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

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Chapter 4

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

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Abstract

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

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

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

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

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

Introduction

Rheumatoid Arthritis (RA) is a chronic, systemic, inflammatory autoimmune disease, characterized by the presence of autoantibodies. Among the autoantibodies described in RA, antibodies directed against citrullinated proteins (ACPA) are highly specific and predictive for RA (1-3) and can be detected in approximately 70-80% of long-standing RA patients. Antigens recognized by ACPA are present in the inflamed joint (4;5). The production of ACPA is most likely dependent on the presence of T cells as ACPA have undergone isotype-switching.

HLA class II alleles are the most important genetic risk factor for RA. Notably HLA-DRB1 molecules sharing a common epitope, R(Q)K(R)RAA, at position 70-74 in the third hypervariable region of the DRB1 chain, the so-called shared epitope (SE), are associated with both susceptibility to and severity of RA (6-9). It has been shown that the SE-containing HLA-DRB1 alleles predispose to $ACPA^+$ disease, but not to $ACPA^-$ RA (10), and that they are associated with the production of ACPA (11). The latter provides an additional indication for the existence of T cell responses underlying the production of ACPA.

Several human proteins were found to be citrullinated *in vivo*, one of which is vimentin, also known as the Sa-antigen (12-15). Citrullinated vimentin has been shown to be present in the synovial fluid of RA patients and to be recognized by ACPA in approximately 40% of RA patients (13;16-18). Furthermore, we have shown that over 90% of $ACPA^+$ RA patients recognizing a citrullinated peptide derived from human vimentin (19) and 80% of $ACPA^+$ RA patients recognizing the Sa-antigen (unpublished data) carry at least one SE-containing HLA-DRB1 allele. These observations not only show that the SE-containing HLA-DRB1 alleles influence the specificity of the ACPA-response, but also suggest that vimentin could be a protein involved in the recruitment of T cell help for ACPA-producing B cells.

Here, we examined whether we could identify $CD4^+$ T cells that are specific for citrullinated peptides from the human vimentin protein presented in the context of the most frequent SE-containing HLA-DRB1 allele, HLA-DRB1*0401. To this end, we used an unbiased approach by testing the capacity of all possible citrullinated peptides derived from human vimentin to induce T cell responses in DR4-transgenic mice. Epitope mapping in HLA-transgenic mice, both for class I and II, has been shown to be a reliable method to identify T cell epitopes that are also recognized by human T cells (20-24), and therefore we anticipate that the epitopes identified in HLA-transgenic mice are prime candidates for recognition by human T cells. We identified two

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

Material and Methods

Mice

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

Patients and Controls

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

Peptides/protein

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

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

Table 1. Patient characteristics

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

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

Immunization protocol and epitope mapping

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

Table 2. Peptides synthesized from the vimentin protein

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

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

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

ELISA

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

Intracellular cytokine staining

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

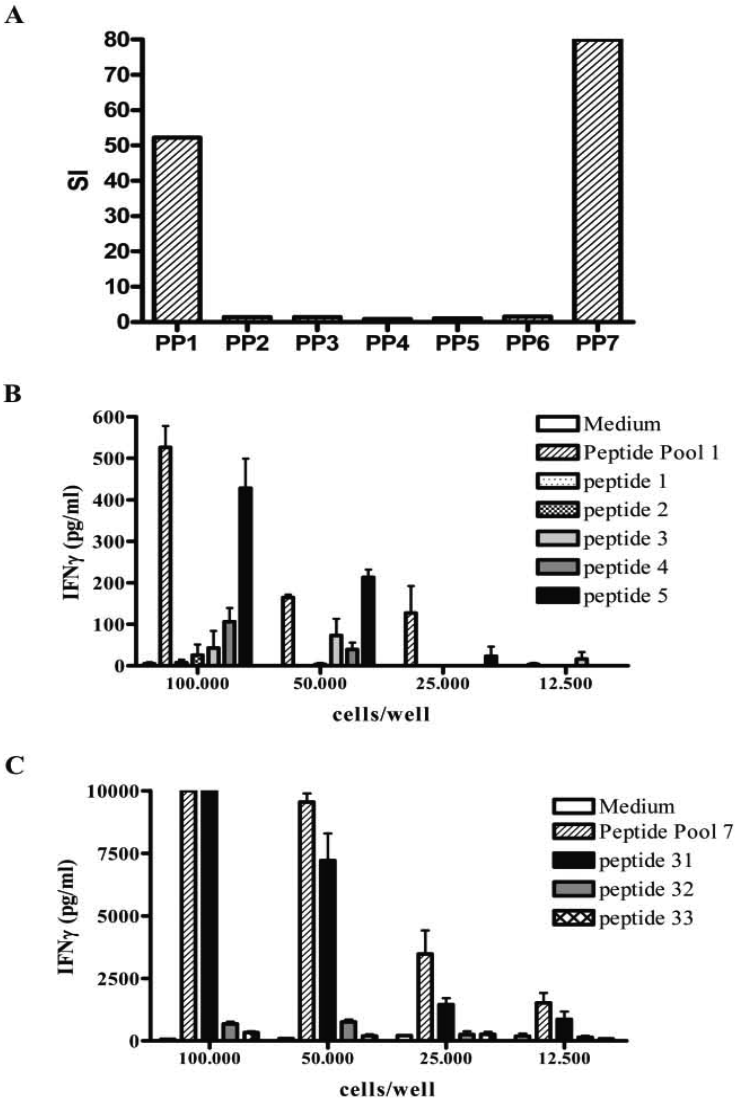

Figure 1. IFNγ production of bulk spleen cell cultures from DR4-transgenic mice immunized with the indicated peptide pools.
A. Reactivity against the different peptide pools (PP; hatched bars) with 100,000 c/w. The bars represent the mean stimulation indices (SI) against each peptide pool of two independent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

Statistics

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

Results

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

Peptide Pools inducing antigen-specific T cell responses

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

Characterization of immunogenic peptides

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

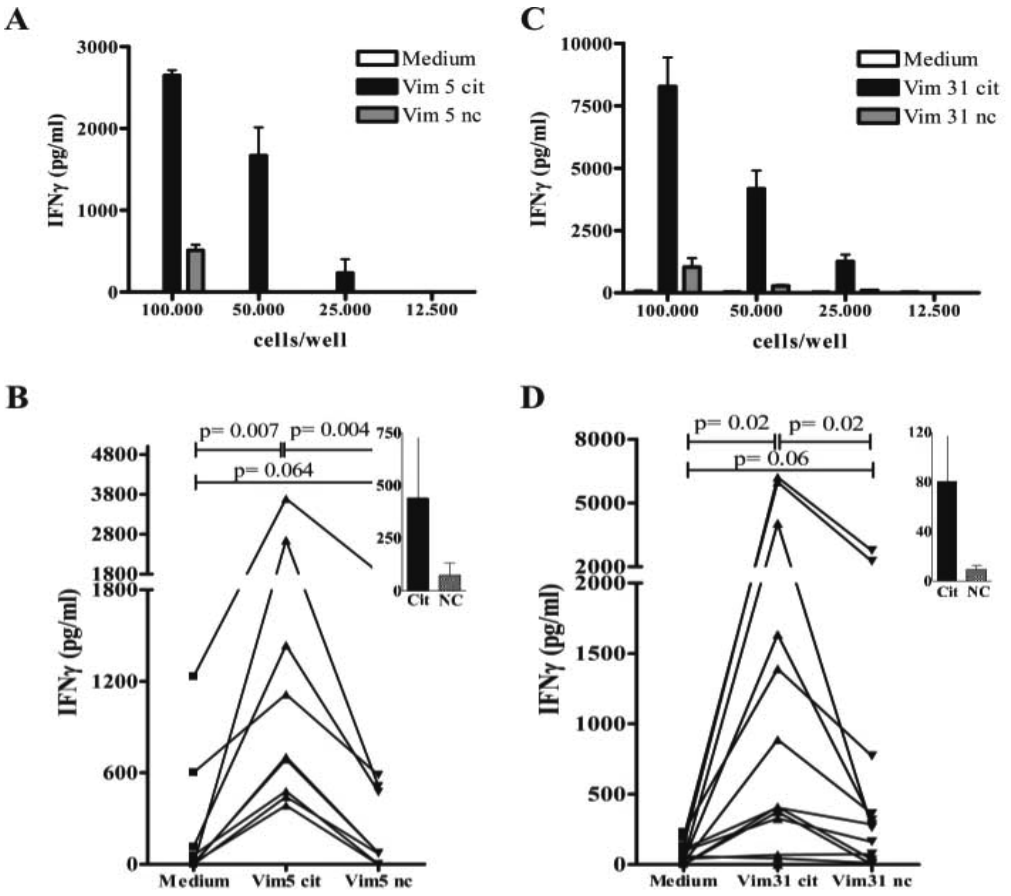

Figure 2. IFNγ response of bulk spleen cell cultures from DR4-transgenic mice immunized with the indicated citrullinated peptides and tested against the citullinated (cit; black bars) and non-citrullinated (nc; grey bars) peptide.
A. Cell titration showing the response to vim5 from one representative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

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

Natural processing of the immunogenetic peptides from citrullinated vimentin protein

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

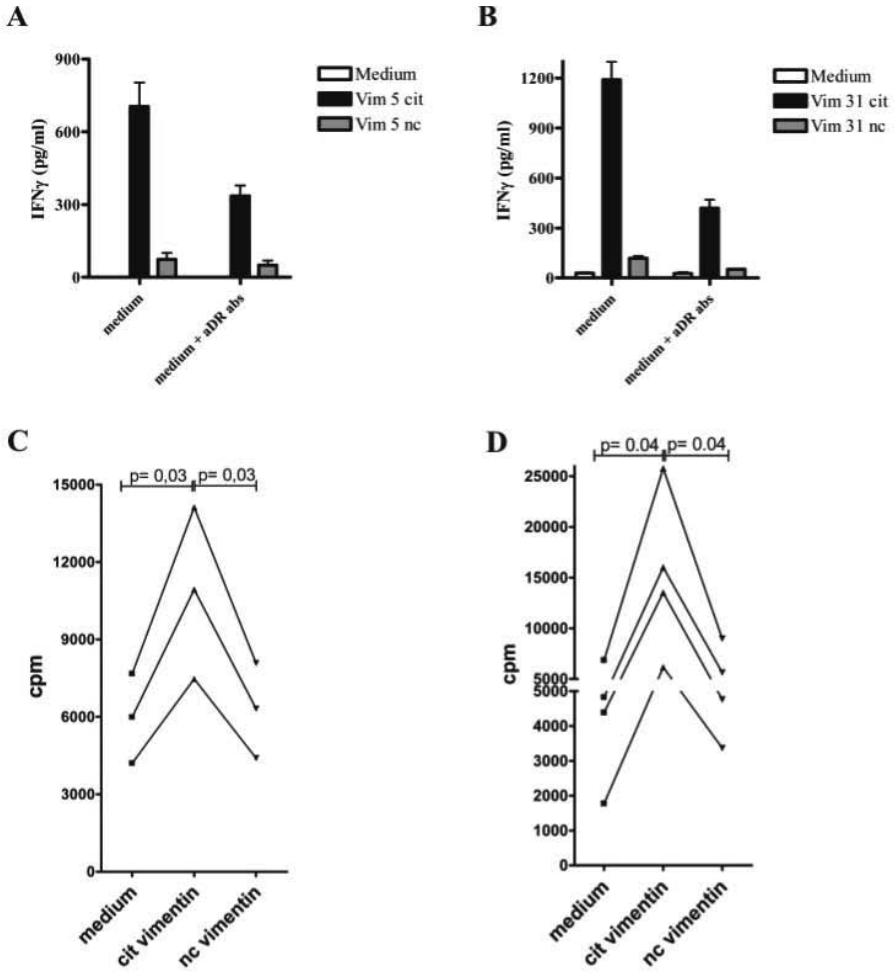

Figure 3. A&B. HLA-restriction of the T cell response. Spleen cells from mice immunized with vim5 Cit (A) or vim31 Cit (B) were restimulated with the indicated peptides in the presence or absence of anti-HLA-DR antibodies. Bars represent the mean of a triplicate measurement in supernatants of cells without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

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

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

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

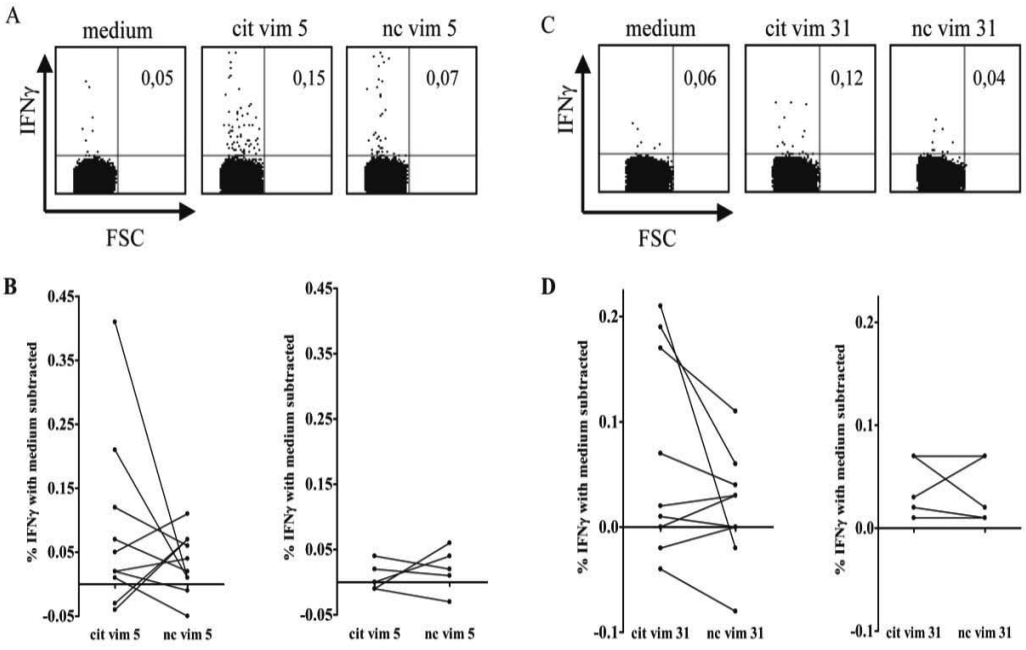

Figure 4. T cell response of PBMC cultures from human individuals. PBMC from ACPA+ HLA-DRB1*04+ RA patients and healthy individuals were cultured and tested against citrullinated and non-citrullinated vim5 (A&B) or 31 (C&D). One representative patient is depicted in the dot plots for reactivity against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

Discussion

At present, only limited data are available on potential T cell epitopes that can be recognized in a "citrulline'-dependent manner by RA patients. Therefore, an unbiased inventory, focusing on relevant autoantigens recognized by ACPA, such as performed in the experiments described in this manuscript is highly relevant.
We examined in this study whether we could identify $CD4^+$ T cells specific for citrullinated peptides derived from human vimentin. In total 33 peptides were synthesized in their citrullinated form and tested for T cell reactivity in DR4-transgenic mice. A "citrulline"-specific response was observed against two of the peptides, vim5 (vim26-44) and vim31 (vim415-433). We have shown that these peptides are naturally processed epitopes of human vimentin and provided data indicating that they can be recognized by T cells with a memory phenotype from RA patients.

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

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

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

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

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

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