TRANSLATIONAL SCIENCE

Isolation of HLA-DR-naturally presented peptides identifies T-cell epitopes for rheumatoid arthritis

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ABSTRACT

Objective Rheumatoid arthritis (RA) immunopathogenesis revolves around the presentation of poorly characterised self-peptides by human leucocyte antigen (HLA)-class II molecules on the surface of antigen-presenting cells to autoreactive CD4 +T cells. Here, we analysed the HLA-DR-associated peptidome of synovial tissue (ST) and of dendritic cells (DCs) pulsed with synovial fluid (SF) or ST, to identify potential T-cell epitopes for RA.

Methods HLA-DR/peptide complexes were isolated from RA ST samples (n=3) and monocyte-derived DCs, generated from healthy donors carrying RA-associated shared epitope positive HLA-DR molecules and pulsed with RA SF (n=7) or ST (n=2). Peptide sequencing was performed by high-resolution mass spectrometry. The immunostimulatory capacity of selected peptides was evaluated on peripheral blood mononuclear cells from patients with RA (n=29) and healthy subjects (n=12) by flow cytometry.

Results We identified between 103 and 888 HLA-DR-naturally presented peptides per sample. We selected 37 native and six citrullinated (cit)-peptides for stimulation assays. Six of these peptides increased the expression of CD40L on CD4 +T cells patients with RA, and specifically triggered IFN-γ expression on RA CD4 +T cells compared with healthy subjects. Finally, the frequency of IFN-γ-producing CD4 +T cells specific for a myeloperoxidase-derived peptide showed a positive correlation with disease activity.

Conclusions We significantly expanded the peptide repertoire presented by HLA-DR molecules in a physiologically relevant context, identifying six new epitopes recognised by CD4 +T cells from patients with RA. This information is important for a better understanding of the disease immunopathology, as well as for designing tolerising antigen-specific immunotherapies.

Key messages

What is already known about this subject?

- Rheumatoid arthritis (RA) is triggered and sustained by CD4 +T cells recognising poorly characterised self-peptides on human leucocyte antigen (HLA)-class II molecules, displayed on the surface of antigen-presenting cells.

What does this study add?

- Our study confers key insights into the repertoire of naturally presented self-peptides in HLA-DR molecules in the context of RA.
- We identified six new epitopes, four native and two citrullinated, recognised by CD4 +T cells from patients with RA.

How might this impact on clinical practice or future developments?

- These results contribute to the understanding of the immunopathogenesis of the disease and the design of tolerising antigen-specific therapeutic strategies.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by synovial infiltration of adaptive immune cells, including T and B cells, as well as specialised antigen-presenting cells (APCs) such as dendritic cells (DCs). It is believed that RA is initiated and perpetuated by autoreactive CD4 +T cells that recognise self-peptides presented by human leucocyte antigen (HLA)-class II molecules displayed by APCs.[1] A group of HLA-class II molecules encoded by *HLA-DRB1* alleles such as *HLA-DRB1*0101*, **0401*, **0404*, **0405*, **1001* and **1402*, known as 'shared epitope' (SE) alleles, has been shown to confer increased susceptibility and severity for RA.[2 3]

SE alleles have also been associated with anti-citrullinated protein/peptide antibody (ACPA) seropositivity in RA.[4] Citrullination is a post-translational modification (PTM) that involves the enzymatic conversion of positively charged arginine to neutral citrulline within a peptide. Since SE molecules share a positively charged peptide-binding pocket 4 (P4), it has been proposed that peptides containing a citrulline in P4 present a higher binding affinity for SE molecules compared with their arginine-bearing counterparts.[5 6] Alternatively, it has been shown that citrulline on certain citrullinated (cit)-peptides can be beneficial in interacting with other SE anchor pockets, or could directly contact the T-cell receptor (TCR), selecting citrulline-specific T-cell clones.[7–9] Cit-peptides from several proteins have been reported to activate CD4 +T cells in patients with RA, which are thought to support the differentiation of ACPA-secreting plasma cells, although definitive evidence on this point is still needed.[10–12]

Numerous autoantigens have been proposed for RA, however, information regarding the epitopes responsible for T-cell activation remains limited. Some CD4 +T cell epitopes derived from proteins that can be found in the synovium, such as type-II collagen, fibrinogen and proteoglycan-aggrecan, have been identified by analysing T-cell responses to peptides encompassing whole protein sequences,[13 14] or in silico predicted HLA-class II-binding peptides.[15 16] Nevertheless, those approaches have mainly focused on proteins previously described as autoantibody targets and have not taken into consideration the restrictions imposed by antigen-processing and peptide-loading onto HLA molecules. These limitations can be circumvented by the isolation of peptide/HLA complexes (pHLA) and further sequencing of naturally presented peptides (NPPs) by liquid chromatography coupled to mass spectrometry (LC-MS/MS), a technology that has already identified several autoimmunity-associated T-cell epitopes,[17–19] as well as post-translationally modified NPPs.[20 21]

So far, only a few reports have described NPPs potentially involved in RA. These studies used synovial tissue (ST),[20 22] and peripheral blood (PB) or synovial fluid (SF) mononuclear cells (PBMCs and SFMCs, respectively)[22] as pHLA sources, and succeeded in identifying two RA T-cell epitopes[23]; however, no cit-peptides were reported. Here, we extend these results by describing six novel T-cell epitopes for RA, including two cit-peptides, out of a repertoire of HLA-DR-bound peptides isolated from ST-resident APCs, as well as SF and ST-pulsed monocyte-derived (Mo)DCs.

METHODS

Study participants

We recruited healthy controls (HC), who donated buffy coats (n=10) and PB (n=12), as well as patients with RA donating SF (n=15), ST (n=3) and PB (n=29), from Hospital Clínico Universidad de Chile, Hospital del Salvador and Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León (online supplemental tables 1 and 2).

Generation of MoDCs

MoDCs were generated from HC PB monocytes as previously described.[24 25] MoDCs were first pulsed with a pool of albumin and IgG-depleted RA SF (SF-DCs) (online supplemental figure 1), or RA ST lysate obtained from a single donor (ST-DCs), and then maturated with lipopolysaccharide. Unpulsed DCs (UP-DCs) were used as controls. After harvesting, MoDCs were characterised by flow cytometry and dry cell pellets were prepared.

Isolation of pHLA-DR complexes

Solubilised membranes from dry cell pellets and ST lysates were subjected to immunoprecipitation with an anti-HLA-DR antibody (clone B8.11.2). HLA-DR-bound peptides were disassembled by acidic elution.

Mass spectrometry analysis

Eluted peptides were fractionated by strong cation-exchange chromatography. Fractions were analysed by LC-MS/MS on an LTQ-Orbitrap-XL mass spectrometer. Additionally, MoDCs, SF and ST lysates were trypsinised and analysed by LC-MS/MS. Fragmentation spectra were searched on the software Proteome Discoverer v1.4.

T-cell stimulation

RA PBMCs were stimulated with pools of 5–6 peptides or single peptides for 12–16 hours, to evaluate CD40L expression on CD4 +T cells by flow cytometry. The peptides with the highest CD40L induction capacity were used to stimulate PBMCs from patients with RA and HC for 5 days, to assess CD4 +T cells IFN-γ production by flow cytometry.

Flow cytometry analysis

Cells were stained with fluorochrome-conjugated antibodies and analysed on a LSR Fortessa X-20 or a FACS Canto flow cytometer. Data analysis was done on FlowJo V.10.4.

Statistical analysis

Statistical analyses were performed on IBM-SPSS Statistics V.27.0. A $p<0.05$ was considered significant.

Detailed experimental methods are available as online supplemental material.

RESULTS

Characterisation of the HLA-DR immunopeptidome generated by a combined approach

HLA-DR-bound peptides were isolated from RA ST, SF-DCs and ST-DCs carrying SE-positive molecules. For the last two approaches, UP-DCs were used as controls. Both, UP-DCs and SF/ST-DCs showed high expression of CD83, CD86 and HLA-DR (online supplemental figure 2B,C), indicating that the exogenous load did not affect DCs maturation state. HLA-DR expression was also detected in ST samples (online supplemental figure 2D), attesting the presence of resident or infiltrating APCs.

The identity of peptides isolated from HLA-DR molecules was defined based on the fragmentation spectra obtained from LC-MS/MS sequencing. Sequences derived from skin-specific proteins were considered contaminants and discarded from further analyses. Between 103 and 888 peptides were obtained per sample (table 1). The number of peptides identified from MoDC samples positively correlated with starting MoDC counts (figure 1A). Over 90% of identified sequences were 9–26

Table 1 Number of HLA-DR-bound peptides isolated from each sample

Sample*	*HLA-DRB1* alleles†	Initial cell no ($x10^6$)	No of peptides‡
UP-DC1	***0101**/*1104	5.0	204
UP-DC2	***0404**/*0407	8.0	493
UP-DC3	***1402**/*1301	5.7	327
UP-DC4	***0101**/*0301	11.3	595
UP-DC5	***0401**/*0301	5.4	509
SF-DC1	***0101**/*1104	10.0	329
SF-DC2	***0404**/*0407	3.9	186
SF-DC3	***1402**/ *1301	2.1	156
SF-DC4	***0101**/*0301	8.5	888
SF-DC6	***0405**/*0701	7.3	724
SF-DC7	***0401**/*1101	1.0	319
SF-DC8	***0401**/*0407	5.0	341
ST-DC9	***0101**/*0301	5.9	585
ST-DC10	***0101/*0401**	4.5	506
ST1	***0401**/*1101–04	N/A	197
ST2	***0405**/*0701	N/A	103
ST3	***0401**/*1602	N/A	305

*DC samples with the same code number derive from the same donor.
†*HLA-DRB1* alleles expressing the SE are highlighted in bold.
‡Number of peptides obtained after discarding sequences derived from skin-specific proteins.
DC, dendritic cells; HLA, human leucocyte antigen; N/A, not assessed; SF-DC, synovial fluid-pulsed DCs; ST, synovial tissue; UP-DC, unpulsed DCs.

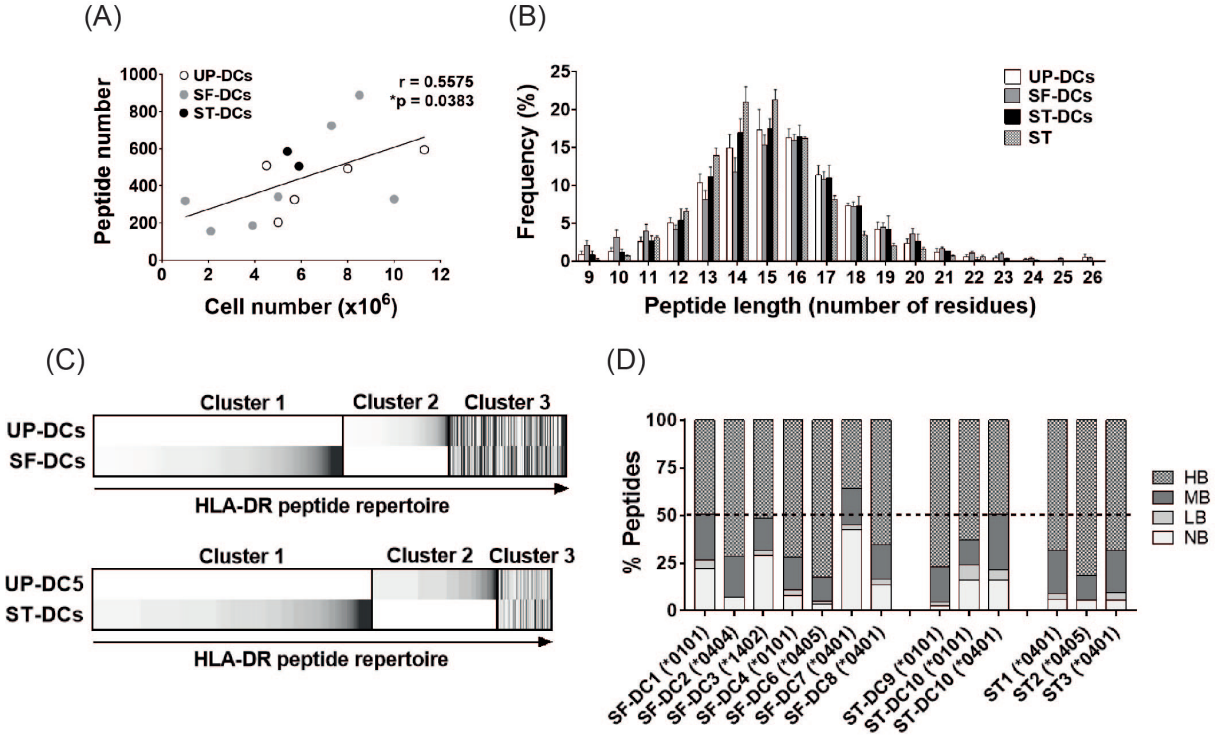


Figure 1 Characterisation of peptides isolated from HLA-DR molecules. HLA-DR/peptide complexes from dendritic cells (DCs) and synovial tissue (ST) samples were immunoprecipitated and peptides were sequenced by liquid chromatography coupled to mass spectrometry. (A) Correlation between the number of isolated peptides and the initial number of DCs (n=14). Data from unpulsed DCs (UP-DCs), synovial fluid-pulsed DCs (SF-DCs) and ST-pulsed DCs (ST-DCs) are shown. (B) Peptide length distribution in UP-DCs, SF-DCs, ST-DCs and ST samples. Bars represent the average peptide length frequency (%)±SD for all donors. (C) Relative abundance of peptides obtained from UP-DCs, SF-DCs and ST-DCs. Grouped data from UP-DC1 to UP-DC4 was compared with grouped data from SF-DC1 to SF-DC8 (5 day DCs), while data from UP-DC5 was compared with grouped data from ST-DC9 and ST-DC10 (7 day DCs). (D) Theoretical binding affinities of selected peptides to the SE-positive HLA-DR molecule present in the respective source sample. Bars show the cumulative percentage of high (HB), medium (MB), low (LB) and non-binder (NB) peptides isolated from each sample.

residues long, with a mode of 15 residues (figure 1B), which is consistent with reported data for HLA-class II peptides.[26]

When comparing NPPs obtained from UP-DCs and SF/ST-DCs, a significant number of differentially isolated peptides was found. This can be partially explained by the low number of peptides retrieved and the omission of less abundant sequences. Nevertheless, with the aim to work with peptides more likely to be derived from synovial proteins, non-redundant sequences obtained exclusively from SF/ST-DCs (cluster 1 in figure 1C) were considered for subsequent analyses.

After discarding peptides derived from HLA chains and those shorter than nine residues (HLA-class II minimum core), the universe of non-redundant sequences was reduced to 731, 348 and 210, for SF-DCs, ST-DCs and ST, respectively. The frequency of peptides belonging to nested sets, families of peptides with a common 9-mer binding core but different C- and N-termini lengths, which are a common attribute of HLA-class II peptides,[26] varied between 21% and 80% (table 2).

Numerous non-redundant sequences and parental proteins were found in more than one sample of the same type (table 2), and across different sample sources (online supplemental figure 3A,B). Furthermore, five non-redundant sequences and 35 parental proteins were present in all three sources (online supplemental figure 3C), suggesting that ST-resident APCs can uptake, process and present local antigens alike ex vivo-pulsed MoDCs. Since cit-proteins are prominent autoantigens in RA, citrullination was included as a variable PTM in the MS/MS database search. As a result, 13 non-redundant cit-sequences were detected and further confirmed by manual inspection (table 2).

According to a theoretical analysis for HLA-DR binding affinity, for most samples, over 50% of peptides were assigned as high binders (HB) and around 25% were medium binders for the SE-positive molecule expressed by the sample donor (figure 1D). Only one donor was SE double-positive (ST-DC10), for whom numerous peptides were considered HB for both alleles (figure 1D). In contrast, lower percentages of HB were assigned for SE-negative molecules (online supplemental figure 4A). Nevertheless, most peptides were predicted to bind to several different HLA-DR molecules (online supplemental figure 4B).

A total of 502, 225 and 177 parental proteins were identified from SF-DCs, ST-DCs and ST samples, respectively (table 2), which were associated with a variety of cellular processes (figure 2A). As expected for HLA-class II molecules, most parental proteins were assigned to subcellular compartments associated with the endocytic processing pathway[27] (figure 2B). Regarding tissue distribution, most proteins were considered ubiquitous or widely expressed, while a minor percentage was regarded as tissue specific or secreted in plasma (figure 2C). Interestingly, over 20% of parental proteins have been reported to be increased in SF, ST, fibroblast-like synoviocytes (FLS) or serum from patients with RA. Also, over 10% have been described as targets for RA autoantibodies, either in their native or PTM

Table 2 Characteristics of peptides isolated from HLA-DR molecules

	SF-DC1	SF-DC2	SF-DC3	SF-DC4	SF-DC6	SF-DC7	SF-DC8	ST-DC9	ST-DC10	ST1	ST2	ST3
Peptides*, n	132	56	42	231	525	155	102	511	336	186	93	280
Non-redundant peptides†, n	107	47	38	174	254	109	79	197	163	88	53	138
Parental proteins, n	98	47	38	149	210	71	68	130	125	74	49	126
Unique peptides‡, (%)	60	68	79	54	27	45	49	20	29	22	35	28
Peptides in nested sets, (%)	40	32	21	46	73	55	51	80	71	78	65	72
Cit-peptides, n	0	0	1	4	0	10	1	0	0	1	1	1
Non-redundant cit-peptides, n	0	0	1	2	0	6	1	0	0	1	1	1
Total non-redundant peptides§, n				**731**				**348**			**210**	
Non-redundant peptides in one sample, n (%)	661 (90.4)							335 (96.3)		147 (70)		
Non-redundant peptides in ≥2 samples, n (%)	70 (9.6)							13 (3.7)		63 (30)		
Total parental proteins§, n				**502**				**225**			**177**	
Parental proteins in one sample, n (%)	383 (76.3)							195 (86.7)		116 (65.5)		
Parental proteins in ≥2 samples, n (%)	119 (23.7)							30 (13.3)		61 (34.5)		
Total non-redundant cit-peptides, n				**10**				**0**			3	
Total parental cit-proteins, n				**8**				**0**			3	

*Number of peptides obtained after discarding sequences present in UP-DCs, derived from HLA chains, or shorter than nine amino acids.
†Non-redundant peptides include unique peptides and peptides belonging to nested sets but as the latter share the same core, the whole family is considered as one in the counting.
‡Unique peptides are sequences not belonging to a nested set.
§The total number indicated is not equal to the sum of peptides/proteins obtained per sample, as some of them were found in more than one sample.
DC, dendritic cells; HLA, human leucocyte antigen; SF-DC, synovial fluid-pulsed DCs; ST, synovial tissue; UP, unpulsed.

forms, and a lower percentage have been recorded as eliciting T-cell responses in patients with RA. No reported association to RA was found for over 50% of parental proteins (figure 2D). To collate the origin of parental proteins, the proteome of MoDC, SF and ST lysates was compared with parental proteins from SF-DCs, ST-DCs and ST NPPs. These analyses showed that parental proteins from SF-DCs and ST-DCs can be found in both, MoDC lysates and the material used to pulse MoDCs (SF and ST lysates, respectively) (figure 2E,F), suggesting that MoDCs were able to uptake and process exogenous synovial proteins. Similar results were obtained when analysing parental proteins from ST NPPs (figure 2G).

Selection of NPPs for T-cell stimulation assays

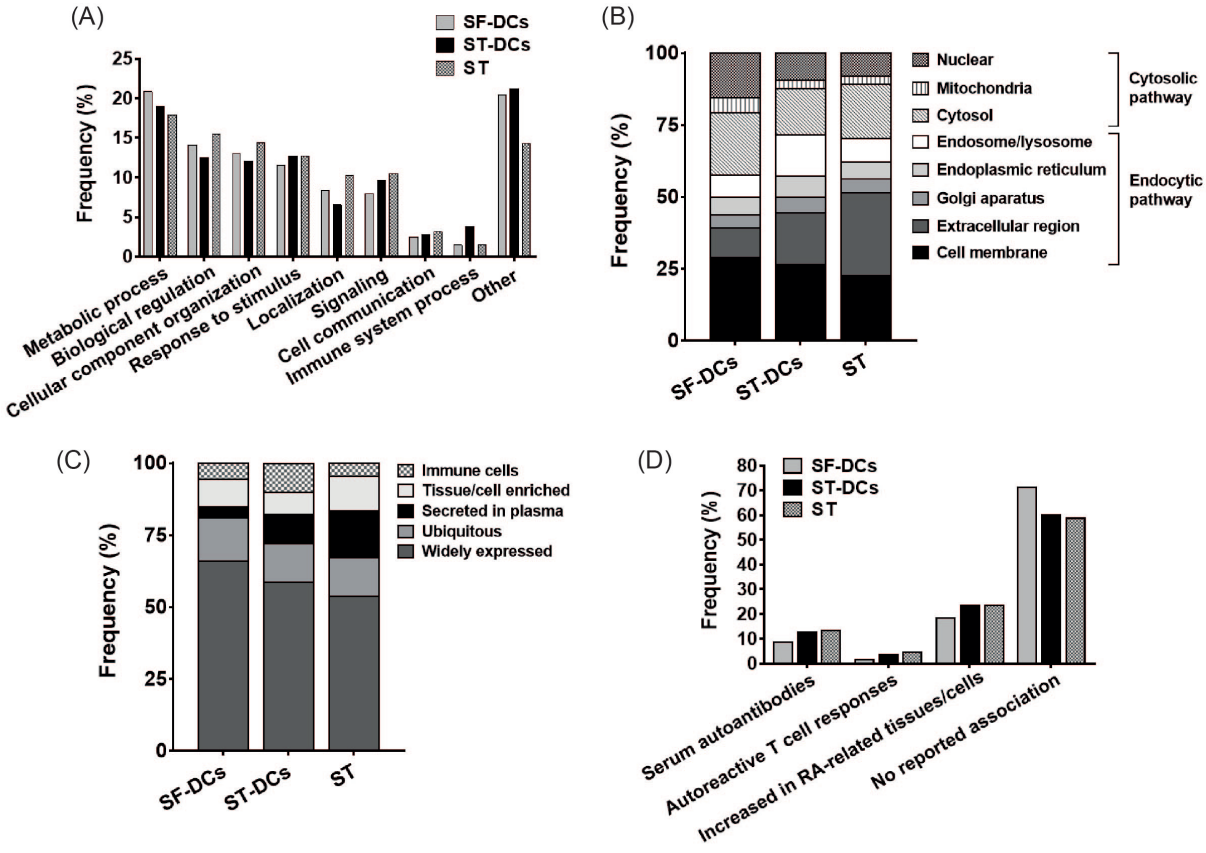
In order to screen for potential T-cell epitopes, a group of 43 NPPs was selected among those peptides that were most frequently detected, showed a higher theoretical affinity for SE-positive molecules and promiscuity for other HLA-DR molecules, and whose parental proteins had been described as autoantigens or highly expressed in patients with RA (table 3). These candidates also exhibited common features of HLA-class II peptides, such as the nature of core-flanking residues and intra-protein localisation[28 29] (online supplemental figure 5A-C). Among these NPPs, six cit-peptides and their native counterparts were included. The presence of citrulline was confirmed by a manual compared analysis of MS/MS spectra from cit-peptides and their eluted native counterparts when available (online supplemental figure 6), as well as from their synthetic versions (online supplemental figure 7).

A predictive analysis assessing the contribution of citrulline on the binding of cit-peptides to the SE-positive alleles displayed by the donors from which these cit-peptides were retrieved (DR*0401 and *0101), suggested that three cit-peptides, deriving from cit-alpha-actin-2, cit-histone H2B and cit-cathepsin Z, could accommodate citrulline in the P4 pocket, which should increase their binding affinity compared with their unmodified versions, according to previous reports[5 6] (online supplemental figure 8A). Instead, for cit-proteoglycan-4 (PG4), citrulline was predicted to match the P9 pocket, whereas for the remaining cit-peptides (cit-fibronectin and cit-gelsolin), citrulline was predicted to be located outside the binding core. Since HLA-DR binding algorithms for cit-peptides have not been widely validated, these predictions should be regarded with caution. To extend these analyses, binding competition assays were performed using soluble HLA-DR molecules. These assays demonstrated that four cit-peptides were able to bind to SE-positive molecules (figure 3). Of note, an increase in experimental affinity of a cit-peptide versus its native counterpart was only observed for cit-PG4, although the difference was very modest (figure 3). In addition, we assessed the binding capacity of three cit-peptides to their corresponding SE-negative allele (DR*1101). While cit-fibronectin was able to bind to DR*0401 but not to DR*1101; cit-histone H2B bound to DR*1101 but not to DR*0401. On the other hand, cit-alpha-actin did not bind to either allele (figure 3).

NPPs activate CD4+ T cells from patients with RA

For an initial set of T-cell stimulation assays, 8 pools consisting of 5–6 peptides each, were designed (table 3). Only pools 5 and 6 increased the expression of CD40L by RA CD4 +T cells relative to unstimulated cells, and above the median increase achieved by an autoantigenic cit-aggrecan peptide[11] (figure 4A and online supplemental figure 9B).

When peptides from pools 5 and 6 were assessed individually, a positive CD40L response was detected against peptides derived from gelsolin, histone H2B, cit-histone H2B, cit-PG4, histone H4 and myeloperoxidase (MPO) (figure 4B, online supplemental figures 9C and 10C). These six peptides were further evaluated in their capacity to trigger T-cell responses in patients with RA versus HC, including both SE-positive and SE-negative donors (online supplemental table 2). All peptides elicited stronger IFN-γ CD4 +T cell responses among patients with RA (figure 4C and online supplemental figure 11), being cit-PG4, gelsolin and MPO the most RA-specific (online supplemental table 3). Although no significant differences on T-cell responses were found between SE-positive and SE-negative patients with RA, among HC the only appreciable responses to any peptide were detected in SE-positive individuals (online supplemental



(E)

Parental proteins (SF-DCs)	DC lysate	SF lysate
HSP-90	■	
Histone H2B.d	■	■
Annexin A2	■	■
Alpha-actin-2	■	
Ceruloplasmin		■
Serotransferrin	■	■
Beta-actin	■	■
Cathepsin Z	■	
CD206		
Histone H2A/s	■	■

(F)

Parental proteins (ST-DCs)	DC lysate	ST lysate
Alpha-2-M	■	■
Cathepsin D	■	■
Complement C3	■	■
Cathepsin Z	■	
Histone H2A/s	■	■
Transthyretin		■
Aminopeptidase N	■	
Vimentin	■	■
Elongation factor 1-alpha 1		■
Cathepsin B	■	

(G)

Parental proteins (ST)	DC lysate	ST lysate
Fibronectin		■
Fibrinogen gamma chain	■	■
Fibrinogen beta chain	■	■
Ig gamma-2 chain C region		■
Fibrinogen alpha chain	■	■
Complement C3	■	■
Transthyretin		■
Moesin	■	■
Calnexin	■	
Myeloperoxidase	■	

Figure 2 Characterisation of parental proteins for peptides isolated from HLA-DR molecules. (A) Frequency of parental proteins associated to different biological processes, based on the gene ontology database annotations for synovial fluid-pulsed dendritic cells (SF-DCs), synovial tissue-pulsed DCs (ST-DCs) and ST samples. (B, C) Frequency of parental proteins classified according to the subcellular compartments from which they are originated (B), or their tissue specificity (C), based on Uniprot and The Human Protein Atlas (THPA) databases. (D) Frequency of parental proteins reported as targets for serum autoantibodies and/or T-cell responses in patients with RA, according to PubMed, and increased in RA-related tissues or cells, based on data from Uniprot and THPA databases. All frequencies were calculated from a total of 502, 225 and 177 parental proteins, obtained from SF-DCs, ST-DCs and ST, respectively. (E–G) The presence (grey) or absence (white) of the 10 most frequent RA-associated parental proteins for peptides isolated from (E) SF-DCs, (F) ST-DCs, and (G) ST, was evaluated in DCs, SF and ST lysates. RA, rheumatoid arthritis.

figure 12). Finally, we observed that the disease activity score in 28 joints (DAS28) positively correlated with IFN-γ CD4 +T cell responses to the MPO peptide (figure 4D), as well as with the number of peptides eliciting T-cell responses (online supplemental figure 13).

DISCUSSION

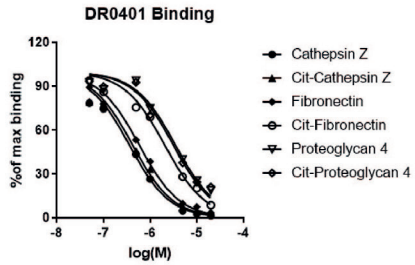
The ST represents the epicentre of RA pathogenesis, a site where infiltrating CD4 +Th1 and Th17 cells lead to the activation of myeloid cells and fibroblasts, and ultimately, to joint destruction.[30] The enrichment of inflammatory APCs and autoantigen-specific memory CD4 +T cells in RA joints,[31 32] together with in vivo evidence of HLA-DR/autoantigen complexes in ST from patients with RA,[33] and in vitro experiments showing the ability of FLS to present synovial autoantigens,[34 35] argue in favour of the synovium as a propitious niche where disease-driving autoantigens could activate self-reactive T cells. One of the main limitations to study T-cell autoreactivity in RA is the paucity of data regarding the epitopes responsible for disease initiation and progression. In this study, we identified multiple HLA-DR-presented NPPs by using MoDCs pulsed ex vivo with synovial material, and directly from ST-resident APCs.

Table 3 Characteristics of peptides selected for T-cell stimulation assays

Parental protein	RA association	Peptide sequence*	Theoretical affinity†	Promiscuity‡	Isolation source	Pool
Alpha-actin-2	Auto-Ab[47]	SGGTTMYPGIADRMQKEITA	LB	12/51	SF-DCs	*
Cit-Alpha-actin-2	Citrullination	SGGTTMYPG**IADrMQKEI**TA	MB	–	SF-DCs	*
Cathepsin Z	↑ ST[48]	SDGTEYWIVRNSWGEPWG	MB	38/51	SF-DCs	*
Cit-Cathepsin Z	Citrullination	SDGTEY**WIVrNSWGE**PWG	HB	–	SF-DCs	*
Fibronectin	Auto-Ab[39]; ↑ ST[49]	APITGYRIVYSPSVEGSS	HB	51/51	ST/ST-DCs	*
Cit-Fibronectin	Citrullination	APITGY**r**IVYSPSVEGSS	HB	–	ST	*
Alpha-2-HS-glycoprotein	Auto-Ab; ↑ SF[50]	SVVYAKCDSSPDSAE	HB	45/51	SF-DCs	†
Aminopeptidase N	↑ SF[51]	INDAFNLASAHKVPV	HB	30/51	ST-DCs	†
Annexin A2	Auto-Ab; ↑ serum[52]	DALNIETAIKTKGVDE	HB	21/51	SF-DCs	†
Calreticulin	Auto-Ab[53]; ↑ SF and plasma[54]	GGGYVKLFPNSLDQT	HB	51/51	SF-DCs	†
Ceruloplasmin	Auto-Ab[50]	VDKEFYLFPTVFDENE	HB	39/51	SF-DCs	†
Clusterin	↑ serum[55]	TVSDNELQEMSNQGSKY	HB	23/51	ST	†
Complement C1q B chain	↑ serum[56]	HVITNMNNNYEPR	HB	16/51	ST	‡
Complement C1r	↑ ST[57]	GDFRYTTTMGVNTY	MB	51/51	ST / ST-DCs	‡
Complement C3	Auto-Ab[50]; ↑ SF[58]	SETRILLQGTPVAQMT	HB	46/51	ST	‡
Elongation factor 1-alpha 1	Auto-Ab[59]	AAGFTAQVIILNHPGQISAG	HB	51/51	ST-DCs	‡
Chaperone BiP	Auto-Ab; ↑ SF and plasma; Auto-T[60]	GVFEVVATNGDTH	HB	16/51	SF-DCs	‡
Ferritin heavy chain	Auto-Ab[61]; ↑ FLS[62]	DDVALKNFAKYFLHQSHE	MB	36/51	SF-DCs	4
Fibrinogen beta chain	Auto-Ab[50]; ↑ SF[63]; Auto-T[14]	TSEMYLIQPDSSVKPY	HB	44/51	ST	4
Ganglioside GM2 activator	↑ SF[64]	TTGNYRIESVLSSSG	HB	51/51	SF-DCs	4
HSP 70–2	Auto-Ab[65]; ↑ SF[63]	EVISWLDANTLAEKD	HB	25/51	ST-DCs	4
HSP 90	Auto-Ab[65]	KELKIDIIPNPQERT	MB	24/51	SF-DCs	4
Gelsolin	Auto-Ab; ↑ SF[50]	DAYVILKTVQLRNGN	HB	51/51	SF-DCs	5
Cit-Gelsolin	Citrullination	DAYVILKTVQL**r**NGN	HB	–	SF-DCs	5
Histone H2B.d	Auto-Ab[66]	MNSFVNDIFERIAGEA	HB	25/51	SF-DCs	5
Cit-Histone H2B.d	Citrullination	MNSFVND**IFErIAGEA**	HB	–	SF-DCs	5
Proteoglycan 4	↑ ST[67]	THTIRIQYSPARLA	HB	45/51	–	5
Cit-Proteoglycan 4	Citrullination	THT**IrIQYSPAr**LA	HB	–	ST	5
Histone H2A/s	Auto-Ab[66]	TAEILELAGNAARDN	HB	18/51	ST-DCs/ SF-DCs	6
Histone H4	Auto-Ab[68]; ↑ SF[63]	DNIQGITKPAIRR	MB	42/51	SF-DCs	6
ITI heavy chain H4	Auto-Ab[50]; ↑ SF[63]	RPSLVPASAENVNK	HB	41/51	SF-DCs/ ST-DCs	6
Moesin	Auto-Ab[69]; ↑ SF[63]	AELEFAIQPNTTGKQ	HB	41/51	ST	6
Myeloperoxidase	Auto-Ab[70]; ↑ SF[64]	SNEIVRFPTDQLTPDQ	MB	46/51	ST / ST-DCs	6
Osteopontin	↑ ST[71]	GAYKAIPVAQDLNAP	HB	30/51	SF-DCs / ST	7
Phosphoglycerate kinase 1	Auto-Ab[72]; ↑ SF[63]	DKIQLINNMLDKV	HB	39/51	SF-DCs	7
Plasma alpha-L-fucosidase	↑ ST[67]	FDPTWESLDARQLPA	HB	19/51	SF-DCs	7
Plasminogen	Auto-Ab[73]	HSIFTPETNPRAGL	HB	12/51	ST	7
Protein S100-A4	↑ ST[67]	DEAAFQKLMSNLDSNR	HB	25/51	SF-DCs	7
Protein S100-A9	↑ ST[67]	IEHIMEDLDTNADKQ	HB	7/51	SF-DCs	8
Pyruvate kinase PKM	↑ SF[63]	ASDPILYRPVAVALDT	MB	50/51	ST-DCs/ SF-DCs	8
Serotransferrin	Auto-Ab[50]	TIFENLANKADRDQ	MB	50/51	SF-DCs	8
Transthyretin	↑ serum[74]	IAALLSPYSYSTTAVVTNPK	HB	28/51	ST-DCs/SF-DCs/ST	8
Vimentin	Auto-Ab[50]; ↑ SF[64]; Auto-T[10]	VPGVRLLQDSVDFSLADAI	HB	36/51	SF-DCs	8

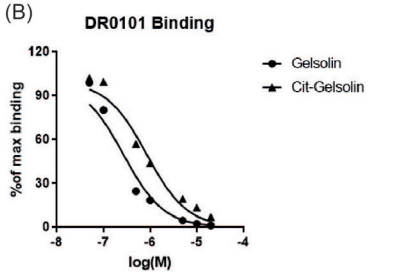
*The 9-mer core of each peptide sequence is underlined and citrulline residues (r) are highlighted in grey.
†Theoretical binding affinities for the corresponding donor SE-positive molecule are shown.
‡Number of HLA-DR molecules to which each peptide is predicted to bind among 51 alleles available in the *Propred* server.
Auto-Ab, autoantibodies; DCs, dendritic cells; FLS, fibroblast-like synoviocytes; HB, high binders; HLA, human leucocyte antigen; LB, low binders; MB, medium binders; RA, rheumatoid arthritis; SF, synovial fluid; ST, synovial tissue.

Human MoDCs are acknowledged as a prototype to study antigen presentation.[36 37] We and others have demonstrated the suitability of using a small number of MoDCs for the characterisation of HLA-DR-associated peptide repertoires and disease-related T-cell epitopes.[28 38] In this study, we obtained abundant non-redundant sequences from MoDCs pulsed with RA SF or ST, which exhibited characteristic features of DCs-derived peptide repertoires in terms of length, presence of nested sets, theoretical affinity, flanking residues properties, intraprotein location and expected degradation pathway.[28] In parallel, we identified 210 peptides from RA ST, which were not significantly different from the MoDCs repertoire, containing a considerable proportion of predicted HB presumably processed through the endo-lysosomal pathway. In addition, we detected an important number of peptides derived from extracellular and tissue-specific proteins, many of which are enriched in RA joints, including previously described autoantigens such as fibronectin[39] and fibrinogen.[14] These findings suggest that HLA-DR-expressing cells in RA ST may correspond to resident or infiltrating cells with antigen uptake, processing and presentation capabilities, as has been shown for autoimmune thyroid glands.[40]



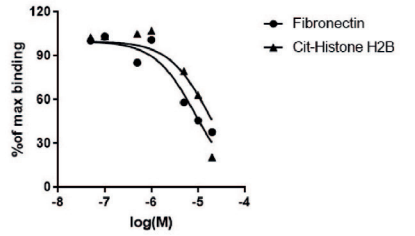
IC50 in µM	Parental protein	Sequence [1]	Donor *HLA-DRB1* haplotype [2]	Predicted binding affinity [3]
>50	Alpha-actin-2	SGGTTMYPGIADRMQKEITA	***0401** / *1101	LB
>50	Cit-Alpha-actin-2	SGGTTMYPGIADrMQKEITA	***0401** / *1101	MB
0.36	Cathepsin Z	SDGTEYWIVRNSWGEPWG	***0401** / *0407	MB
0.44	Cit-Cathepsin Z	SDGTEYWIVrNSWGEPWG	***0401** / *0407	HB
0.57	Fibronectin	APITGYRIVYSPSVEGSS	***0401** / *1101-04	HB
2.12	Cit-Fibronectin	APITGYrIVYSPSVEGSS	***0401** / *1101-04	HB
>50	Histone H2B	MNSFVNDIFERIAGEA	***0401** / *1101	HB
>50	Cit-Histone H2B	MNSFVNDIFErIAGEA	***0401** / *1101	HB
3,57	Proteoglycan 4	THTIRIQYSPARLA	***0401** / *1602	HB
3.04	Cit-Proteoglycan 4	THTIrIQYSPArLA	***0401** / *1602	HB

(B)



IC50 in µM	Parental protein	Sequence [1]	Donor *HLA-DRB1* haplotype [2]	Predicted binding affinity [3]
0.23	Gelsolin	DAYVILKTVQLRNGN	***0101** / *0301	HB
0.74	Cit-Gelsolin	DAYVILKTVQLrNGN	***0101** / *0301	HB

(C)



IC50 in µM	Parental protein	Sequence [1]	Donor *HLA-DRB1* haplotype [2]	Predicted binding affinity [3]
>50	Alpha-actin-2	SGGTTMYPGIADRMQKEITA	***0401** / *1101	MB
>50	Cit-Alpha-actin-2	SGGTTMYPGIADrMQKEITA	***0401** / *1101	MB
8.72	Fibronectin	APITGYRIVYSPSVEGSS	***0401** / *1101-04	HB
>50	Cit-Fibronectin	APITGYrIVYSPSVEGSS	***0401** / *1101-04	HB
>50	Histone H2B	MNSFVNDIFERIAGEA	***0401** / *1101	HB
12.75	Cit-Histone H2B	MNSFVNDIFErIAGEA	***0401** / *1101	HB

Figure 3 Peptide binding competition assays. Citrullinated (cit)-peptides selected for CD4 +T cell stimulation assays, along with their native counterparts, were tested in binding competition assays for shared epitope (SE)-positive molecules expressed by their respective source sample (A, B), or for HLA-DR*1101, the most frequent SE-negative allele among the sample donors for these peptides (C). Binding curves for peptides with IC50 values <50 µM (highlighted in grey in the tables) for HLA-DR*0401 (A), HLA-DR*0101 (B), and HLA-DR1101 (C) are shown. The IC50 values for all analysed peptides are shown in the tables. [1] the predicted 9-mer core of each sequence is underlined and citrulline residues (r) are highlighted in BOLD; [2] SE-positive alleles from the sample donor are highlighted in BOLD; [3] predicted binding affinities for the corresponding donor SE-positive molecule are shown, except for (C), where the theoretical affinity for the HLA-DR*1101 molecule is shown. [4] The native version of the proteoglycan four peptide was not detected among the HLA-DR-associated peptides; however, for these analyses we considered the haplotype of the donor from which its corresponding cit-version was isolated. HLA, human leucocyte antigen; LB, low binders; MB, medium binders; HB, high binders.

Previous studies analysing the RA ST HLA-DR immunopeptidome described a significant number of sequences,[20 22] of which only those derived from *N*-acetylglucosamine-6-sulfatase and filamin-A were reported to activate RA CD4 +T cells.[23] Our results show that peptides deriving from gelsolin, histone H2B, cit-histone H2B, cit-PG4, histone H4 and MPO were able to stimulate RA CD4 +T cells, measured as an increase in the expression of CD40L, which has been shown to be a reliable marker of antigen-activated T cells.[41] In addition, these peptides elicited a higher IFN-γ production by RA CD4 +T cells compared with HC. Interestingly, we showed that T-cell responses to the MPO-derived peptide were correlated with DAS28 score. Supplementary longitudinal studies in a larger patient cohort will be needed to validate the relevance of T-cell responses to these epitopes in disease activity and progression, as well as the relationship with the emergence of autoantibodies against their parental proteins.

To our knowledge, this is the first successful attempt to sequence cit-NPPs from RA samples. This task has proven to be a major challenge in the past given the relatively low amount of peptides retrieved from pHLA and the infrequent occurrence of citrullination in vivo.[22 42] In this study, we obtained both, cit-peptides and their native counterparts, allowing us to manually compare their spectra and validate the presence of citrulline.

It has been proposed that citrullination can contribute to break self-tolerance in RA by enabling peptides to accommodate into the P4 pocket of SE-positive molecules.[5] While this model can be applicable for certain peptides,[6 43] it might not always be the case, as suggested by studies showing that citrulline could also interact with other SE anchor pockets, or be placed at positions available for TCR recognition, thus activating cit-specific T-cell clones.[7–9] In consonance with these reports, our competition binding assays, involving six cit-NPPs and the SE-positive molecule expressed by the corresponding sample donor, revealed that citrullination exerts limited influence, or can even be disadvantageous, for the binding of peptides to HLA-DR*0401 or *0101, as has been shown for α-enolase cit-peptides.[8] Moreover, one cit-peptide only bound to its corresponding SE-negative molecule. These findings, together with a considerable number of predicted promiscuous peptides and HB for SE-negative molecules among our HLA-DR immunopeptidome, suggest that a proportion NPPs, and even some cit-NPPs, could have been isolated from SE-negative molecules, including HLA-DRB4/B3 molecules (although the latter possibility seems less likely, according to our preliminary prediction affinity analysis; data not shown). Our results showing no significant differences in T-cell responses to NPPs between SE-positive and SE-negative

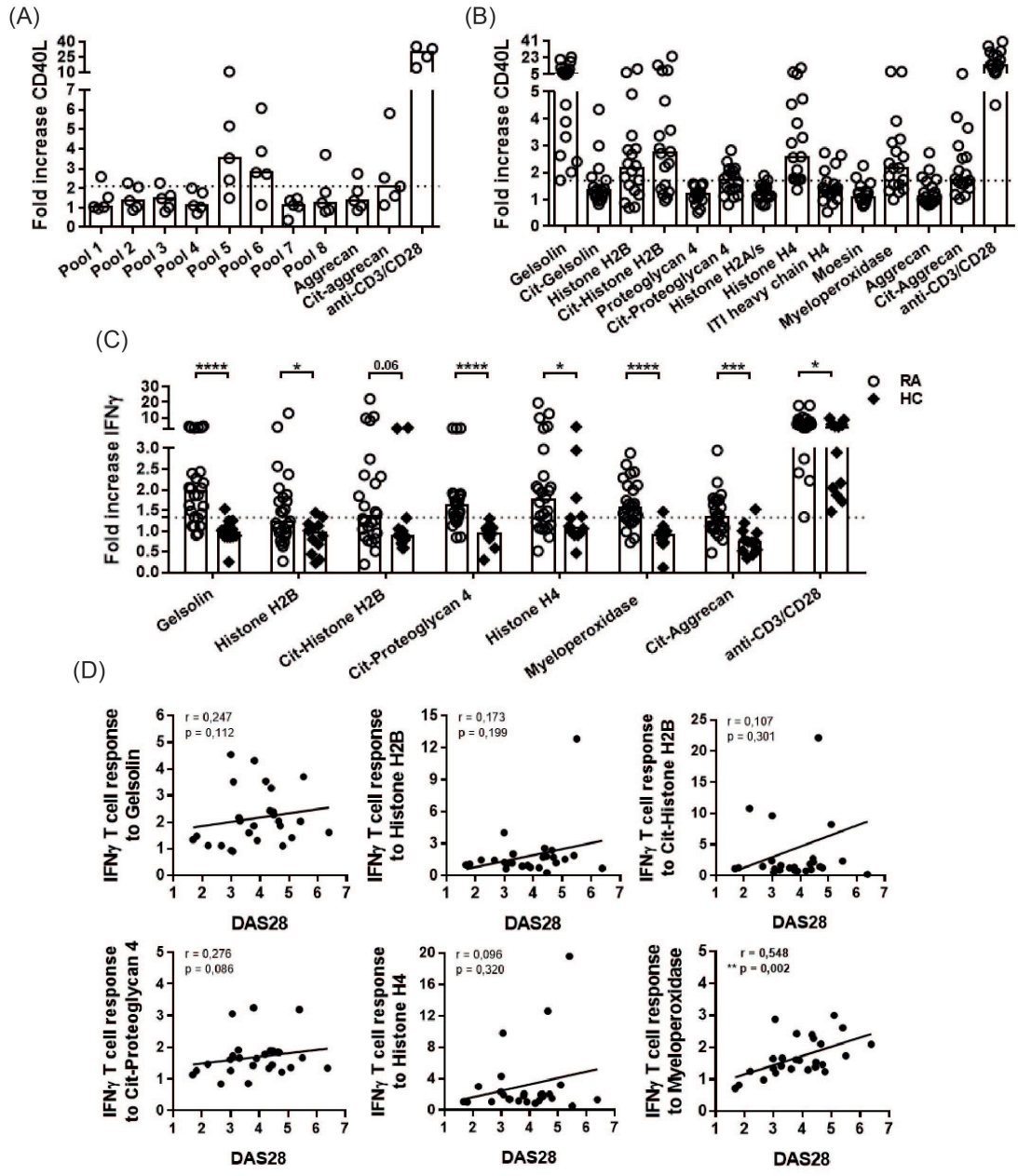


Figure 4 Responsiveness of CD4 +T cells from patients with RA after in vitro stimulation with HLA-DR-associated peptides. (A) Peptides were pooled and used to stimulate PBMCs from patients with RA (n=5). Unstimulated PBMCs and PBMCs stimulated with anti-human-CD3/CD28 beads were used as negative and positive controls, respectively. A previously described autoantigenic aggrecan peptide was included in its native and citrullinated versions. A positive CD4 +CD40L+T cell response was defined as a greater than the median response observed against the cit-aggrecan control peptide in relation to the unstimulated controls. (B) The previous strategy was repeated, using peptides belonging to pools 5 and 6 to stimulate PBMCs from patients with RA (n=18). (C) Peptides with the highest T-cell immunostimulatory capacity in (B) were used for stimulating PBMCs from patients with RA (n=26) and HC (n=12), and IFN-γ production was evaluated. A positive CD4 +IFN-γ+T cell response was defined as greater than the median response observed against the cit-aggrecan control peptide in relation to the unstimulated controls. (D) Correlation between IFN-γ T-cell responses to peptides (fold increase in the percentage of CD4 +IFN-γ+T cells) in patients with RA (n=26) and the disease activity measured by the DAS28 score. *P<0.05; **p<0.01; ***p<001; ****p<0.0001. DAS28, disease activity score in 28 joints; HC, healthy control; HLA, human leucocyte antigen; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis.

patients with RA, regardless of the presence of citrulline, further support the interpretation that the presentation of some of these peptides may be promiscuous; this assumption should be verified in experiments including a larger set of patients from both groups. Likewise, the chance that these peptides could be presented to CD4 +T cells by other HLA-class II molecules (DP, DQ) requires a more thorough examination.

Altogether, these results provide valuable information about the immunopeptidome displayed by HLA-DR molecules loaded ex vivo and in vivo with RA material, revealing autoantigenic T-cell epitopes that could have an impact in disease initiation and/or progression, both in SE-positive and SE-negative patients. Despite the fact that some of these epitopes have been previously isolated from HLA-class II molecules,[44] none of them have been

previously described as RA T-cell epitopes. Given the exploratory character of this study, we only test the antigenicity of a few peptides. We are currently undertaking a comprehensive screening of T-cell epitopes throughout the whole list of NPPs described herein. We believe that this information could be useful for the development of antigen-specific therapies aimed at restoring self-tolerance in patients with RA, such as those based on antigen-loaded tolerogenic DCs[45] and peptide-specific regulatory T cells.[46]

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Contributors JCA, DC and JM participated in the conception and design of the study. JM, MC, DC, JCA, OA, JA, DJ and EJ worked on analysis and interpretation of data. JM, DC and JCA participated in manuscript preparation and redaction. JM, MC and EJ performed experiments and data acquisition. LS, ON and MC participated in the recruitment of subjects for this study. JCA acts as guarantor.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by—'Comité Ético Científico o de Investigación del Hospital Clínico de la Universidad de Chile', Project Ref. OAIC 735-15 (approval certificate #33), 979-18 (approval certificate #44), 962-18 (approval certificate #21) and 964-18 (approval certificate #24)—'Comité de Ética Científico del Servicio de Salud Metropolitano Oriente', Project Ref. 20-10-2018- 'Comisión de Ética en la Experimentación Animal y Humana de la Universitat Autònoma de Barcelona', Project Ref. CEEAH 3112 (collaboration agreement with 'Biobanco del Centro de Hemoterapia y Hemodonación de Castilla y León', Ref. UAB-DJ-19-01). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article. All data relevant to the study are included in the article or uploaded as online supplemental information.

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