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Molecular basis for increased susceptibility of Indigenous North Americans to seropositive rheumatoid arthritis

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Abstract

Objective—The pathogenetic mechanisms by which *HLA-DRB1* alleles are associated with anticitrullinated peptide antibody (ACPA)-positive rheumatoid arthritis (RA) are incompletely understood. RA high-risk *HLA-DRB1* alleles are known to share a common motif, the ‘shared susceptibility epitope (SE)’. Here, the electropositive P4 pocket of HLA-DRB1 accommodates self-peptide residues containing citrulline but not arginine. HLA-DRB1 His/Phe13 β stratifies with ACPA-positive RA, while His13 β Ser polymorphisms stratify with ACPA-negative RA and RA protection. Indigenous North American (INA) populations have high risk of early-onset ACPA-positive RA, whereby HLA-DRB1*04:04 and HLA-DRB1*14:02 are implicated as risk factors for RA in INA. However, HLA-DRB1*14:02 has a His13 β Ser polymorphism. Therefore, we aimed to verify this association and determine its molecular mechanism.

Methods—HLA genotype was compared in 344 INA patients with RA and 352 controls. Structures of HLA-DRB1*1402-class II loaded with vimentin-64Arg_{59–71}, vimentin-64Cit_{59–71} and fibrinogen β -74Cit_{69–81} were solved using X-ray crystallography. Vimentin-64Cit_{59–71}-specific and vimentin_{59–71}-specific CD4+ T cells were characterised by flow cytometry using peptide-histocompatibility leukocyte antigen (pHLA) tetramers. After sorting of antigen-specific T cells, TCR α and β -chains were analysed using multiplex, nested PCR and sequencing.

Results—ACPA⁺ RA in INA was independently associated with *HLA-DRB1*14:02*. Consequent to the His13 β Ser polymorphism and altered P4 pocket of HLA-DRB1*14:02, both citrulline and arginine were accommodated in opposite orientations. Oligoclonal autoreactive CD4+ effector T cells reactive with both citrulline and arginine forms of vimentin_{59–71} were observed in patients with HLA-DRB1*14:02⁺ RA and at-risk ACPA⁻ first-degree relatives. HLA-DRB1*14:02-vimentin_{59–71}-specific and HLA-DRB1*14:02-vimentin-64Cit_{59–71}-specific CD4+ memory T cells were phenotypically distinct populations.

Conclusion—HLA-DRB1*14:02 broadens the capacity for citrullinated and native self-peptide presentation and T cell expansion, increasing risk of ACPA+ RA.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease with peak incidence in the sixth decade and prevalence of 1% in Caucasians, linked to *HLA-DRB1*. *HLA-DRB1* alleles associated with anticitrullinated peptide antibody (ACPA)-positive RA in Caucasians share a common motif in the third hypervariable region, the ‘shared susceptibility epitope (SE)’, which was shown to accommodate citrullinated (Cit) self-epitopes.^{1–3} Citrulline is post-translationally modified from arginine during inflammation, endoplasmic reticulum (ER) stress and autophagy.⁴⁵ Various RA-associated Cit-autoantigens, recognised by ACPA, are present in inflamed sites, including joint tissues. Low-titre ACPA develops in healthy individuals associated with environmental risk factors including smoking and periodontitis, but are unrelated to *HLA-DR SE*.^{6–11} In the immediate pre-RA period, ACPA isotype diversity and titre increase — a process associated with antigen-specific CD4+ T cell help for affinity maturation in germinal centres.⁸ *HLA-DR SE* is associated with ACPA⁺ RA rather than ACPA, implying that presentation of Cit-autoantigens bound to HLA-DR SE molecules to

CD4⁺ T cells is associated with RA development in at-risk individuals carrying *HLA-DR SE*.

Based on genome-wide studies (GWAS) in patients of predominantly Caucasian and Asian ethnicity, *HLA* alleles associated with ACPA-positive RA, including *HLA-DRB1*04:01*, *HLA-DRB1*04:05* and *HLA-DRB1*01:01* (ORs of 2.17–4.44), were found to share a common motif at amino acid positions 11, 13, 71 and 74, influencing the P4 antigen-binding pocket of DRβ.² Moreover, Val11βSer and His13βSer polymorphisms within that motif were found in genomic studies to stratify with ACPA-negative RA in Caucasians.³ The discovery that P4-Cit was accommodated but the positively charged Arg was excluded from the electropositive P4 pocket of HLA-DRB1*04:01/04 suggested that preferential presentation of Cit-autoantigens might underpin the association of ACPA⁺ RA with the SE.¹² While HLA-DRB1*04:01-restricted CD4⁺ memory T cells recognising Cit-autoantigens have been reported in patients with RA,^{12 13} their role in disease development is unclear. To date it has not been determined how autoreactive T cells respond to Cit-autoantigen in RA and whether they undergo clonal expansion due to antigen experience in patients with RA or in HLA-SE⁺ at-risk first-degree relatives (FDRs).

Furthermore, in unravelling antigen presentation by HLA-DR molecules in RA, the ethnic mix of the samples included in GWAS may skew interpretations of amino acids contributing to binding motifs made from genomic studies. The Indigenous North American (INA) population has a twofold to threefold higher prevalence of RA than Caucasians, with RA onset peaking earlier in the fourth decade of life.¹⁴ Moreover, 90% of INA patients with RA are ACPA⁺. In this population, FDRs have a high prevalence of joint symptoms and of ACPA positivity, predisposing them to RA.¹⁵¹⁶ To date, the *HLA* association with RA in INA has been sought in studies of <100 patients.^{17–19} These studies suggested that the *HLA-SE* alleles also predispose to RA in INA. *HLA-DRB1*04:04* is the most frequent *SE* allele in these populations, followed by *HLA-DRB1*14:02*. Since HLA-DRB1*14:02 carries the β13Ser residue, which was interpreted to stratify with ACPA-negative RA,³ we investigated the genetic and underlying molecular bases for the increased risk of severe ACPA⁺ RA in INA.

MATERIALS AND METHODS

Study participants

RA cases, non-RA controls and FDRs were recruited from INA populations in Central Canada (Cree, Ojibway and Ojicree) and Alaska Native people (from Southcentral and Southeast Alaska). DNA for HLA typing, serum and peripheral blood mononuclear cells (PBMC) were isolated.

HLA-DRB1*14:02 expression and purification

Peptide-loaded HLA-DRB1*14:02 molecules were purified, crystallised and structures determined.

Multiplex ACPA assay

Serum levels of antibodies targeting 40 putative RA-associated autoantigens were measured using a custom bead-based immunoassay on a Bio-Plex platform, as previously described.²⁰

Tetramer staining and analysis of TCR repertoire

Tetramer staining and T cell receptor (TCR) repertoire analysis used previously published methods, with some modifications.¹²²¹²²

Details of all methods are available in online supplementary methods, supplementary table 1 and supplementary figure 1.

RESULTS

*HLA-DRB1*14:02* is independently associated with ACPA⁺ RA in INA

Although very rare in Caucasians and Asians, *HLA-DRB1*14:02* has a prevalence of up to 80% in some INA populations, suggesting a particular survival advantage against pathogens.^{17–19} To determine the *HLA-DRB1* association with ACPA⁺ RA in INA, we genotyped the largest cohort available, comprising 344 INA patients with RA and 352 controls. Rheumatoid factor (RF) and ACPA status was known in 241/344 patients with RA: 90% were seropositive (RF and/or ACPA⁺). In patients with seropositive RA, 32% carried *HLA-DRB1*14:02* and 45% *HLA-DRB1*04:04*. One-third of patients carrying *HLA-DRB1*14:02* carried an additional *SE* allele. In healthy controls (HCs), 28% carried *DRB1*14:02*, 22% *HLA-DRB1*04:04* and 17% carried an additional *SE* allele. After stratifying patients with RA according to HLA status, *HLA-DRB1*14:02* was a risk factor for seropositive RA (OR 2.38) independent of other *SE* alleles (table 1). In INA patients with RA, the most commonly associated other *SE* allele is *HLA-DRB1*04:04* (table 1). In INA, RA risk was associated with *HLA-DRB1* alleles with a conserved SE motif at 71 and 74 in all RA (OR=2.48, 95% CI 1.70 to 3.60, p<0.0001) and in seropositive patients with RA (OR=2.46, 95% CI 1.58 to 3.81, p = 0.0001) (table 1). We note that without genome-wide genotyping, we cannot rule out the possibility of confounding due to case-control differences in ancestry.

To stratify ACPA response with genotype, sera of 232 INA patients with RA were tested in a multiplex ACPA antigen array and given an ACPA score (sum of all normalised ACPA titres divided by number of epitopes).²³ ACPA score was higher in INA patients with RA who were either *HLA-DRB1*14:02* homozygotes or *HLA-DRB1*14:02/HLA-SE* compound heterozygotes than those who were *HLA-SE*-negative (p<0.05). ACPA scores in *HLA-DRB1*14:02*⁺ patients were equivalent to those in *HLA-SE*⁺ patients lacking *HLA-DRB1*14:02*. ACPA specificities increased among *HLA-DRB1*14:02*⁺ patients and included vimentin-64Cit_{58–77}, filaggrin-56Cit_{48–65} and fibrinogen- α -573Cit_{556–575} (figure 1A,B). Thus, despite polymorphisms of Val11 β Ser and His13 β Ser, INA individuals carrying *HLA-DRB1*14:02* develop a broad ACPA response whether or not they carry other *SE* alleles. This implies binding and presentation of a variety of Cit-autoantigens by *HLA-DRB1*14:02* to autoreactive T cells.

Accommodation of arginine and citrulline residues within the P4 pocket of HLA-DRB1*14:02

The HLA-DRB1 chain contains 12 polymorphic residues that have been directly implicated in peptide binding.²⁴ HLA-DRB1*14:02 differs from HLA-DRB1*04:01, HLA-DRB1*04:04 or HLA-DRB1*01:01 in eight of these residues, which shape P4, P6, P7 and P9 pockets (figure 2A). To test whether HLA-DRB1*14:02 presents autoantigens differently from HLA-DRB1*04:01, HLA-DRB1*04:04 or HLA-DRB1*01:01, we compared the capacity of each HLA binding pocket to accommodate Cit and Arg residues using the influenza-derived HA₃₀₅₋₃₁₉ peptide.²⁵⁻²⁸ Conversion of Arg to Cit at peptide positions interacting with P4 enhanced peptide binding affinity to HLA-DRB1*01:01 by twofold and to HLA-DRB1*04:01 by tenfold. Conversion of Arg to Cit at peptide positions interacting with the P6 and P9 pockets enhanced peptide binding affinity to HLA-DRB1*01:01, and P7 to HLA-DRB1*04:01. In contrast, peptides containing an Arg or Cit residue at positions interacting with P4 of HLA-DRB1*14:02 had similar binding affinity, and peptides containing Arg at positions interacting with P6 and P9 had increased affinity relative to Cit peptides (figure 2B). While the IC₅₀ of the self-peptide, vimentin-64Cit₅₉₋₇₁, was decreased by 1.3-fold relative to the 64-Arg variant for HLA-DRB1*01:01, and the 64-Arg variant did not bind HLA-DRB1*04:01, HLA-DRB1*14:02 bound both 64-Arg and 64-Cit variants with IC₅₀ of 19 μ M (figure 2C), indicating that both residues could be accommodated within P4 of HLA-DRB1*14:02, or that HLA-DRB1*14:02 presented in differing peptide binding registers.

Structural basis of peptide presentation by HLA-DRB1*14:02

We solved the structure of HLA-DRB1*14:02 in complex with vimentin-64Arg₅₉₋₇₁, vimentin-64Cit₅₉₋₇₁ and fibrinogen β 74Cit₆₉₋₈₁ (online supplementary table 3, figure 3A–D). These HLA-DRB1*14:02 structures overlaid closely (figure 3E), ruling out markedly differing binding modes to accommodate these differing epitopes. Within the HLA-DRB1*14:02-vimentin-64Cit₅₉₋₇₁ and HLA-DRB1*14:02-vimentin-64Arg₅₉₋₇₁ structures, Tyr, Ser and Arg occupied the P1, P6 and P9 pockets of HLA-DRB1*14:02, respectively (figure 3B,C). In the HLA-DRB1*14:02-fibrinogen β 74Cit₆₉₋₈₁ complex, Tyr, Ala and Ala occupied the P1, P6 and P9 pockets of HLA-DRB1*14:02, respectively (figure 3D). The largest structural differences between the peptides in each binary complex centred on the residue occupying the P4 pocket of HLA-DRB1*14:02, namely P4-Cit and P4-Arg in vimentin-64Cit₅₉₋₇₁, fibrinogen β 74Cit₆₉₋₈₁ and vimentin-64Arg₅₉₋₇₁, respectively. These structures clearly show that P4-Arg and P4-Cit are presented in alternative orientations, whereby the P4-Arg projects inwards, whereas the P4-Cit projects outwards from the HLA-DRB1*14:02 Ag-binding cleft (figure 3F–H). The P4-Arg is buried in the pocket to avoid interactions with the positively charged β 71Arg residue. This orientation is promoted by β 11Ser and β 13Ser, whereupon these two small polar residues allow the accommodation of Arg by providing the necessary space and H-bonding partners (figure 3F). Larger residues in HLA-DRB1*04:01 (β 11Val and β 13His) and HLA-DRB1*01:01 (β 11Leu and β 13Phe) as well as the charge repulsion of β 13His in HLA-DRB1*04:01 would prevent the accommodation of Arg at P4.¹² The P4-Arg residue is stabilised by H-bonds with Ser11 β , Ser13 β and Tyr30 β , and a salt bridge with Glu28 β (figure 3F). In contrast, the P4-Cit sits upright in both Cit-epitopes, similar to its orientation in P4-Cit from Cit epitopes presented

by HLA-DRB1*04:01/04:04 (figure 3G,H).¹² The P4-Cit is stabilised by H-bonds with Arg71 β and Gln70 β (figure 3G,H). Accordingly, we demonstrate a conserved positioning of the citrulline residue in two distinct epitopes that contrast the orientation of the non-Cit residue within the P4 pocket. Similar to its orientation in HLA-DRB1*04:01 and HLA-DRB1*04:04,¹²²⁹ P4-Cit was solvent-exposed in HLA-DRB1*14:02 and could potentially interact with TCR.

Antigen-experienced, oligoclonally expanded T cells recognise Arg and Cit variants of vimentin₅₉₋₇₁ presented by HLA-DRB1*14:02

Although both vimentin-64-Arg and 64-Cit variant peptides bound HLA-DRB1*14:02, only P4-Cit sits upright, and thereby potentially able to interact with the TCR. Thus, we addressed whether autoreactive T cells with TCRs recognising one or both epitopes were present in the periphery and displayed evidence of *in vivo* expansion in response to antigen presentation. We analysed T cells recognising HLA-DRB1*14:02-vimentin-64Cit₅₉₋₇₁ and HLA-DRB1*14:02-vimentin₅₉₋₇₁ tetramers in 10 HLA-DRB1*14:02⁺ INA patients with RA, 10 HLA-DRB1*14:02⁺ ACPA⁻ FDRs and 6 HLA-DRB1*14:02⁺ non-INA HC subjects. FDRs in the INA population have a high burden of environmental risk factors for RA, a high level of background HLA-DR SE genes, high inflammatory C-reactive protein (CRP) and a high prevalence of joint symptoms.¹⁶ Among the 13 FDRs studied (online supplementary table 3), 85% were past smokers and 31% had an abnormal CRP >8. Therefore we could compare T cells recognising vimentin or Cit-vimentin in individuals both with RA and with high risk of future RA. Similar frequencies of CD4⁺ T cells recognised vimentin 64-Arg and 64-Cit variant peptides in HLA-DRB1*14:02⁺ FDRs and patients with RA, and with similar tetramer staining intensity (mean fluorescence intensity (MFI)) (figure 4A,B). In all individuals, HLA-DRB1*14:02-vimentin-64Cit₅₉₋₇₁-reactive and HLA-DRB1*14:02-vimentin₅₉₋₇₁-reactive CD4⁺ T cells were significantly enriched in CD25⁺CD127⁺ effector (Teff) and CD25⁺ CD127⁻ regulatory T cells (Treg)³⁰ compared with the total CD4⁺ PB T cell pool (Treg $p < 0.0001$, Teff $p < 0.05$; figure 4C), consistent with antigen experience *in vivo*. This enrichment did not differ between patients with RA and FDRs, indicating that antigen experience and formation of memory develop before the onset of ACPA in INA. The numbers of circulating HLA-DRB1*14:02-vimentin-64Cit₅₉₋₇₁-reactive and HLA-DRB1*14:02-vimentin₅₉₋₇₁-reactive CD4⁺ T cells were correlated in each individual patient with RA and each FDR ($r^2 = 0.74$, $p < 0.0001$; figure 4D).

T cell effector function is balanced by the suppressive activity of Treg.³¹ The ratio of HLA-DRB1*14:02-vimentin₅₉₋₇₁Cit-64-reactive and HLA-DRB1*14:02-vimentin₅₉₋₇₁-reactive Teff/Treg was significantly lower than that of the total CD4⁺ T cell pool in patients with RA and FDRs ($p < 0.001$, figure 4E), consistent with active regulation of the autoreactive T cells. To understand the particular role of CD4⁺ T cells of each vimentin specificity further, we analysed an additional six HLA-DRB1*14:02⁺ INA individuals (three with RA, three FDRs; clinical details in online supplementary table 3) and six HLA-DRB1*14:02⁺ HC for markers of memory T cell activation and differentiation relative to total CD4⁺ T cells. In INA patients with RA and FDRs, the proportion of CD28⁺ memory T cells was significantly higher among vimentin₅₉₋₇₁-reactive CD4⁺ T cells ($p < 0.05$; figure 4F). Moreover, cells expressing CD69 were significantly enriched among vimentin₅₉₋₇₁-reactive CD4⁺ T cells in

these individuals. Some CD69⁺ vimentin₅₉₋₇₁-reactive memory CD4⁺ T cells also expressed NKG2D (figure 4G). In contrast, HC vimentin-64-Cit₅₉₋₇₁-reactive and vimentin₅₉₋₇₁-reactive CD4⁺ T cells did not differ in phenotype from total CD4⁺ T cells (online Supplementary figure 2). Thus, in INA FDRs and patients with RA but not HC, vimentin-64-Cit₅₉₋₇₁-reactive and vimentin₅₉₋₇₁-reactive CD4⁺ T cells reflect antigen-driven activation and differentiation.

TCR bias and oligoclonal TCR reactive with vimentin-64-Cit₅₉₋₇₁ and vimentin₅₉₋₇₁

To obtain evidence of in vivo expansion in HLA-DRB1*14:02⁺ INA, we used multiplex PCR to sequence the TCRs from a total of 53 single vimentin₅₉₋₇₁-reactive and 71 vimentin-64Cit₅₉₋₇₁-reactive tetramer-positive CD4⁺ T cells derived from five patients with HLA-DRB1*14:02⁺ RA and five HLA-DRB1*14:02⁺ FDRs. *TRAV* and *TRBV* gene usage among CD4⁺ T cells reactive to each vimentin epitope was generally diverse in RA and FDR (online supplementary table 4). However, *TRBV20-1* and *TRBV30* were preferentially used variable gene segments for recognition of vimentin 64-Arg and 64-Cit variant peptides bound to HLA-DRB1*14:02 (figure 4H). *TRAV13-2* and *TRAV26-1* were preferentially used variable gene segments among both vimentin₅₉₋₇₁-reactive and vimentin-64Cit₅₉₋₇₁-reactive TCRs. Use of preferential variable gene segments in the repertoires of T cells recognising each epitope was observed in both patients with RA and FDRs and suggested underlying oligoclonality of the autoreactive T cell populations when compared with the total CD3⁺ T cell population (figure 4H). Indeed, multiple vimentin-64Cit₅₉₋₇₁-reactive and vimentin₅₉₋₇₁-reactive CD4⁺ T cells bearing the same CDR3 α and/or CDR3 β sequences were identified among the single cells sorted from two of the patients with RA and two FDRs (table 2). In two FDRs, CD4⁺ T cells bearing the same TRBV30 CDR3 sequences were identified multiple times among single cells reactive for vimentin 64-Arg and 64-Cit variant peptides bound to HLA-DRB1*14:02. In one patient with RA, the same TRBV2 CDR3 sequences were identified multiple times among single vimentin-64Cit₅₉₋₇₁-reactive CD4⁺ T cells, and in another patient the same TRBV10-3 CDR3 sequences were identified multiple times among single vimentin₅₉₋₇₁-reactive CD4⁺ T cells. These repeated CDR3 α and CDR3 β sequences indicate antigen-reactive clonal expansion within the blood of these HLA-DRB1*14:02⁺ patients and at-risk FDRs (table 2). In all cases, clonally expanded tetramer⁺ T cells were CD4⁺CD25⁺CD127⁺ Teff, as determined by index sorting. Individuals in whom any oligoclonal sequences were detected in peripheral blood (PB) were more likely to be *HLA-DRB1*14:02* homozygous or *HLA-DRB1*14:02/*04:04* compound heterozygous than individuals without oligoclonal expansion ($p < 0.05$, X^2 test). Remarkably, a common TRBV30 CDR3 sequence (SI/VGAGNQPQ) was expanded in the blood of two individual FDRs, which in each case encoded TCRs recognising vimentin₅₉₋₇₁ as well as vimentin-64Cit₅₉₋₇₁. Of 34 sorted antigen-reactive T cells yielding productive TRBV gene sequences from FDRs, 23.5% contained this CDR3 β sequence. We used retroviral vectors encoding HLA-DR14:02-vimentin₅₉₋₇₁-restricted TCR P2F3 (online supplementary table 4, bold) to transduce the $\alpha\beta$ TCR-deficient SKW-3 cell line.³²³³ When stimulated with HLA-DRB1*14:02-vimentin₅₉₋₇₁ or vimentin-64Cit₅₉₋₇₁ tetramers, P2F3 SKW-3 cells upregulated phospho- extracellular signal-related kinases (pERK) relative to HLA-DRB1*14:02-CLIP tetramers (figure 4I), confirming that TCR identified from cells with

HLA-DRB1*14:02-vimentin₅₉₋₇₁ tetramer reactivity recognises vimentin₅₉₋₇₁ and vimentin-64Cit₅₉₋₇₁ in the context of HLA-DRB1*14:02.

The biased TCR usage suggests a structural requirement for conserved amino acid sequences to recognise vimentin₅₉₋₇₁ and vimentin-64Cit₅₉₋₇₁. Nucleotide sequences encoding TRBV30 CDR3 reveal that although the second N region at the D–J junction in each TCR is different, they encode the same amino acid sequence (table 2). These data implicate convergent recombination events in the selection of this sequence during TCR gene rearrangement.³⁴

DISCUSSION

*HLA-DRB1*14:02* and *HLA-DRB1*04:04* are shown to be independent risk alleles for ACPA⁺ RA in the INA population. Analysis of the structures and T cell responses to Cit and non-Cit epitopes shows that, consequent to the His13βSer polymorphism and altered P4 pocket, HLA-DRB1*14:02 can present both variant peptides. This contrasts with structures of HLA-DRB1*04:01 and *04:04, in which Cit but not Arg can be accommodated in P4.¹²²⁹ Presentation of both 64-Cit and 64-Arg vimentin₅₉₋₇₁ variants promoted autoreactive CD4⁺ T cell activation and differentiation to Teff and Treg, and clonal expansion of Teff in patients with *HLA-DRB1*14:02*⁺ RA and at-risk FDRs. In *HLA-DRB1*14:02*⁺ HC, we observed no activation or differentiation of antigen-specific T cells above the background total CD4⁺ T cells. Previous studies of CD4⁺ T cells in individuals carrying Caucasian SE alleles show that T cell responses to Cit-autoantigenic peptides are increased relative to Arg-variant peptides,¹²²⁹³⁵³⁶ reinforcing that T cell function aligns with HLA-DR-peptide structure.

We show preferential variable gene segments and clonally expanded TCR among vimentin₅₉₋₇₁-reactive and vimentin-64Cit₅₉₋₇₁-reactive CD4⁺CD25⁺CD127⁺ Teff sorted from patients with RA and FDRs, including a public TRBV CDR3. These data suggest that presentation of vimentin self-epitopes in vivo continues in genetically predisposed individuals before and after onset of RA, and selects T cells making productive TCR rearrangements, as identified by the nucleotide sequences, for antigen recognition and T cell expansion.

Although limited by small sample numbers, the phenotypic profiles of 64-Cit and 64-Arg variant-specific autoreactive T cells appeared to be different. The fibroblast antigen, vimentin, has widespread tissue expression.³⁷ Vimentin 64-Arg-specific memory CD4⁺ T cells specifically expressed CD69. CD69 is a marker of recent activation or tissue residency and exposure to cytokines such as tumour necrosis factor (TNF),³⁰ suggesting that vimentin₅₉₋₇₁-reactive T cells are activated in tissue inflammatory sites. NKG2D signifies Teff costimulatory function and is TNF-activated.³¹ Intriguingly, our data suggest cross-reactivity of some TCRs for the 64-Cit and 64-Arg variants: cell frequency of each specificity was correlated, the public clonotype occurred in T cells reactive with each variant, and T cells expressing TCR P2F3 were activated with both tetramers. In general, coexpansion of T cells recognising Cit and Arg variants might be advantageous, for example, for cross-protection against infectious antigens requiring rapid immunity.^{38–40} This suggests a hypothesis for persistence of *HLA-DRB1*14:02* in the INA population, even

though its molecular structure permits presentation of multiple self-antigens driving ACPA⁺ RA.

Low-titre ACPA develops in healthy individuals independent of HLA-DR SE, particularly in inflammatory contexts.^{6–81011} In HLA-DR SE⁺ individuals, memory T cells driven by antigen experience would provide required B cell help for increased titres and epitope spreading in patients developing RA.⁸⁴¹⁴² Since HLA-DRB1*14:02 broadens capacity for autoantigen presentation and T cell expansion, our study provides a mechanism for enhanced risk of early onset of ACPA⁺ RA in INA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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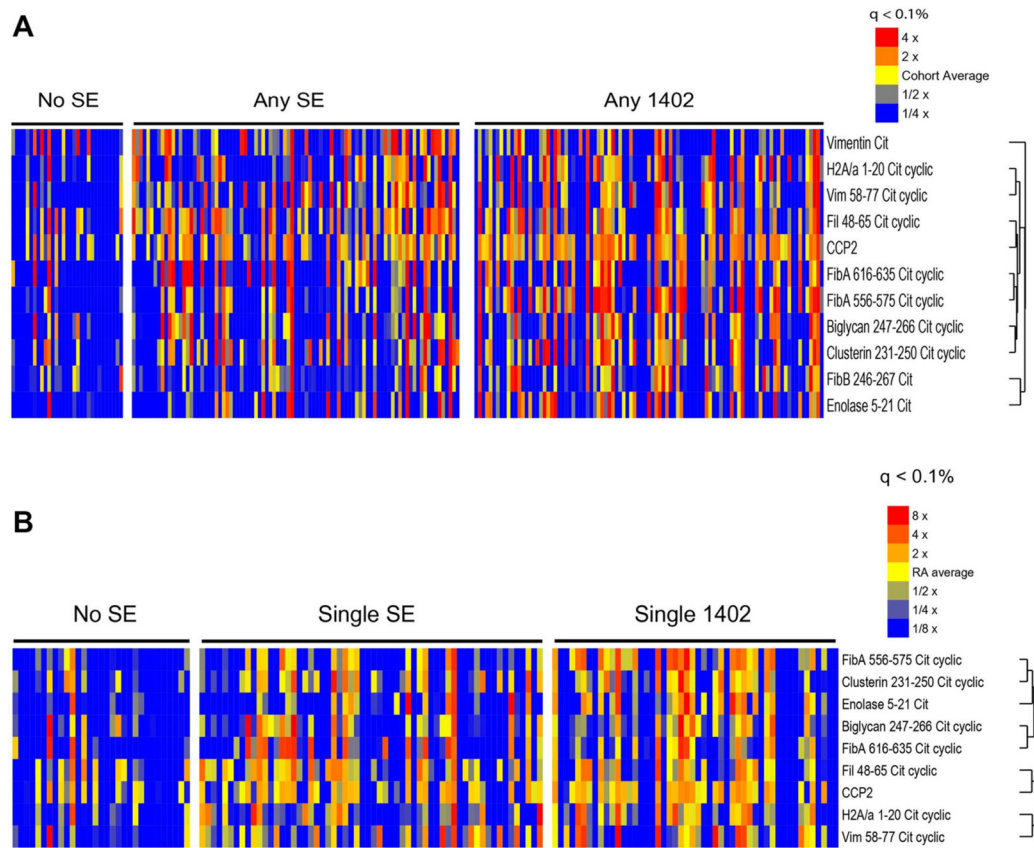


Figure 1. HLA-DRB1*14:02 is associated with a broad ACPA response. Serum levels of antibodies targeting RA-associated Cit-autoantigens were measured in serum from 232 INA patients with RA using a custom bead-based fluorescence immunoassay. Fluorescence intensities for 10 Cit-autoantigens and CCP were clustered (A) according to the presence or absence of 1 or 2 HLA-DR-SE alleles or HLA-DRB1*14:02 with any HLA-DR-SE allele, and (B) according to presence or absence of a single HLA-DR-SE or HLA-DRB1*14:02 allele, as shown. Each column represents one patient sample. ACPA, anticitrullinated peptide antibody; CCP, cyclic citrullinated peptide; Cit, citrullinated; RA, rheumatoid arthritis; SE, susceptibility epitope.

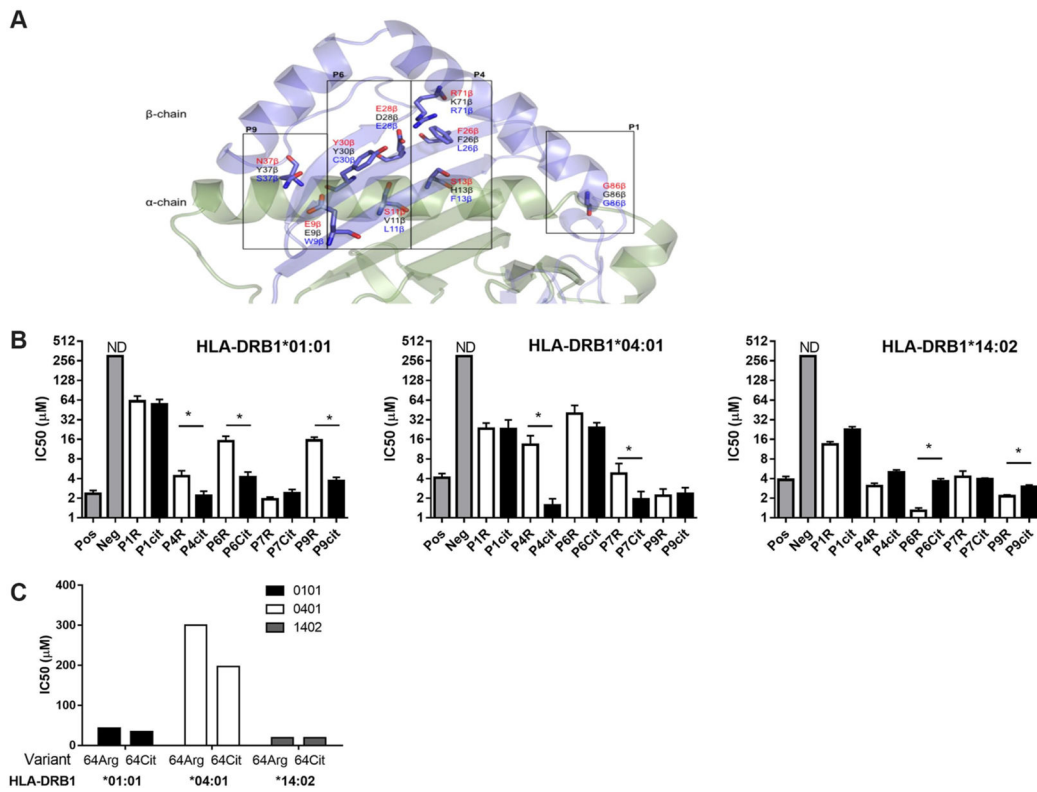


Figure 2.

Accommodation of arginine and citrulline residues by HLA-DRB1*01:01, HLA-DRB1*04:01 and HLA-DRB1*14:02. (A) The peptide binding groove of an HLA-DR molecule is shown in cartoon with polymorphic residues shown as sticks corresponding to the residues present in HLA-DRB1*14:02, which is associated with RA among the INA. Schematic representation of the differences in pockets between HLA-DRB1*14:02 (red font), HLA-DRB1*04:01 (black font) and HLA-DRB1*01:01 (blue font). (B) Competitive binding of a biotin-labelled HA₃₀₅₋₃₁₉ peptide with an unlabelled HA₃₀₅₋₃₁₉ peptide or HA₃₀₅₋₃₁₉ variants with citrulline or arginine residues in P1, P4, P6, P7 and P9 to HLA-DRB1*01:01, HLA-DRB1*04:01 and HLA-DRB1*14:02. ND, non-detectable binding affinity. Pooled binding data from at least three experiments are shown and error bars depict the variation between experiments. (C) Competitive binding of a biotin-labelled HA₃₀₅₋₃₁₉ peptide with unlabelled vimentin₅₉₋₇₁ and vimentin-64Cit₅₉₋₇₁ to HLA-DRB1*01:01, HLA-DRB1*04:01 and HLA-DRB1*14:02. INA, Indigenous North American; RA, rheumatoid arthritis.

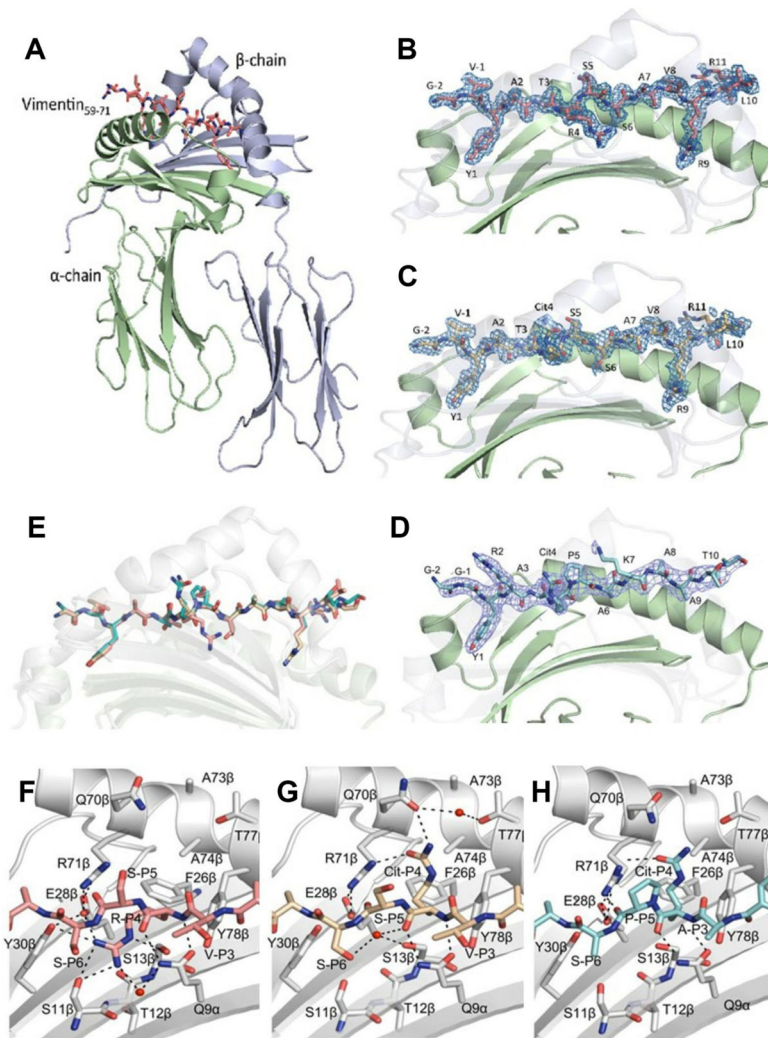


Figure 3. Crystal structure of HLA-DRB1*1402 in complex with vimentin₅₉₋₇₁, vimentin-64Cit₅₉₋₇₁ and fibrinogen β 74Cit₆₉₋₈₁. The α -chains and β -chains of (A) the HLA-DRB1*1402 in complex with vimentin₅₉₋₇₁ are shown in cartoon representation and coloured in light green and light blue, respectively, while peptide is displayed as sticks. Side view of (B) HLA-DRB1*1402-vimentin₅₉₋₇₁, (C) HLA-DRB1*1402-vimentin-64Cit₅₉₋₇₁ and (D) HLA-DRB1*1402-fibrinogen β 74Cit₆₉₋₈₁. The carbons are coloured deep salmon, light orange and teal for vimentin₅₉₋₇₁, vimentin-64Cit₅₉₋₇₁ and fibrinogen β 74Cit₆₉₋₈₁, respectively; nitrogens are coloured in blue and oxygens are coloured in red. The β -chain is transparent to help visualise the peptide. The unbiased 2Fo-Fc electron density map of the peptides is shown in blue and contoured to 1 σ . (E) Superposition of the three peptides bound to HLA-DRB1*1402. The P4 pocket of HLA-DRB1*1402 bound to (F) vimentin₅₉₋₇₁, (G) vimentin-64Cit₅₉₋₇₁ and (H) fibrinogen β 74Cit₆₉₋₈₁. The P4-Arg in the vimentin₅₉₋₇₁ peptide is buried in the P4 pocket. The P4-Cit of the vimentin-64Cit₅₉₋₇₁ and fibrinogen β 74Cit₆₉₋₈₁ peptide adopts an upright conformation.

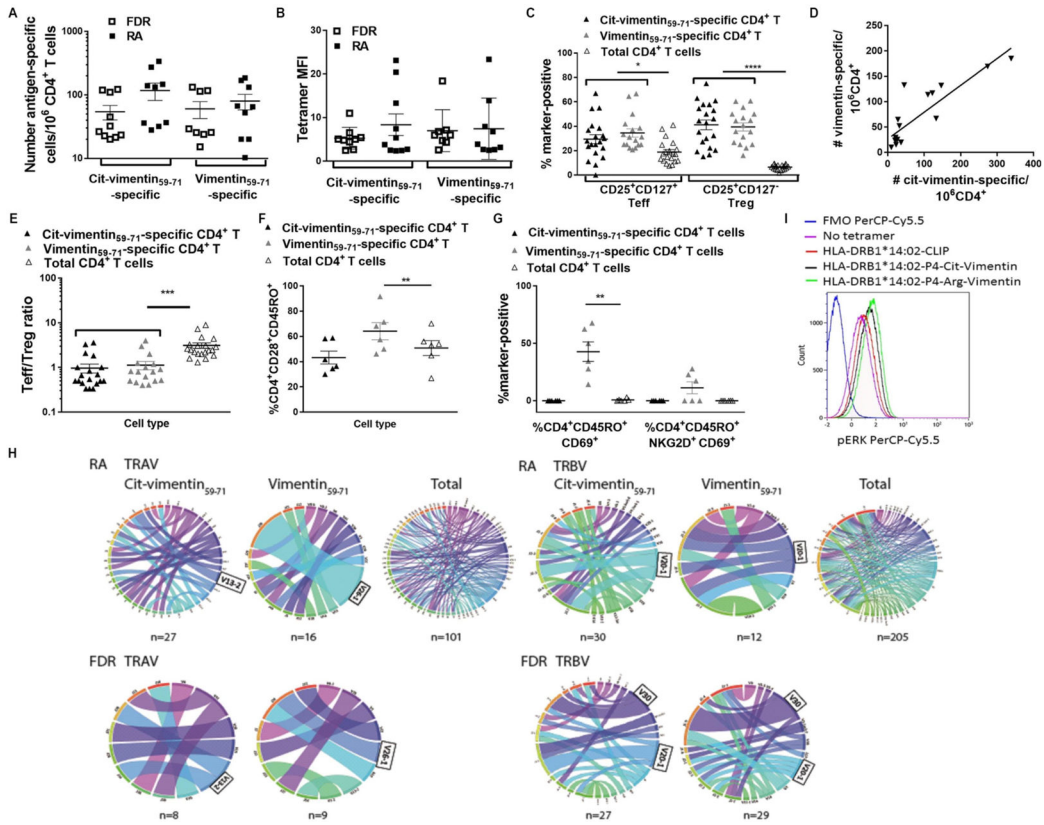


Figure 4. Presentation of vimentin₅₉₋₇₁ and vimentin-64Cit₅₉₋₇₁ self-antigens in context of HLA-DRB1*14:02 to CD4⁺ T cells in individuals with and at high risk of ACPA⁺ RA. (A–D) PBMC from HLA-DRB1*14:02⁺ RA patients W1:1–10 and HLA-DRB1*14:02⁺ FDR W1:1–10 were stained with PE-labelled HLA-DRB1*1402-vimentin64Cit₅₉₋₇₁ or HLADRB1*14:02-vimentin₅₉₋₇₁ tetramer, CD4-APC, CD25-PE/Cyt, and CD127-BV421. Live CD4⁺ FITC-lineage-negative tetramer⁺ T cells were gated based on an FMO sample stained with unlabelled primary and conjugated secondary antibodies. The number calculated relative to the CD4⁺ T cell count, (A) and mean fluorescence intensity (MFI) (B) of tetramer⁺ cells within the total CD4⁺ population is shown for RA patients and FDR; (C) the proportion of CD4⁺CD25⁺CD127⁺ Teff and CD4⁺CD25⁺CD127⁻ Treg within vimentin64Cit₅₉₋₇₁-reactive and vimentin₅₉₋₇₁-reactive CD4⁺ T cell gates and the total CD4⁺ gate is shown for pooled RA patients and FDR. (D) The numbers of vimentin64Cit₅₉₋₇₁-reactive and vimentin₅₉₋₇₁-reactive CD4⁺ T cells were correlated in pooled RA patients and FDR (R^2 0.74, $p < 0.0001$). (E) The Teff/Treg ratio of vimentin64Cit₅₉₋₇₁ and vimentin₅₉₋₇₁-reactive CD4⁺ T cells is plotted relative to total CD4⁺ T cell ratio in pooled RA patients and FDR; (F) CD45RO⁺CD28⁺, (G) CD45RO⁺CD69⁺ and CD45RO⁺CD69⁺ NKG2D⁺ vimentin₅₉₋₇₁-reactive CD4⁺, vimentin64Cit₅₉₋₇₁-reactive CD4⁺ and total CD4⁺ T cells are plotted in pooled RA patients W2:1–3 and FDR W2:1–3. (H) Frequencies of V-J pairing for TCR α and TCR β among vimentin64Cit₅₉₋₇₁-specific and vimentin₅₉₋₇₁-specific CD4⁺ T cells from HLADRB1*14:02⁺ RA patients (n=5) and FDR (n=5), and tetramer-negative CD3⁺ T cells sorted from DRB1*14:02⁺ RA patients (n=3).

The width of the bands is proportional to the frequency of TCR sequences with a particular V-J pairing. Details of productive TCR α and TCR β sequences in online supplementary table 2 and 4. The figures were generated using the Circos online Table Viewer software (<http://mkweb.bcgsc.ca/tableviewer/>). (I) 10^6 P2F3 SKW3 cells were stimulated with 5 ng/mL unlabelled HLA-DRB1*14:02-vimentin₅₉₋₇₁ or HLA-DRB1*14:02-vimentin-64Cit₅₉₋₇₁ tetramer for 4 hours at 37°C. Cells were fixed and permeabilised, then stained with PerCP/Cy5.5-ERK1/2 and PB-CD3. Live CD3+ cells expressing the GFP marker gene were gated. Representative of 2 experiments.

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Table 1

Association of HLA-DRB1*14:02 with all RA and seropositive RA in INA patients with RA and controls

	HC, n (%)	All RA, n (%)	OR (CI)	p	Seropositive RA, n (%)	OR (CI)	p
SE ⁻	106 (30)	51 (15)	Ref		32 (15)	Ref	
14:02/Other SE ⁻	64 (18)	80 (23)	2.60 (1.63 to 4.15)	0.0001	46 (21)	2.38 (1.38 to 4.12)	0.0019
14:02-/Other SE ⁺	147 (42)	178 (52)	2.52 (1.69 to 3.75)	<0.0001	117 (54)	2.64 (1.66 to 4.19)	<0.0001
14:02-/Other SE ⁺	35 (10)	35 (10)	2.08 (1.17 to 3.70)	0.0127	23 (11)	2.18 (1.13 to 4.20)	0.02
Any SE ⁺	246 (70)	293 (85)	2.48 (1.70 to 3.60)	<0.0001	186 (85)	2.46 (1.58 to 3.81)	0.0001

Patients and controls were stratified according to the presence of SE. SE-positive individuals were further stratified as shown. Other SE alleles included DRB1*01:01, DRB1*01:02, DRB1*04:01, DRB1*04:04, DRB1*04:05, DRB1*04:08, DRB1*04:10, DRB1*04:13 and DRB1*10:01.

HC, healthy controls; INA, Indigenous North American; RA, rheumatoid arthritis; SE, susceptibility epitope.

Repeated TCR TRAV, TRBV and TRAV/TRBV clonotypes used by HLA-DRB1*14:02-restricted vimentin₅₉₋₇₁ or vimentin-64Cit₅₉₋₇₁-reactive CD4⁺T cells

Table 2

Subject	Tetramer	TRAV	CDR3a	TRAJ	Frequency number/Total, (%)	HLA-DRB1			
RA W1:1	Cit-vimentin	13-2	SQPGTAL	15	2/17 (11.8%)	14:02, 14:02			
RA W1:2	Vimentin	26-1	SGAGSYQL	28	5/13 (38%)	04:04, 14:02			
Subject	Tetramer	TRBV	CDR3β	TRBJ	Frequency* (%)				
RA W1:1	Cit-vimentin	2	SEAADNEQ	2-1	2/14 (14.2%)	14:02, 14:02			
RA W1:2	Vimentin	10-3	GGTRTESSYEQ	2-7	2/5 (40%)	04:04, 14:02			
FDR W1:3*	Cit-vimentin	30	SIGAGNQPQ	1-5	1/19 (5.2%)	04:04, 14:02			
FDR W1:3*	Vimentin	30	SIGAGNQPQ	1-5	3/7 (42.8%)				
FDR W1:5 [†]	Cit-vimentin	30	SVGAGNQPQ	1-5	2/2 (100%)	13:02, 14:02			
FDR W1:5 [†]	Vimentin	30	SVGAGNQPQ	1-5	2/6 (33%)				
Subject	Tetramer	TRAV/TRBV	CDR3a/CDR3β	TRAJ/TRBJ	Frequency* (%)				
RA W1:1	Cit-vimentin	13-2/2	SQPGTAL/SEAADNEQ	15/2-1	2/12 (16.6%)	14:02, 14:02			
Subject	TRBV								
FDR W1:3 [†]	S	I	G	A	G	N	Q	P	Q
	agt	atc	ggg	ggc	ggc	aat	cag	ccc	cag
FDR W1:5 [†]	S	V	G	A	G	N	Q	P	Q
	agt	gtg	ggg	gca	ggc	aat	cag	ccc	cag

TCR repertoire analysis was undertaken in RA patients W1:1-5 and FDR W1:1-5. Productive single TRAV and TRBV clonotypes detected from two patients with RA and three FDRs are shown.

* Frequency reflects the frequency of the repeated TRAV or TRBV clonotype divided by the total number of tetramer[†] cells with productive TRAV or TRBV sequence, for each individual.

[†] Nucleotide sequences encoding each of the public CDR3β amino acid sequences, which require a minimal number of N or P additions to be produced. Nucleotides attributed by the germline Vβ, Dβ and Jβ genes are shown in blue, red and green, respectively. N-additions are in black and P-additions in purple text. The nucleotides at the D-J junction encoding the same amino acid are underlined in each case.

FDR, first-degree relatives; RA, rheumatoid arthritis.