 15% SDS-PAGE gel and ran for 10 minutes. The protein-containing parts of the gel were cut out and *in-gel* digested with trypsin as described elsewhere Citation: Chirivi RGS, Jenniskens GJ, Raats JMH (2013) Anti-Citrullinated Protein Antibodies as Novel Therapeutic Drugs in Rheumatoid Arthritis. J Clin Cell Immunol S6: 006. doi:10.4172/2155-9899.S6-006

[14]. Proteolytical peptides were extracted from the gel pieces and subsequently desalted and concentrated using STAGE tips [15] prior to LC-MS/MS analysis. Measurements were performed by nanoflow reversed-phase C18 liquid chromatography (Agilent 1100 series) coupled online to a 7T linear ion trap Fourier-Transform ion cyclotron resonance mass spectrometer (Thermo Fisher Scientific 7T LTQ FT Ultra) via a nanoflow electrospray ionization source. Chromatographic separations were performed using an in-house packed fused silica electrospray emitter (New Objective, PicoTip Emitter, Tip: 8 ± 1 μm, ID: 100 μm, FS360-100-8-N-5-C15) packed with reversed phase ReproSil-Pur C18AQ resin (Dr. Maisch, 3µm particles, 120 Å pore size). Peptides were loaded directly onto the analytical column at 600 nl/min using 0.5% HAc and were gradually eluted from the column using a 60 min gradient of 10-35% acetonitrile at a flow rate of 300 nl/ min. The mass spectrometer was programmed to acquire a single survey scan in the ICR cell with parallel acquisition of data dependent collision induced dissociation (CID) fragmentation spectra by the linear ion trap for the top 4 most abundant precursor ions. Survey scans by the ICR cell were performed at 100.000 FWHM resolving power at m/z400 using 1E6 ions for the mass range of 350-1600 m/z. Fragmentation spectra acquired by the linear ion trap used the following settings: CID fragmentation, normalized collision energy: 27%, 3Th isolation width, activation q=0.250, activation time: 30 ms. Dynamic exclusion with early expiration was enabled to prevent re-analysis of precursor ions. Doubly and triply charged precursor ions were exclusively selected for fragmentation analyses. Protein and peptide identifications were performed via database searches (NCBInr database, Homo sapiens) using Mascot software (Matrix Science, v2.1). Search parameters specified 20 ppm precursor mass tolerance, 0.8 Da fragment ion mass tolerance, tryptic specificity with up to 1 missed cleavage, ESI-TRAP instrument type and carbamideomethylation (Cys) as fixed modification. The following variable modifications were allowed: Oxidation (Met), Deamidation (NQ), and Deamidation (R). Here, deamidation of arginines was used to identify citrunillated peptides as both modifications result in a mass increase of 1 Da. Validation criteria specified a minimum Mascot ion score of 50 or better for peptides from proteins covered by a single peptide match whereas proteins with greater peptide coverage required a Mascot ion score of 30 or better [16]. The exponentially modified protein abundance index (emPAI) values were calculated for identifying proteins according to Ishihama et al. [17]. The emPAI values provide an approximate, label-free, relative quantitation of the proteins in a mixture, based on protein coverage by the peptide matches in a database search result. This technique enabled us to identify deiminated proteins that preferentially bind to RhmAb2.102 (Table 2). Mass-spectrometry analysis was performed by the Radboud Proteomics Centre, Nijmegen, The Netherlands.

#### Histone protein and histone peptide ELISA

Human recombinant histones H1, H2A, H2B, H3, and H4 (100  $\mu$ g; New England Biolabs, Ipswich, MA, USA) were incubated with or without 53,4 mU human PAD2 or PAD4, at 37°C for 3 hours (ModiQuest Research B.V.; cat. no. MQ16.201 and MQ16.203, respectively). Deiminated as well as non-deiminated histones were coated on 96-well ELISA plates (0.3  $\mu$ g/well) by overnight incubation at 4°C. Wells were washed 5 times with PBS-Tween 20 (PBS-T) and blocked by incubation with PBS-T/1% bovine serum albumin (BSA) at RT, for 1 hour. After 5 washes with PBS-T, wells were incubated with serial dilutions of RhmAb2.101, RhmAb2.102, or RhmAb2.104 (diluted in PBS-T/1% BSA, starting at a concentration of 10  $\mu$ g/ well) at RT, for 1 hour. Wells were washed 5 times with PBS-T and incubated with rabbit-anti-human IgG-HRP antibody (1:2000; Dako, Glostrup,

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Protein ID	Protein	Ratio 102/ 101
gi 4503841 ref NP_001460.1	ATP-dependent DNA helicase II, 70 kDa subunit	~
gi 4504279 ref NP_002098.1	H3 histone, family 3A	~
gi 4504263 ref NP_003512.1	H2B histone family, member E	~
gi 16306566 ref NP_003518.2	Histone H2B	~
gi 10800130 ref NP_066409.1	Histone 1, H2ad	~
gi 4501955 ref NP_001609.1	Poly (ADP-ribose) polymerase family, member 1	~
gi 60097902 ref NP_002007.1	Filaggrin	~
gi 13399298 ref NP_064455.1	Immunoglobulin lambda-like polypeptide 1 isoform a precursor	~
gi 113414893 ref XP_001127175.1	Similar to lactotransferrin	~
gi 62122917 ref NP_001014364.1	Filaggrin 2	~
gi 4557581 ref NP_001435.1	Fatty acid binding protein 5 (psoriasis-associated)	~
gi 13775212 ref NP_112583.1	Polyamine modulated factor 1 binding protein 1	~
gi 21614544 ref NP_002955.2	S100 calcium-binding protein A8	~
gi 4758170 ref NP_004397.1	Deleted in malignant brain tumors 1 isoform a precursor	~
gi 4503143 ref NP_001900.1	Cathepsin D preproprotein	~
gi 77539758 ref NP_001029249.1	Histone cluster 2, H4b	30.2
gi 4501883 ref NP_001604.1	Alpha 2 actin	3.2
gi 12056468 ref NP_068831.1	Junction plakoglobin	2.8
gi 4501885 ref NP_001092.1	Beta actin	2.7
gi 58530840 ref NP_004406.2	Desmoplakin isoform I	2.2

Mass spectrometry identification of a series of citrullinated proteins that preferentially bind to RhmAb2.102. The ratio RhmAb2.102/RhmAb2.101 in the right-hand column indicates the ratio by which proteins bind to RhmAb2.102, compared to RhmAb2.101.

 $\sim:$  Indicates that the corresponding protein was only found in the RhmAb2.102 and not in the RhmAb2.101 precipitated sample.

Table 2: Mass spectrometry data (Homo sapiens taxonomy).

Denmark) at RT, for 1 hour, followed by 5 washes with PBS-T and 3 washes with PBS. Staining was performed with TMB substrate for 10-15 minutes, and the substrate reaction was stopped with 2M  $H_2SO_4$ . Next, the optical density was measured by 450 nm.

To further characterize the RhmAb2.102 epitope, ELISA was performed on an array of Histone-2A-derived citrullinated peptides. ELISA plates were coated with neutravidin (0.1 µg/well) at 4°C, overnight. Wells were washed 5 times with PBS-T and blocked with PBS-T/1% BSA at RT, for 1 hour. Following 5 washes with PBS-T, wells were incubated with histone-derived citrulline- and biotin-containing peptides at RT, for 1 hour (0.3 µg/well). After 5 washes with PBS-T, wells were incubated with serial dilutions of RhmAb2.101, RhmAb2.102, or RhmAb2.104 in PBS-T/1% BSA (starting at a concentration of 10 µg/well) at RT, for 1 hour. Wells were washed 5 times with PBS-T and incubated with rabbit-anti-human-HRP (1:200) at RT, for 1 hour, followed by 5 washes with PBS-T and 3 wash steps with PBS. Staining was performed with TMB substrate for 10-15 minutes, and the substrate reaction was stopped with 2M  $H_2SO_4$ . Next, the optical density was measured by 450 nm.

# Results

#### Prophylactic anti-inflammatory effect of ACPA

Our goal was to investigate the influence of different ACPAs on paw and ankle swelling in our CAIA mouse model. For this purpose, we used the first generation of ACPAs that were selected by phage display panning (RhmAb2.101, RhmAb2.102, and RhmAb2.104) against citrullinated cyclic peptide (CFC1) [12], as well as commercially available anti-citrullinated fibrinogen antibodies (MmAb13.101, MmAb13.102, and MmAb13.103).

In a typical CAIA mouse experiment, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice (CAIA-mix; ModiQuest Research B.V., Oss, the Netherlands). Three days later mice received another i.p. injection containing 25 µg LPS to synchronize the inflammation between mice. Typically, inflammation in the front and hind paws became visible as from 2 days after LPS injection (i.e. day 5). Simultaneous with LPS, mice received 1 mg ACPA (RhmAb2.101, RhmAb2.102, or RhmAb2.104), commercially available ACPA (anti-citrullinated fibrinogen; MmAb13.101, MmAb13.102, or MmAb13.103), non-related isotype-matched control antibody (RhmAb2.201), or physiological salt solution (0.9% NaCl). The degree of swelling in the paws was scored from day 0, for a time period of two weeks. We found that a subset of ACPA (a mix of MmAb13.101, MmAb13.102, and MmAb13.103) was able to slightly enhance the arthritis score (data not shown), confirming their potential pathogenic effect as was previously shown by others [18,19]. Mice that were treated with RhmAb2.102 or RhmAb2.104 showed a significantly reduced inflammation in their paws, as compared to mice treated with RhmAb2.101, control antibody (RhmAb2.201), or physiological salt solution (Figure 1A). Moreover, RhmAb2.102 completely abolished the inflammation. The therapeutic potential of RhmAb2.102 was confirmed in the CIA mouse model (Figure 1B). Upon LPS injection,



A) Collagen antibody induced arthritis (CAIA): On day 0, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice, followed by an i.p. injection containing 25 µg LPS on day 3 with or without (no Ab) first generation ACPA or unrelated antibody (1 mg/mouse). B) Collagen induced arthritis (CIA): On day 0, 100 µg chicken type II collagen was injected intradermally at the tail base, followed by an i.p. booster with 100 µg chicken type II on day 21. On day 22 mice received an i.p. injection containing 25 µg LPS. Mice received 6 mg RhmAb2.102 or placebo (no Ab) i.p. on days 22 and 30. Mice were scored daily for signs of inflammation in their paws according to Table 1. Shown are the means ± SEM (CAIA, n=5; CIA, n=9).

Figure 1: Prophylactic anti-inflammatory effect of first generation ACPAs in CAIA and CIA.

mice development an inflammatory response quickly, resulting in lower motility, ceased grooming, and an overall neglected appearance. When comparing the different groups of mice, we observed that the mice treated with RhmAb2.104 showed less symptoms of neglected appearance, whereas mice treated with RhmAb2.102 looked healthy. These mice did not seem to suffer at all from the injection of CAIAmix and LPS, nor did they develop inflammation in their paws (Figure 1). The above experiment was repeated with various batches of RhmAb2.102, resulting in the same outcome.

The unexpected observation that injection of specific tACPAs effectively prevents RA-like symptoms in the CAIA mouse model, made us realize that we found a potential new treatment for inflammation in RA.

# Therapeutic anti-inflammatory effect of the lead tACPA: RhmAb2.102

After we had repeatedly observed the prophylactic capacity of the strongest inflammation-preventing antibody RhmAb2.102, we continued to investigate the therapeutic potential of this particular ACPA. For this purpose, we induced inflammation in DBA/J1 mice as described above by injecting anti-collagen antibodies on day 0, LPS on day 3, and subsequently treated mice by injecting them with 1 mg RhmAb2.102 i.p. on day 7 when inflammation was already present and nearly at its maximum (Figure 2A). We observed that RhmAb2.102 was not able to quickly reduce the swelling that was already present. However, it prevented further increase of the swelling, thus stabilizing the inflammation at its current level (Figure 2A). A typical CAIA experiment has a running time of 2 weeks, but in this



On day 0, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice to induce collagen antibody induced arthritis (CAIA), followed by an i.p. injection containing 25  $\mu$ g LPS on day 3, and RhmAb2.102 or placebo (No Ab) i.p. injection on day 7 (1 mg/mouse). Shown are the means  $\pm$  SEM (n=5). Mice were scored daily for signs of inflammation in their paws. Scoring was performed according to the method outlined in Table 1. **A**) Mean arthritis score of all 4 paws of all mice per group. **B**) Mean arthritis score of the right hind paws of all mice per group. Right hind paws were used for histological analysis (Figures 3 and 4).

Figure 2: Therapeutic RhmAb2.102 treatment.

particular experiment we ran the experiment longer to investigate whether the mean arthritis score in the RhmAb2.102 treated group would drop faster than in the placebo-treated group. This turned out not to be true in the acute CAIA arthritis model. The swelling levels in both experimental groups dropped in the same order of magnitude. To investigate the effect of the RhmAb2.102 antibody on joint damage, mice were sacrificed on day 35, and their right hind paws were dissected, fixed in 4% formaldehyde, decalcified, dehydrated, embedded in paraffin, sectioned, and processed for histological analysis. Figure 2B shows the mean arthritis score of the right hind paws that were used for histology. In this figure, error bars are bigger than in figure 2A, because it depicts the mean arthritis score of 5 right hind paws of 5 mice instead of all 20 paws. It is important to note that in the right hind paws the mean arthritis score for both experimental groups is similar at the end of the experiment. Figures 2B and 3A show that the macroscopical arthritis score in the right hind paws is similar between both experimental groups on day 35. Most surprisingly, it was observed that all known parameters for joint erosion were significantly decreased. When scoring cartilage and bone erosion, inflammatory cell influx, cartilage PG depletion, and chondrocyte death, a dramatic decrease was observed in the experimental group that was treated with RhmAb2.102 on day 7 (Figure 3B-3F). Thus, RhmAb2.102 has a strong therapeutic potential in regard to preventing joint damage during inflammation. Figure 4 shows representative pictures of bone structures in the right hind paws of the RhmAb2.102-treated and nontreated (placebo) mice, compared to mice that were not injected with CAIA antibodies (control). In the non-treated CAIA group a massive inflammatory cell influx was observed (asterisk in HE- and SO-stained sections). RhmAb2.102-treated joints had little to no inflammatory cell influx, whereas the control mice were void of inflammatory cells. When looking at the SO-stained sections (blue=bone; red=GP in the cartilage), it becomes obvious that RhmAb2.102-treated mice are largely protected from massive bone and cartilage destruction.

Now that we found RhmAb2.102 to exhibit both prophylactic and therapeutic potential, we tried to enhance its therapeutic efficacy by performing a combination therapy with the corticosteroid dexamethasone that is also used for the treatment of RA patients. Treating CAIA-challenged mice with RhmAb2.102 alone, after the onset of inflammation (day 7), prevented further increase of the inflammation compared to a slight rise in inflammation of the untreated control group, but could not reduce the swelling in the paws (Figure 2). However, most importantly, joint damage was strongly reduced in the treated mice (Figures 3 and 4). With the combination therapy we aimed to supress the arthritis score to zero using dexamethasone, and prevent the inflammation from reappearing with RhmAb2.102. For this purpose, mice that had been challenged with CAIA antibodies received dexamethasone treatment on day 7, 8, and 9 (1 mg/kg/day),



On day 0, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice to induce CAIA, followed by an i.p. injection containing 25 µg LPS on day 3, and RhmAb2.102 or placebo i.p. injection on day 7 (1 mg/mouse). Panel (A) shows that macroscopic arthritis score in the right hind paws between experimental groups was similar on day 35. Paraffin sections were made from right hind paws and consecutive sections were stained with either Haematoxylin-Eosin or Safranin O staining. Sections were scored for the following parameters: cartilage erosion (B), bone erosion (C), inflammatory cell influx (D), cartilage PG depletion (E) and chondrocyte death (F). All parameters were scored on a scale from 0-3, with 0 meaning no pathological signs present, and 3 indicating maximum pathological signs. Figure 3: RhmAb2.102 prevents joint damage during inflammation.

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On day 0, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice to induce CAIA, followed by an i.p. injection containing 25 µg LPS on day 3, and RhmAb2.102 or placebo i.p. injection on day 7 (1 mg/mouse). Paraffin sections were made from right hind paws and consecutive sections were stained with either Haematoxylin-Eosin staining (HE) or Safranin O staining (SO). Control mice did not receive CAIA antibodies. Blue and red staining indicates bone and cartilage respectively. \* indicates inflammatory infiltrate. Bar represents 100 µm.





On day 0, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice to induce CAIA, followed by an i.p. injection containing 25 µg LPS on day 3. On day 7, when the mean arthritis score almost reached its maximum level, a single dose of tACPA RhmAb2.102 (1 mg/mouse) was injected i.p. Dexamethasone treated groups received three consecutive i.p. injections on days 7, 8, and 9 (Dex; 1 mg/kg). Shown are the means  $\pm$  SEM (n=5). Mice were scored daily for signs of inflammation in their paws. Scoring was performed according to the method outlined in Table 1.

Figure 5: Combination therapy with RhmAb2.102 and dexamethasone.

and a single dose of RhmAb2.102 (1mg/mouse) on day 7 (Figure 5). In mice that received the combination therapy the arthritis scores remained low, as compared to mice that received dexamethasone only. Moreover, the inflammation reappeared and increased rapidly over time in mice treated with dexamethasone alone. These data imply that combination therapy of tACPA in combination with corticosteroids should be considered when designing new treatment strategies for RA.



HEK293 cells were lysed and citrullinated with human PAD4. Citrullinated cell lysates were subsequently immunoprecipitated with ACPA RhmAb2.101 or tACPA RhmAb2.102 coupled to Protein A Sepharose beads, or with Protein A Sepharose beads only. Beads were washed, and bound proteins were eluted and subjected to SDS-PAGE (15%), followed by protein staining using Coomassie Brilliant Blue.

Figure 6: SDS-PAGE of RhmAb2.101 and RhmAb2.102 immunoprecipitation on huPAD4 citrullinated HEK293 cell lysates.

Citation: Chirivi RGS, Jenniskens GJ, Raats JMH (2013) Anti-Citrullinated Protein Antibodies as Novel Therapeutic Drugs in Rheumatoid Arthritis. J Clin Cell Immunol S6: 006. doi:10.4172/2155-9899.S6-006



Homing in on the specificity of ACPA RhmAb2.101 (**A**, **B**), RhmAb2.102 (**C**, **D**) and RhmAb2.104 (**E**, **F**). ELISA of ACPA dilution series on various citrullinated and non-citrullinated histone proteins (A, C, E) and citrulline-containing histone-derived peptides (B, D, F). All ACPA react strongly with PAD2-deiminated human histone-2A (H2A/p2; A, C, E) and to a somewhat lesser extent with PAD2-deiminated human histone-4 (H4/ p2; A, C, E). As with their specific citrulline-dependent reactivity towards various histone isoforms, ACPA showed selective reactivity towards citrulline-containing peptide-1\*, -4, -6, and -7 (B, D, F). CFC1 was used as a positive, and CFC0 as a negative control in ELISA experiments. **Figure 7:** Homing in on the anti-histone specificity of ACPA.

J Clin Cell Immunol

Immunotherapies and Rheumatoid arthritis

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Peptide Number	Amino-acid sequence
1	SGXGKQGGKARA
2	AKAKSXSSRAGL
3	KSRSSXAGLQFP
4	QFPVGXVHRLLR
5	VGRVHXLLRKGN
6	VHRLLXKGNYSE
7	GNYSEXVGAGAP
8	AGNAAXDNKKTR
9	DNKKTXIIPRHL
10	TRIIPXHLQLAI
11	LQLAIXNDEELN
12	NKLLGXVTIAQG

Synthesized peptides of all domains in histone-2A in which an arginine could be enzymatically changed into a citrulline.

Table 3: Histone-2A citrulline containing peptides.



Citrullin-dependent reactivity of tACPA against citrullin-containing antigenic peptide-1 (P1-C) versus its arginin-containing counterpart (P1-R). ELISA was performed as described, using 0.5  $\mu g$  of purified ACPA per well.

Figure 8: Citrullin-dependent reactivity of tACPA for antigenic peptide-1.



On day 0, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice to induce collagen antibody induced arthritis (CAIA), followed by an i.p. injection containing 25  $\mu$ g LPS on day 3 together with second generation tACPA (1 mg/mouse) or without antibody (no Ab). Shown are the means  $\pm$  SEM (n=5). Mice were scored daily for signs of inflammation in their paws. Scoring was performed according to the method outlined in Table 1.

Figure 9: Second generation tACPAs identified by phage display.



On day 0, 3.2 mg anti-collagen-II antibody mix was injected i.p. in DBA/J1 mice to induce collagen antibody induced arthritis (CAIA), followed by an i.p. injection containing 25 µg LPS on day 3 together with MmAb2.101, RhmAb2.102 (1 mg/mouse) or without antibody (no Ab). Shown are the means ± SEM (n=5). Mice were scored daily for signs of inflammation in their paws. Scoring was performed according to the method outlined in Table 1. **Figure 10:** Second generation tACPA identified by hybridoma generation.

# Identification of citrullinated Histone-2A as the main differentiating therapeutic target for RhmAb2.102

To identify the therapeutic epitope recognized by RhmAb2.102, we performed IPs on human PAD4 deiminated HEK-293 cell lysates. Hereto, ACPA antibodies RhmAb2.101 and RhmAb2.102 were coupled to protein A Sepharose. Protein A Sepharose without antibodies coupled to it was used as negative control. Before subjecting the immunoprecipitated samples to mass spectrometry analysis, a small amount was analyzed by SDS-PAGE. Coomassie Brilliant Blue stained gel showed different staining patterns for non-therapeutic RhmAb2.101 and therapeutic RhmAb2.102 immunoprecipitated samples. The most prominent feature in the RhmAb2.102-IP was a doublet of bands running at approximately 15 and 17 kiloDalton (Figure 6). The proteins running at 50 and 25 kiloDalton are the antibody heavy and light chains from RhmAb2.101 and RhmAb2.102. IP performed with empty Protein A Sepharose alone (negative control) did not yield any visible bands. We envisaged that tACPA-bound proteins (designated p15 and p17) are key suspects for the therapeutic potential of RhmAb2.102.

Mass spectrometry analysis identified a series of citrullinated proteins that preferentially bind to RhmAb2.102 (Table 2). The ratio RhmAb2.102/RhmAb2.101 in the right-hand column of Table 2 indicates the ratio by which peptides of a given protein were immunoprecipitated by RhmAb2.102, as compared to RhmAb2.101. A diacritical mark (~) in the ratio-column indicates that the corresponding protein was only found in the RhmAb2.102 and not in the RhmAb2.101 precipitated sample. Notably, RhmAb2.102 precipitates a variety of citrullinated proteins, with histones being among the most prevalent.

Now that the main differentiating therapeutic target for RhmAb2.102 was identified as citrullinated histones, we verified this in ELISA. Human recombinant histones H1, H2A, H2B, H3.1, and H4 were first citrullinated using human PAD2 or PAD4. Both PAD enzymes were used since they are known to be present in inflamed RA tissues in macrophages and granulocytes respectively. Citrullinated as well as non-citrullinated histones were coated on ELISA plates and incubated with serial dilutions of RhmAb2.101, RhmAb2.102, or RhmAb2.104. We looked for a histone recognition pattern that could

explain the prophylactic capacity of tACPA as seen in Figure 1, with RhmAb2.102 preventing swelling in CAIA, RhmAb2.104 reducing maximal swelling by 50% and RhmAb2.101 having no effect on swelling prevention. In ELISA, RhmAb2.102 bound human PAD4 citrullinated histone-2A with high affinity, RhmAb2.104 bound with lower affinity, whereas RhmAb2.101 did not bind at all (Figures 7A, 7C, and 7E). The binding capacities of RhmAb2.101, RhmAb2.102, and RhmAb2.104 to PAD4 citrullinated histone-2A perfectly reflected the antibodies prophylactic tendencies as, observed in the CAIA experiment depicted in Figure 1, thus revealing the potential therapeutic relevance of this target. tACPA RhmAb2.102 recognized PAD2 citrullinated histone-2A and PAD2-citrullinated histone-3.1 and histone-4 as well.

To further characterize the therapeutic epitope in histone-2A to which RhmAb2.102 binds, we synthesized peptides of all domains of histone-2A in which an arginine could be enzymatically changed into a citrulline (Table 3).

For the ELISA experiments in figure 7 we added three neutrally charged alanine residues to the N-terminal peptide of histone-2A in order to position its citrulline residue in the middle of the peptide (peptide 1\*). In ELISA, only the N-terminal containing peptide of histone-2A clearly differentiated between the therapeutic ACPAs (RhmAb2.102 and RhmAb2.104), and the non-therapeutic ACPA RhmAb2.101 (Figures 7B, 7D, and 7F). Thus identifying the citrulline-containing N-terminal peptide from human histone-2A as the main differentiating therapeutic target for tACPAs RhmAb2.102 and RhmAb2.104. It is important to note that histone-2A is highly conserved between mouse and man, with the N-terminal part being 100% identical.

#### Histone-2A specific anti-inflammatory tACPA

Following the identification of the differentiating target for tACPA (citrullinated N-terminus of histone-2A), we validated this epitope by using it for the production of second generation tACPAs.

Second generation tACPAs were selected in a similar way as described for first generation tACPAs, using human-derived scFv phage display libraries, and by mouse immunizations. scFv phage display libraries were affinity selected against antigenic peptide-1 from histone-2A (Table 3), hPAD2- or hPAD4-deiminated human histone-2A and -4, and CFC1. Antibody clones were picked based on their citrulline-dependent reactivity with antigenic peptide-1, hPAD2- and hPAD4-deiminated human histone-2A and -4, and lack of reactivity with non-deiminated histone-2A and -4.

Second generation tACPA (RhmAb2.108, -109, -110, -111, and -112), as well as first generation ACPA (RhmAb2.101, -102, and -104) were cloned to full-size recombinant IgG. IgG-ACPA and -tACPA were produced in transiently transfected HEK293 cells and in stably transfected CHO-K1 cell lines, and purified over Mab Select affinity columns on an Akta-FPLC system. All IgG-tACPA strongly interact with antigenic peptide-1 in ELISA (Figure 8). Second generation tACPA, as well as RhmAb2.102, were subsequently tested for their prophylactic potential in the CAIA mouse model. Hereto, CAIA was induced in DBA/J1 mice, and mice were prophylactically treated with tACPA as described above. Transiently produced second generation tACPA showed a better anti-inflammatory response, as compared to transiently produced RhmAb2.102. tACPA RhmAb2.109 and RhmAb2.110 completely abolished inflammation, whereas RhmAb2.112 nearly abolished, and RhmAb2.111 and RhmAb2.108 strongly reduced the signs of inflammation (Figure 9). In this experiment, transiently produced RhmAb2.102 showed a lower activity as compared to the stably produced RhMAb2.102 that was used in earlier experiments. By using transiently produced tACPA in comparative CAIA experiments, the prophylactic efficacy among tACPA could be compared (Figure 9).

Additional proof that the citrullinated N-terminus of histone-2A is the differentiating target for the tACPAs, was generated by immunization of DBA/J1 mice with antigenic peptide-1. When serum titers for citrulline-dependent reactivity were sufficient, splenocytes were harvested and fused with mouse myeloma cell-line NS-1. Supernatants of the resulting hybridoma cell lines were analyzed for citrulline-dependent reactivity on antigenic peptide-1, and antibodies were purified over Mab Select affinity columns on an Akta-FPLC system. Two tACPA-producing hybridoma cell-lines were generated: MmAb22.101 and MmAb22.102. Both hybridoma-derived tACPAs possess a strong immuno-reactivity towards antigenic peptide-1 (Figure 8) and have a strong prophylactic anti-inflammatory capacity in the CAIA mouse model. Figure 10 shows a CAIA experiment in which the prophylactic effect of MmAb22.101 is compared to RhmAb2.102.

### MmAb22.101 human tissue array analysis

To probe for possible side effects of tACPA therapy in humans, non-diseased human tissue arrays were stained with MmAb22.101. Histological analysis revealed staining of white blood cells (granulocytes and macrophages) in the placenta and intestine (weak), in the liver (moderate), and in pancreas and spleen (strong; data not shown). Staining of granulocytes and macrophages can be explained by their high expression of PAD enzymes.

## Discussion

Citrullination is a post-translational modification of arginine residues, in which peptidylarginine is converted into peptidylcitrulline. Citrullination is catalyzed by PAD enzymes in a calcium-dependent manner [1], and has important roles in the normal functioning of the immune system, skin keratinization, insulation of neurons, gene regulation, and in the plasticity of the central nervous system [2].

The disease in which a close link between citrullination and pathology was first established is RA. Citrullinated proteins present in the joints are thought to sustain inflammation in RA [20-22]. Moreover, antibodies against citrulline-containing epitopes (ACPAs) are diagnostic markers for this disease. Such antibodies can be found up to nine years prior to the manifestation of the actual disease [9,23,24], with periods of increased concentrations of ACPA as the individuals approach the onset of disease. The key issue to anti-citrulline immunity is the question whether the observed autoantibodies are the consequence, or the cause of the disease [23]. Likewise, little is known about ACPA epitope specificity during arthritis development. Information on ACPA occurrence and specificity may help to elucidate the role of different ACPAs in pathology. The occurrence of ACPA prior to the onset of the disease and, in particular, the finding that only very few patients develop ACPA after disease onset, provide indirect evidence for the contribution of anti-citrulline immunity in the pathogenesis of ACPA-positive RA.

Recently, indirect [25,26] and direct evidence [20,21] became available in support of the pathogenicity of anti-citrulline antibodies in RA mouse models. Hill et al. showed that arthritis can be induced in DR4-IE mice with citrullinated fibrinogen. Their data directly implicate citrullinated fibrinogen as arthritogenic in the context of RA-associated MHC class II molecules [25]. Kuhn et al. showed that transfer of monoclonal anti-citrullinated fibrinogen antibodies enhanced arthritis when co-administered with a submaximal dose of anti-collagen II antibodies [18], and a more recent study shows that antibodies specific for citrullinated collagen II are a pathogenic factor in the development of RA in mice [19].

Another major factor that plays an important role in the (early) inflammatory processes in RA is the infiltration of immune cells, such as macrophages and neutrophils, which contain PAD2 and PAD4 respectively. The activation of PAD4 and the subsequent citrullination of different proteins (including histones) plays a major role in the inflammatory process [27]. Citrullination of histones results in the exposure of inflammatory neo-epitopes to the immune system, and interferes with many processes in which histones are involved. One process in which citrullinated histones play a pivotal role, is the formation and stabilisation of NETs [4,28,29]. Modified histones may be involved in the anchoring of various compounds into NETs, such as the stimulators of immune response LL37, HMGB-1, elastase, and myeloperoxidase. It is thought that NETs function as attractants for phagocytes or other inflammatory immune cells. Finally, modified histones might activate or reduce the inflammatory response by acting on innate pattern recognition receptors [4].

Recently, various studies have shown that interfering with the peptidyl-citrulline pathway results in a decreased inflammation in RA and in inflammatory bowel disease [22,30-32]. Inhibition of the PAD enzymes that are responsible for arginine citrullination is an obvious approach by which one can interfere with the peptidyl-citrulline pathway and its various roles in inflammation [31,32]. Specific targeting of PAD inhibitors to cells or tissues might reduce the risk of negative side effects and cytotoxicity of these compounds, whereas a systemic application of PAD inhibitors requires a balance between obtained benefits and negative side effects. This problem will be more severe when non-specific PAD inhibitors are used.

An alternative, more focussed, approach avoiding the above mentioned pitfalls, is the use of antibodies to block specific citrullinated epitopes that play critical roles in the inflammatory process.

In this study, we investigate the influence of different ACPAs on paw and ankle swelling in the CAIA and CIA mouse arthritis models. ACPAs were selected from recombinant human antibody phage display libraries made from the immune repertoire of RA patients, as well as through mouse hybridoma generation. Both approaches yielded a panel of ACPAs that recognize a variety of citrullinated peptides, which could be divided in three groups. I) ACPAs that have no effect on paw swelling in the CAIA mouse model (i.e. RhmAb2.101; Figure 1). II) ACPA that slightly enhance the swelling in the paws (a mix of MmAb13.101, MmAb13.102, and MmAb13.103; data not shown), and III) tACPAs that prevent the swelling (RhmAb2.102 and RhmAb2.104; Figure 1). The fact that a subset of ACPAs slightly enhances the swelling indicates that some ACPAs might have a pathogenic effect in RA-like diseases, as was also shown by others [18,19].

This study aims to focus on the specific tACPAs that prevent or diminish the inflammatory swelling and subsequent bone and cartilage destruction in RA mouse models. The successful prophylactic use of RhmAb2.102 in CAIA or CIA challenged mice made us question whether RhmAb2.102 could also be used as a therapeutic agent. We demonstrate that RhmAb2.102 treatment of CAIA challenged mice on day 7, when swelling was present, stabilizes the inflammatory swelling (Figure 2). Histological analysis reveals RhmAb2.102's capacity to diminish the inflammation and prevent joint damage (Figures 3 and 4). Furthermore, combination treatment with steroids and RhmAb2.102 on day 7 reduces inflammatory joint swelling and subsequent flares without detectable side effects (Figure 5).

We identified the citrullinated histone N-terminal domains as the differentiating therapeutic target proteins for RhmAb2.102. Differential affinities towards citrullinated proteins were observed for the non-therapeutic RhmAb2.101 and the therapeutic RhmAb2.102 in IP on PAD-deiminated HEK293 lysates (p15/p17; Figure 6) and in subsequent mass spectrometry analysis (Table 2). To further define the differentiating epitope for the tACPAs, ELISA experiments were performed using RhmAb2.101, RhmAb2.102, and RhmAb2.104 on an array of human PAD2 and PAD4 citrullinated recombinant histones (H1, H2A, H2B, H3.1, and H4). Human PAD4 citrullinated histone-2A was identified as a major target for the tACPA RhmAb2.102 (Figures 7A, 7C and 7E). Using citrulline containing peptides from histone-2A in similar ELISA experiments (Figures 7B, D and F) we were able to identify the citrullinated N-terminus of histone-2A as the differentiating therapeutic target epitope for tACPA RhmAb2.102 (Table 3; Peptide 1). It is important to note that histone-2A is highly conserved between mouse and man, with the N-terminal part being 100% identical.

To prove that the epitope in the N-terminal region of histone-2A is indeed the therapeutic target for tACPA in RA-like diseases, we selected novel tACPA against this epitope through phage display and hybridoma generation. Second generation tACPAs that were selected against antigenic peptide 1 (RhmAb2.108 to RhmAb2.112, MmAb22.101, and MmAb22.102) show stronger prophylactic antiinflammatory capacities, when compared to the best first-generation tACPA, RhmAb2.102 (Figures 9 and 10). Thus, upon the identification and characterization of the target antigen that is recognized by first generation tACPA, we were able to select second generation tACPA against this target antigen, which have significantly improved *in vivo* efficacy.

The current RA treatment regimens with Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) and Disease Modifying Anti-Rheumatic Drugs (DMARDs) show severe side effects. Biological DMARDs like anti-TNF antibodies interfere with the immune system. RA patients that are treated with anti-TNF antibodies (e.g. Etanercept, Adalimumab, or Infliximab) risk bacterial infections, virus infection, tuberculosis, neuropathy, weight gain, and hair loss [33,34]. Adverse effects from anti-CD20 (Rituximab) treatment, which targets B-cells, include the development of late-onset neutropenia, defects of immune reconstitution with associated immune compromise, infections, progressive multifocal leuko-encephalopathy, reactivation of hepatitis, intestinal perforation, and interstitial pneumonitis [35]. NSAIDs are not without health risks either, since gastrointestinal bleeding, fluid retention, and increased risk of heart disease are some of their possible side effects [36,37].

To screen for side effects that might occur in tACPA therapy, we examined the immunohistological staining of MmAb22.101 on human tissue arrays. We found no staining in any of the healthy tissues that could imply possible tACPA side effects if used as a treatment in humans. Immunohistological staining was only detected in granulocytes and macrophages [2,38]. Staining of granulocytes and macrophages can be explained by their high expression of PAD enzymes. When tissues are being processed for histological analysis, calcium influx may activate PAD enzymes, and proteins might become citrullinated, implying that

the visualized citrullinated proteins are not present in healthy humans. Another possibility might be that intact circulating granulocytes and macrophages contain intracellular citrullinated proteins, which are not exposed to the immune system. Based on the observed MmAb22.101 staining on human tissue arrays, we do not foresee any adverse effects of tACPA therapy in humans.

As described in recent papers, citrullinated histone epitopes are involved in very early processes during the onset of inflammatory responses [4,28,29]. We believe that shielding of the N-terminal citrulline of histone-2A from the immune system is an excellent alternative approach to treat RA-like diseases, for which we anticipate no side effects. It is very important to treat patients as early as possible, which can be achieved through early identification of CCP positive RA patients using the anti-CCP2 test [39-41]. Early identification of RA patients using the CCP2 test, followed by immediate tACPA therapy may prevent joint damage in a very early stage of the disease, allowing a prolonged time span without inflammatory flares and joint damage, thus reducing healthcare costs and enhancing the quality of life for these patients. Furthermore, we anticipate that tACPA therapy in combination with steroids, biologicals, or other anti-inflammatory medication can be used to prevent and treat flares.

In conclusion, we demonstrate that novel tACPAs can be used to block a specific N-terminal citrullinated epitope in histone-2A, which is only present extracellularly during inflammation. By shielding this citrullinated epitope from the immune system we prevent its role in the inflammatory response, and thus prevent inflammation and joint damage. We anticipate that tACPA will have a broad applicability in the prevention and treatment of a wide range of inflammatory diseases such as RA and multiple sclerosis.

#### Acknowledgements

Authors would like to gratefully acknowledge Prof. Dr. Walther J. van Venrooij and Prof. Dr. Ger J. M. Pruijn for helpful discussions, Dr. Astrid Alewijnse for interpretation of histological experiments, Dr. Marije Koenders and Birgitte Walgreen for help with the CIA mouse model and histological analysis. We want to thank the following past and present colleagues from ModiQuest B.V.: Dr. Jos van Rosmalen for managing antibody productions. Tom van der Donk for help with phage display selections and antibody characterization. Gonny Schmets, Marijn Driessen, Nienke Smits, Léon Peeters and Lisa Vos for hybridoma generation and cell culture. Rob Arts, Diane de Groot, Linda Hoeijmakers and Bram Maassen for antibody purification. Kalok Cheung for ELISA experiments.

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**Citation:** Chirivi RGS, Jenniskens GJ, Raats JMH (2013) Anti-Citrullinated Protein Antibodies as Novel Therapeutic Drugs in Rheumatoid Arthritis. J Clin Cell Immunol S6:006. doi:10.4172/2155-9899.S6-006

This article was originally published in a special issue, **Immunotherapies** and Rheumatoid arthritis handled by Editor(s). Dr. Hongkuan Fan, Medical University of South Carolina, USA