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Key interactions in the trimolecular complex consisting of the rheumatoid arthritis-associated DRB1*04:01 molecule, the major glycosylated collagen II peptide and the T-cell receptor

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ABSTRACT

Objectives Rheumatoid arthritis (RA) is an autoimmune disease strongly associated with the major histocompatibility complex (MHC) class II allele DRB1*04:01, which encodes a protein that binds self-peptides for presentation to T cells. This study characterises the autoantigen-presenting function of DRB1*04:01 (HLA-DRA*01:01/HLA-DRB1*04:01) at a molecular level for prototypic T-cell determinants, focusing on a post-translationally modified collagen type II (Col2)-derived peptide.

Methods The crystal structures of DRB1*04:01 molecules in complex with the peptides $HSP70_{289-306}$, citrullinated $CILP_{982-996}$ and galactosylated $Col2_{259-273}$ were determined on cocrystallisation. T cells specific for $Col2_{259-273}$ were investigated in peripheral blood mononuclear cells from patients with DRB1*04:01-positive RA by cytofluorometric detection of the activation marker CD154 on peptide stimulation and binding of fluorescent DRB1*0401/$Col2_{259-273}$ tetramer complexes. The cDNAs encoding the T-cell receptor (TCR) α-chains and β-chains were cloned from single-cell sorted tetramer-positive T cells and transferred via a lentiviral vector into TCR-deficient Jurkat 76 cells.

Results The crystal structures identified peptide binding to DRB1*04:01 and potential side chain exposure to T cells. The main TCR recognition sites in $Col2_{259-273}$ were lysine residues that can be galactosylated. RA T-cell responses to DRB1*04:01-presented $Col2_{259-273}$ were dependent on peptide galactosylation at lysine 264. Dynamic molecular modelling of a functionally characterised $Col2_{259-273}$-specific TCR complexed with DRB1*04:01/$Col2_{259-273}$ provided evidence for differential allosteric T-cell recognition of glycosylated lysine 264.

Conclusions The MHC-peptide-TCR interactions elucidated in our study provide new molecular insights into recognition of a post-translationally modified RA T-cell determinant with a known dominant role in arthritogenic and tolerogenic responses in murine Col2-induced arthritis.

Key messages

What is already known about this subject?

- Rheumatoid arthritis (RA) is closely associated with HLA-DRB1*04:01-encoded major histocompatibility complex II molecules.
- DRB1*04:01-restricted $CD4^+$ T-cell responses to citrullinated autoantigens and post-translationally modified collagen II (Col2) have been described.

What does this study add?

- This study contributes new crystal structure information that reveals the DRB1*04:01 function in presenting post-translationally modified antigenic determinants to T cells in patients with RA.
- Through comparative analysis of RA T cells, T cell receptor (TCR) cloning and molecular modelling of a prototypic trimolecular complex, we gained insights into TCR recognition of unmodified and glycosylated Col2 in a DRB1*04:01 context.

How might this impact on clinical practice or future developments?

- Our findings contribute to a better understanding of the role of posttranslational Col2 modification for TCR recognition in $CD4^+$ T cells and has potential implications for induction of tolerance and onset of pathogenic autoimmunity.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease targeting diarthrodial cartilaginous joints. The disease is believed to be initiated by the development of autoantibodies to various altered self-antigens, predominantly modified by citrullination for yet unknown reasons,[1–4] followed years later by onset of joint inflammation and spreading of autoimmune responses to new structures, including cartilage proteins.[5–7] The strong association of RA with major histocompatibility complex class II (MHCII) suggests activation of T cells and a maturation of an autoreactive B cell response, capable of orchestrating the immune attack on the joints.[8] The origin of the T-cell activation is unknown but is likely to be dependent on presentation of antigenic peptides bound to the MHCII molecules. These peptides

could have been derived from non-self-proteins, for example, from infectious agents giving help to autoreactive B cells, or could be modified self-peptides that have escaped tolerance selection.

The MHCII association with RA has been mapped to the DRB1 locus,[9 10] which encodes a DR β-chain (DRB) and forms a peptide-binding receptor together with an invariant α-chain (DRA).[11] According to a popular hypothesis, alleles of the highly polymorphic DRB1 locus associated with RA encode a shared peptide binding pocket for a selected set of self-peptides, thereby predisposing individuals to pathogenic T-cell activation.[12 13] Most of the RA-associated DRB1 molecules have positively charged amino acids at position 71, favouring interactions with peptides that contain a negatively charged amino acid at the P4 position. Although a favoured binding of peptides with citrulline at this position (in contrast to a positively charged arginine) has been proposed,[13 14] this hypothesis could not be confirmed in studies on larger sets of peptides.[15 16]

A potentially relevant self-antigen in arthritis pathogenesis is type II collagen (Col2) due to its abundance in cartilage and proven role as an arthritogenic immunogen in experimental arthritis.[17] Moreover, antibodies to native and citrullinated Col2[18–22] and Col2-specific T cells[18] are detectable in patients with RA. Interestingly, RA T-cell responses directed to the dominant $Col2_{259\text{-}273}$ peptide were restricted by the RA-associated DR alleles 0401 and 0101, which were demonstrated to confer susceptibility to collagen-induced arthritis on transgenic expression in mice.[23–25] However, it is crucial to consider that the major $Col2_{259\text{-}273}$ peptide can be both hydroxylated and galactosylated at lysine residues and that RA T cells predominantly recognise the galactosylated form.[18] Initial insight into the positioning of the galactosylated (gal) $Col2_{259\text{-}273}$ peptide ($galCol2_{259\text{-}273}$) in the binding pocket of the DRB1*04:01 molecule was provided by molecular modelling.[26 27] The same target peptide and its post-translational modification (PTM) is also recognised by arthritogenic T cells in mice, provided that they express the natural murine antigen MHCII allele or transgenic human DRB1*04:01 or DRB1*01:01 molecules.[18 23–25] Critical differences in central tolerance induction of T cells based on their specificity for unmodified ($nCol2_{259\text{-}273}$) or galactosylated $Col2_{259\text{-}273}$ ($galCol2_{259\text{-}273}$)[28] and in the tolerogenic potential of Col2 peptide vaccination against experimental arthritis have been observed in mice.[29]

To further elucidate the role of PTM in Col2 recognition by human T-cell receptors (TCR), we performed a comparative study of $Col2_{259\text{-}273}$ recognition in either galactosylated or unmodified form using binding of tetramerised recombinant DRB1*04:01/Col2-peptide complexes and analysed Col2 peptide-induced T-cell activation in peripheral blood mononuclear cells (PBMCs) from patients with DRB1*04:01-positive RA. Moreover, we solved the X-ray crystallographic structure of DRB1*04:01(HLA-DRA1*01:01/HLA-DRB1*04:01) complexed with $galCol2_{259\text{-}273}$ to gain the first molecular insights into the structural basis of PTM-dependent differential Col2 recognition. Together with sequence information obtained from a cloned human galCol2-specific TCR, these crystal structures allowed us to perform molecular modelling on the trimolecular MHCII/peptide/TCR complex.

MATERIALS AND METHODS

See online supplemental material.

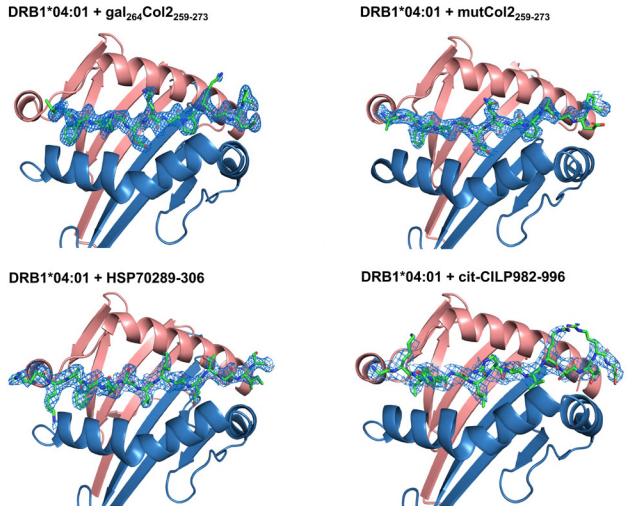

Figure 1 Top view of the DRAxDRB1*04:01 molecule binding peptides. The DRA (alpha chain) is shown in pink and the DRB (beta-chain) shown in blue. The $2F_o$-F_c electron density map of each peptide is shown in blue mesh contoured at 1 σ.

RESULTS

The molecular complex of DRB1*04:01 with bound peptides

The investigated peptides $nCol2_{259\text{-}273}$(GIAGFKGEQGPKGET),[18] heat shock protein (HSP) $HSP70_{289\text{-}306}$(TRKPFQSVIADTGISV) and citrullinated (cit) cartilage-intermediate protein (CILP) $citCILP_{982\text{-}996}$ (GKLYGI[Cit]DV[Cit]STRDR) represent autoantigenic determinants recognised by T cells in RA.[30 31] To establish the structural basis of their autoimmune recognition, we solved the crystal structures of DRB1*04:01 complexed with $Col2_{259\text{-}273}$, mutated $Col2_{259\text{-}273}$ containing alanine replacements at 264 (K264A, at P2) and 270 (K270A, at P8), $HSP70_{289\text{-}306}$ and the $CILP_{982\text{-}996}$ peptide citrullinated at positions 988 and 991 (figures 1 and 2, and online supplemental figure S1). All investigated T cell determinants, either unmodified or altered by citrullination or galactosylation, were bound in a conserved linear and extended conformation located in the classic binding groove of DRB1*04:01, thereby closely resembling previously solved DRB1*04:01 structures.[32]

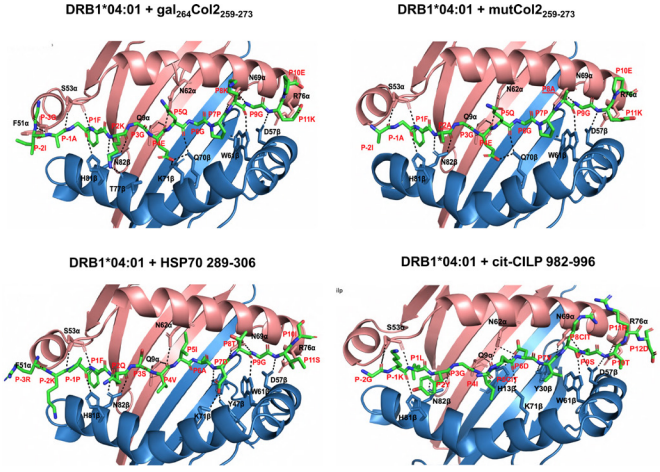

Figure 2 Interaction of peptides with the DRAxDRB1*04:01 molecule. Hydrogen bonds are indicated by dashed black lines. Peptide residues are numbered in accordance with the numbering of the binding pockets. The residues from both α and β chains important for contacts with the peptide are represented as sticks.

The crystal structure of $Col2_{259-273}$-bound DRB1*04:01 was determined at 1.9 Å resolution (online supplemental table S1). A weakly bound mutated human CLIP peptide (PVSKARMATGALAQA) occupying the DRB1*04:01 binding groove was exchanged with a synthetic glycosylated $Col2_{259-273}$ (mono-[β-D-galactopyranosyl]-moiety at a lysine [K264] side chain) prior to protein crystallisation. However, we did not observe electron density for the galactose moiety electron density in the structure, most likely because it is mobile at its solvent-exposed position on the protein surface. As expected, the peptide occupied the P1, P4, P6, P7 and P9 pockets with P1-Phe, P4-Glu, P6-Gly, P7-Pro and P9-Gly, respectively, whereas the potential TCR contact residues are P2-Lys, P5-Gln and P8-Lys. We also determined DRB1*04:01 in complex with covalently attached mutated $Col2_{259-273}$ (online supplemental table S1, figures 1 and 2, online supplemental figure S1 and figure S2). To investigate a possible influence on peptide binding by the two potential TCR contact residues P2-Lys and P8-Lys, we mutated them to Ala. We have previously found that the mutation of P2 from Lys to Ala slightly decreased the affinity of the peptide with DRB1*04:01, but also abolished T cell reactivity.[25] As can be seen from the structure, the mutated peptide mimics the conformation and location of the wildtype peptide (online supplemental figure S2), but its P4-Glu did not engage with Lys71β, thus explaining the reduced affinity.

The $HSP70_{289-306}$ peptide binds in a linear, extended manner with P1-Phe, P4-Val, P6-Ala and P9-Gly occupying the P1, P4, P6 and P9 pockets of DRB1*04:01, respectively, whereas P2-Gln, P5-Ile, P7-Asp, P8-Thr and P10-Ile represented potential TCR contact sites. The Gln70β within the shared epitope motif does not contact P6-Ala as seen in DRB1*04:01 where Gln70β hydrogen bonds to both P4E and P6G of $Col2_{259-273}$. In contrast, P7-D is bound by both Lys71β and Tyr47β.

As expected, the $citCILP_{982-996}$ peptide also binds in a linear, extended manner with P1-L, P4-I, P6-D and P9-S interacting with the DR molecules. Thus, the citrulline does not bind to the P4 pocket but instead is likely to face the TCR.

An overall comparison of the binding sites confirmed that the strongest DR binding site had a hydrophobic amino acid in the P1 position, whereas a considerable degree of flexibility was allowed at other positions. An important DR binding site is P4, in which an acidic side chain (glutamic acid) is favoured as it interacts with basic amino acids at position 71 in the beta chain, in line with the known association with RA. In contrast, the citrulline side chains of the CILP peptide did not bind within the P4 pocket. The TCR recognition sites for the Col2 peptide were the lysines at P2 and P10 as well as the glutamine at P5.

Detection of T lymphocytes specific for $Col2_{259-273}$

DRB1*04:01 allele carriers (patients with RA and healthy donors) were investigated for the presence of $Col2_{259-273}$ peptide (nCol2 or $gal_{264}Col2$)-specific $CD4^+$ T cells in peripheral blood by flow cytometric analysis of tetramer-stained PBMCs. A representative result in figure 3A shows the detection of Col2 epitope-specific cells that stain double-positive for two identical but differently fluorescence-labelled DRB1*04:01/Col2 tetramer complexes. The frequency of double-positive cells was 0.030%

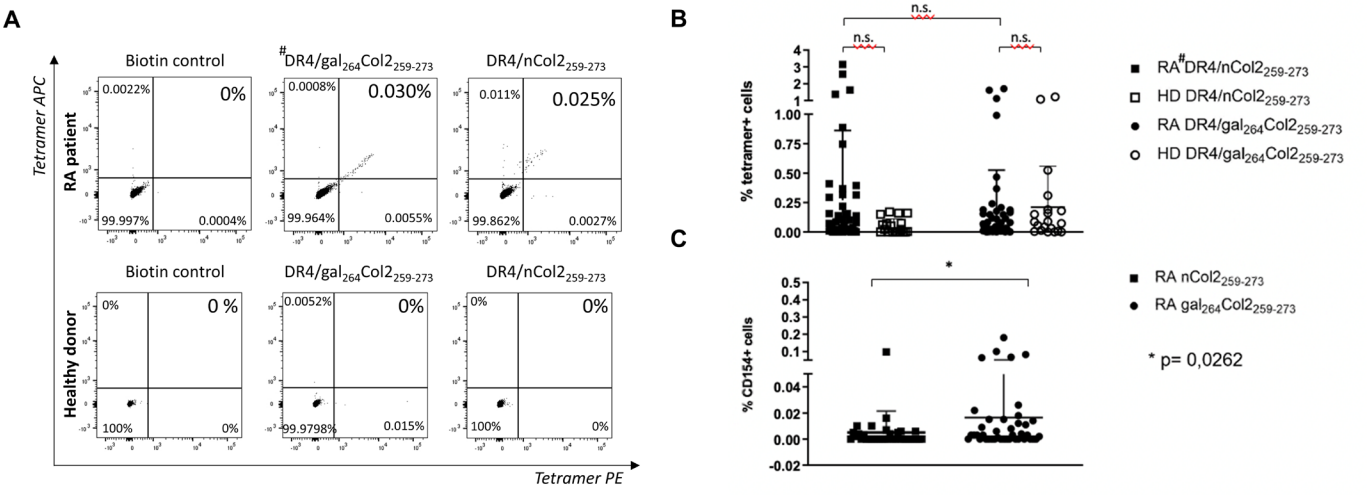

Figure 3 Detection of human antigen-specific T cells in the peripheral blood of HLA-DRB1*04:01 carriers by flow cytometry using #DR4 (HLADRB1*04:01)-tetramers with specificity for the $gal_{264}Col2_{259-273}$ or $nCol2_{259-273}$ peptide. (A) Representative dot blots: the $CD4^+$ enriched T cells from PBMCs were stained with a dead/live marker and DRB1*04:01/Col2 peptide tetramers conjugated with two different fluorophores (PE and APC). Subsequent flowcytometric analysis reveals the Col2-specific cells in the live double positive stained subpopulation. Representative dot blots of specific DR4/gal_{264}Col2 and DR4/nCol2 tetramer binding to T helper cells in samples from a patient with RA and a HD are shown. Biotin-streptavidin complexes without a specific peptide conjugate served as a negative control. (B) Specific tetramer binding of T helper cells from patients with RA (n=55) and HD (n=20) using #DR4 (DRB1*04:01)-tetramers with different peptide specificity (gal_{264}Col2 vs nCol2). In PBMCs of patients with RA, the frequencies of DR4/nCol2 and DR4/gal_{264}Col2 staining CD4+T cells do not differ significantly (n.s.). Depicted values represent processed data in which for each tetramer staining datapoint the respective biotin background has already been subtracted from the raw value. (C) Detection of antigen-specific T cells in PBMCs of patients with RA (HLA-DRB1*04:01) on in vitro stimulation by synthetic Col2 peptides using flow cytometry. PBMCs from patients with RA were stimulated with a Col2 peptide (gal_{264}Col2, nCol2) and anti-CD40 for 7 hours. Subsequently, peptide-induced upregulation of the activation marker CD154 on the surface of the live CD4+T cell population was detected by flow cytometry. The frequency of CD154-positive CD4-positive T helper cells in PBMCs was significantly elevated in response to vitro challenge by the galCol2 peptide (n=41) compared with unmodified nCol2 (n=35, p=0.0262). Statistical significance was determined using the Mann-Whitney test. APC, allophycocyanin; HD, healthy donors; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; RA, rheumatoid arthritis; TCR, T-cell receptor.

for the $gal_{264}Col2_{259-273}$-peptide and 0.025% for the $nCol2_{259-273}$-peptide containing tetramers in the total $CD4^+$ T-lymphocyte population. In a concomitantly analysed blood sample from a healthy donor, no DRB1*04:01/Col2-specific T cells could be identified.

The studies on our entire sample size using DRB1*04:01 tetramers containing either unmodified nCol2 or $gal_{264}Col2_{259-273}$ peptide confirmed detectability of antigen-specific T cells in PBMCs from patients with RA at the expected low precursor frequencies. The frequencies of DRB1*04:01/nCol2-staining and DRB1*04:01/gal_{264}Col2-staining $CD4^+$ T cells staining $CD4^+$ T cells were identical: 27.27%. Staining positivity was defined by a value exceeding the threshold set by the mean of negative biotin control +3 SD (see Methods section). Interestingly, $CD4^+$ T cells staining with the DRB1*04:01/nCol2-tetramers as well the DRB1*04:01/gal_{264}Col2-tetramers were also detectable at a percentage of 5% and 30%, respectively, in the small cohort of healthy DRB1*04:01 carriers (figure 3B). No significant differences were detectable between the RA and healthy donor groups (figure 3B). In this respect, the small sample size constitutes a certain constraint of our study mainly due to limited access to biomaterial from HLA-typed healthy blood donors.

Interestingly, the DRB1*04:01/Col2-tetramer-positive T-cell population detectable in the peripheral blood of patients with RA exhibited a difference in responsiveness to in vitro stimulation of PBMCs with synthetic Col2 peptides. Stimulation with the gal_{264}Col2 peptide resulted in an elevated frequency of antigen-activated $CD4^+$ T lymphocytes compared with nCol2 as determined by peptide-induced upregulation of the activation marker CD154 detected by flow cytometry (figure 3C). Taken together, the results demonstrate a functional impact of the Col2 peptide structure presented in the context of a DRB1*04:01-encoded MHCII molecule on autoimmune recognition by $CD4^+$ T cells in the peripheral blood of patients with RA.

Analysis of a cloned TCR derived from a single sorted Col2-reactive T lymphocyte

We next aimed to characterise TCRs with DRB1*04:01-restricted recognition of $Col2_{259-273}$. Single cells of in vitro expanded $gal_{264}Col2_{259-273}$ peptide-reactive $CD4^+$ T lymphocytes from patients with DRB1*04:01-positive RA were sorted according to staining with DRB1*04:01/$gal_{264}Col2_{259-273}$ tetramers and used for Vα-TCR and Vβ-TCR gene amplification by PCR. A prototypic TCR (TCR#16), for which we obtained the complete cDNA sequence of the paired α-chain and β-chain (see online supplemental figures S3 and S4), was further characterised by recombinant expression in the TCR-deficient Jurkat 76 cell line on lentiviral gene transfer (see online supplemental figure S5 for studies on two additionally transduced human TCRs). As shown in figure 4A, TCR-transduced Jurkat cells exhibited a specific positive staining with the DRB1*04:01/gal_{264} $Col2_{259-273}$ tetramers and at a clearly reduced level with DRB1*04:01/$nCol2_{259-273}$ tetramers, whereas control constructs consisting either of MHCII complexes in which DRB1*04:01 is replaced by the murine analogue A^q (A^q/$gal_{264}Col2_{259-273}$) or DRB1*04:01 complexed with the influenza hemagglutinin (HA) peptide ($HA_{306-318}$: PKYVKQNTLKLAT) (DRB1*0401/HA-peptide) remained negative. In addition, Jurkat cells transduced with a human HA-specific TCR (HA1.7)[32] stained positive with DRB1*04:01/HA306-318 while remaining negative when stained with the DRB1*04:01/$gal_{264}Col2_{259-273}$ tetramer (data not shown).

Subsequent functional studies using lentiviral gene transfer from a single sorted $gal_{264}Col2_{259-273}$-specific T-cell of a patient with HLA-DRB1*04:01-positive RA demonstrated the selective capability of recombinant monomeric DRB1*04:01/Col2-peptide complexes to induce IL-2 production in TCR-reconstituted Jurkat 76 cells (figure 4B). The challenge with monomeric DRB1*04:01/$gal_{264}Col2_{259-273}$ induced the strongest IL-2 response. Stimulation with DRB1*04:01 complexes containing the nCol2 peptide resulted in a considerably reduced IL-2 release that nevertheless clearly exceeded the levels induced by DRB1*04:01/HA306-318 or A^q/$gal_{264}Col2_{259-273}$ control complexes (figure 4B). To confirm this result, we tested antigen-presenting cells (APCs) homozygously expressing DR*0401 obtained from DRB1*04:01 knock-in mice as well as APCs from the peripheral blood of DR*0401individuals (online supplemental figure S6). The presentation of $gal_{264}Col2_{259-273}$ in a DRB1*04:01 context on the surface of either murine or human fixed APC after preloading with peptides was specifically recognised by TCR#16 mRNA transfected nuclear factor of activated T cells (NFAT) luciferase Jurkat reporter cells and associated with stronger NFAT activation compared with the stimulatory effect of the nCOL2 peptide under identical conditions. The CLIP control peptide did not lead to any activation of the TCR mRNA transfected Jurkat reporter cells. Accordingly, these results are in agreement with the studies on the specificity of tetramer-induced IL-2 responses via the recombinantly expressed TCR #16 in lentivirally transduced Jurkat 76 cells lacking an endogenous TCR.

Modelling of molecular interactions in the trimolecular complex of the DRB1*04:01, $Col2_{259-273}$ peptide and TCR

Based on the identified sequence of the human Col2-specific TCR#16 and the solved crystal structure of the DRB1*04:01/$Col2_{259-273}$ complex, molecular modelling was performed using the template of a published TCR cocrystallised with an influenza peptide-containing DRB1*04:01 molecule (HA1.7).[32] The overview of the entire modelled 3D structure of the multicomponent system consisting of the DRB1*04:01/Col2 peptide/TCR complex is shown in online supplemental figure S7. More detailed insights into critical amino acid residues involved in interactions between the unmodified or gal_{264}Col2 peptides and TCR #16 variable regions in the DRB1*04:01 complex are provided in figure 5, which depicts the superimposition of the minimised starting geometries for the two trimolecular complexes. Molecular interaction of the Col2 peptide with the TCR occurs via three side chain bonds irrespective of $Col2_{259-273}$ galactosylation. Two interactions involve the CDR3 region of the TCRα-chain (Asp94—Lys264 [$Col2_{259-273}$] and Asn97—Glu266 [$Col2_{259-273}$]) and an additional salt bridge involves the CDR1 region of the TCRβ-chain (Asp29—Lys270 [$Col2_{259-273}$]). The galactose residue at Lys264 is in close contact with the TCRα-CDR3 backbone but is not involved in side chain interactions.

Additional insight was provided by comparative molecular dynamic simulations of both trimolecular complexes (unmodified or galactosylated Col2 peptide). Snapshots at 950 ns of dynamic modelling revealed critical differences imposed by the galactosyl residue at lysine 264 in the Col2 peptide (figure 6). TCR#16 interaction with the complex containing nCol2 at the initial salt bridge Lys264—Asp94 (TCRα-CDR3) caused the complex to open up to allow neoformation of a bond to Asn97 (TCRα-CDR3). Consequently, Glu266 of $Col2_{259-273}$ formed a new salt bridge with Lys71 in the β-chain of DRB1*04:01 at expense of the initial bonding to Asn97 (TCRα-CDR3)

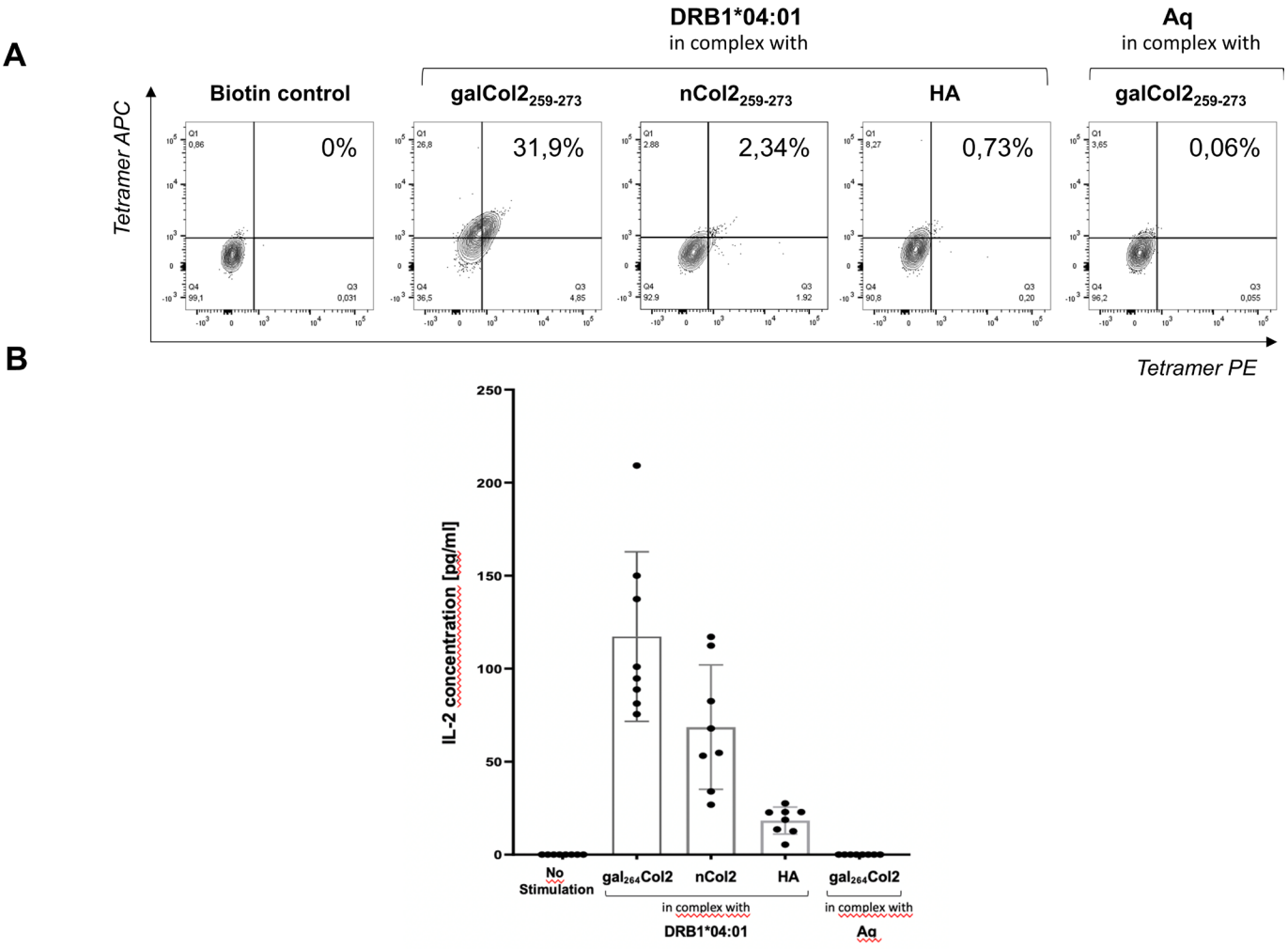

Figure 4 Binding of DRB1*04:01 tetramers to TCR-deficient Jurkat cells and induction of IL-2. (A) Flow cytometric analysis of binding of DRB1*04:01/gal$_{264}$Col2$_{259-273}$ tetramers to TCR-deficient Jurkat 76 cells after gene transfer of the cloned α and β chains of TCR #16. Transduction of a TCR-deficient Jurkat 76 cell line was performed with a lentiviral vector encoding the α and β chains of TCR #16 cloned from a single-cell sorted CD4$^+$ gal$_{264}$Col2-specific T cell. Transduced Jurkat cells were stained with a dead/live marker and DRB1*04:01/peptide tetramers conjugated with two different fluorophores (PE and APC). Flow cytometric analysis revealed tetramer-specific cells in the live double-positive stained subpopulation. A biotin-streptavidin complex without a specific peptide conjugate served as negative control. (B) Induction of specific IL-2 responses in transduced Jurkat 76 cells expressing the human TCR #16 receptor by stimulation with DRB1*04:01/Col2$_{259-273}$ peptide complexes. Transduced Jurkat cells were incubated with soluble DRB1*04:01/peptide complexes for 24 hours. Specific activation of cells via the TCR was measured by induced IL-2 release specific capture ELISA. Unstimulated cells served as a negative control. The MHCII restriction of TCR was performed by stimulation with the murine Aq/gal$_{264}$Col2 peptide complex. Bars indicate mean values, lines indicate SD and dots represent separate experiments. APC, allophycocyanin; Aq, murine MHCII allele; HA, influenza hemagglutinin 306–318; IL, interleukin; PE, phycoerythrin; TCR, T-cell receptor.

(figure 6A). By contrast, the presence of a galactose residue at position 264 allowed formation of a hydrogen bond to the TCRα-CDR3 backbone, also resulting in the stabilisation of both side chain bonds likely due to limitation in lysine 264 mobility (figure 6B). The stabilising allosteric effect is depicted in figure 7, which shows a comparative overview of all intermolecular bonds formed in the trimolecular complexes consisting of TCR#16 and DRB1*04:01 associated with either nCol2 or gal$_{264}$Col2. The graphic illustrates that the galactose is the only moiety bonded to all three molecules (the Col2 peptide, the DRB1*04:01 β-chain and the TCR). The molecular dynamics simulations for a 1000 ns period exhibited a rather low degree of molecular fluctuation in the TCR V-regions contacting the DRB1*04:01/Col2 peptide complex (figure 8). An exception was a peak of molecular mobility detectable in a solvent-exposed loop with reduced protein contacts carboxyterminal of TCRβ-CDR2 in the complex with nCol2-bound DRB1*04:01 (figure 8). Even more remarkable was the increase in molecular fluctuations in the constant region of TCR#16 affecting a Cβ domain just proximal to the so-called FG-loop and a Cα region that included the AB-loop.[33] The molecular flexibility of these functional domains, which localise near ectodomains of the signal-transducing CD3 membrane complex,[33] was clearly more pronounced in the trimolecular complex containing gal$_{264}$Col2 (figure 8).

DISCUSSION

Our studies characterised molecular details of antigen presentation by the DRB1*04:01 molecule, an allelic variant strongly associated with RA in Caucasian patients, by structure determinations following co-crystallisation with several peptides

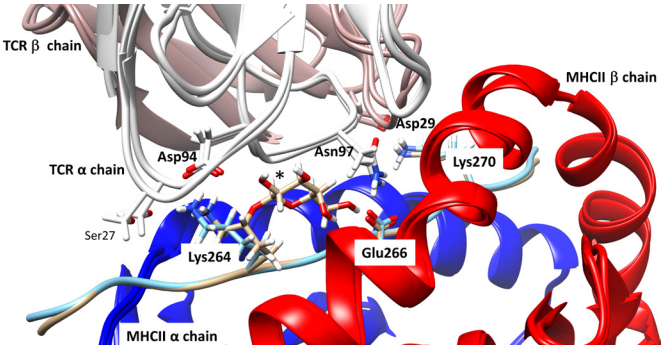

Figure 5 Molecular model of the three-dimensional structure of the multicomponent DRB1*04:01/Col2 peptide/TCR complex. Superimposition of minimised starting geometries for the trimolecular complexes of TCR#16 with DRB1*04:01 containing either the unmodified (K264) or galactosylated (gal264) version of the $Col2_{259-273}$ peptide. The image depicts three side chain bonds irrespective of $Col2_{259-273}$ galactosylation: two involving the CDR3 region of the TCRα-chain, Asp94 - Lys264 ($Col2_{259-273}$) and Asn97—Glu266 ($Col2_{259-273}$) and one in the CDR1 region of the TCRβ-chain, Asp29—Lys270 ($Col2_{259-273}$). The galactose (*) residue at Lys264 is not involved in side chain interactions with the TCR. TCR, T-cell receptor.

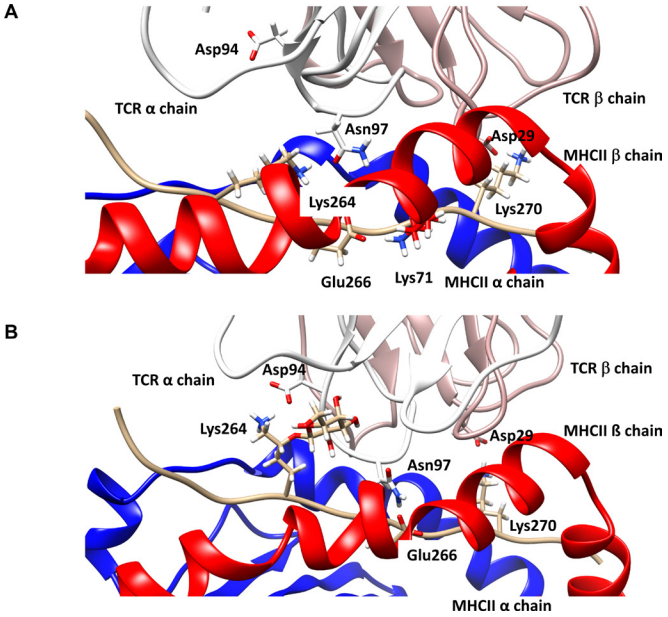

Figure 6 Geometry of the trimolecular complexes after 950 ns of molecular dynamics simulation. (A) Trimolecular complex consisting of TCR#16 and DRB1*04:01 complexed with the unmodified $Col2_{259-273}$ peptide. The initial salt bridge Lys264 - Asp94 (TCRα-CDR3) breaks to allow for neoformation of a bond between Lys264 of $Col2_{259-273}$ and Asn97 (TCRα-CDR3). Glu266 of $Col2_{259-273}$, which initially interacted with Asn97 in the TCRα (figure 5), now forms a salt bridge with Lys71 in the β-chain of DRB1*04:01, whereas the initial bond between Lys270 ($Col2_{259-273}$)-Asp29 (TCRβ-CDR1) remains preserved. (B) Trimolecular complex consisting of TCR#16 and DRB1*04:01 complexed with the $gal_{264}Col2_{259-273}$ peptide. The initial salt bridge Lys264 - Asp94 (TCRα-CDR3) remains intact, likely due to the stabilising impact of the sugar ring by decreasing the mobility of Lys264. The galactose ring in close contact to the CDR3 backbone of TCRα forms a hydrogen bond to the backbone. The two other side chain bonds of the starting geometry (figure 5), Glu266 ($gal_{264}Col2_{259-273}$)-Asn97 (TCRα-CDR3) and Lys270 ($gal_{264}Col2_{259-273}$)-Asp29 (TCRβ-CDR1), remain detectable. CDR, complementarity determining region; MHC, major histocompatibility complex; TCR, T-cell receptor.

known to trigger autoreactive RA T cells. A crucial residue for DR molecules associated with RA is position 71 in the β-chain. This residue, which is a lysine in DRB1*04:01, critically interacts with glutamic acid in position 266 of the Col2 peptide. However, there is some freedom in this interaction as other peptides derived from HSP and CILP contain different amino acids in position 266. The residues of valine 11 (V11b) and histidine 13 (H13b) of the β chain in HLA-DRB1*04:01 have been shown previously to be associated with susceptibility of seropositive RA in genome-wide association studies.[11] Our crystal structure analysis reveals that both residues likely contribute to the stability of the MHCII molecule as they are located where α and β chains pair in the beta-plated sheet. Moreover, a likely contribution to antigen presentation of the $CILP_{982-996}$ peptide is provided by the residue H13β that forms a hydrogen bond with the carboxyl group of the aspartic acid residue in the P6 pocket (P6D) of the CILP peptide. This hydrogen bond is missing in the other three complexed MHCII/peptide crystals, as the P6A ($HSP70_{289-306}$) and P6G ($Col_{259-273}$) lack the corresponding carboxyl group. In addition, the side chains of A74β orientate toward the P4 pocket, likely influencing the binding specificity of the P4 residue.

Similar to its interaction with murine A^q,[34] the DRB1*04:01 bound $Col2_{259-273}$ peptide exposes two lysine residues that are physiologically modified by hydroxylation and glycosylation, and these modified variants can be recognised by T cells.[18 35] Whereas in the mouse Col2 immunisation activates $Col2_{259-273}$ -specific T cells, thereby inducing a severe form of erosive arthritis, it is not clear to what extent MHCII-restricted Col2-specific T cells play a regulatory role in humans. In this context, there has been a long-standing question concerning why these T cells are not deleted from the repertoire by central tolerance. A possible explanation could be that TCR affinity for PTM variants of Col2 peptides promotes escape from thymic selection; this hypothesis is supported by our previous finding that non-modified Col2, but not glycosylated Col2-determinants, could be expressed by mouse or human thymic epithelium.[29] Notably, earlier studies have detected glycopeptide-reactive T cells in patients with DRB1*04:01-positive and DRB1*01:01-positive RA,[18 36] and the present investigation provides new evidence that peripheral T cells of healthy individuals also express TCRs with binding affinity for DRB1*01:01/ $gal_{264}Col2$ complexes in respective MHCII allele carriers.

The present investigation of human PBMCs from RA and healthy DRB1*04:01 carriers, which used tetramer staining as well as parallel peptide-induced T cell activation assays, provides new insight into the impact of PTM on Col2 T-cell recognition. In contrast to a comparable prevalence of $CD4^+$ T cells that recognise nCol2 or $gal_{264}Col2$ in DRB1*04:01 tetramer complexes, T-cell responsiveness was increased on peptide challenge with the galactosylated variant. The mechanism for this glycosylation-dependent impact on T-cell activation is not entirely clear and might be multifactorial, but one attractive hypothesis is that an altered TCR interaction with the DRB1*04:01-bound galactosylated Col2-peptide affects TCR signal transmission. Accordingly, our functional and structural characterisation of a prototypic human TCR and its interaction with nCol2 or $gal_{264}Col2$ in the context of DRB1*04:01 presentation provide first experimental support for this hypothesis. The comparative dynamic modelling of the respective trimolecular complexes containing either the galactosylated or unmodified

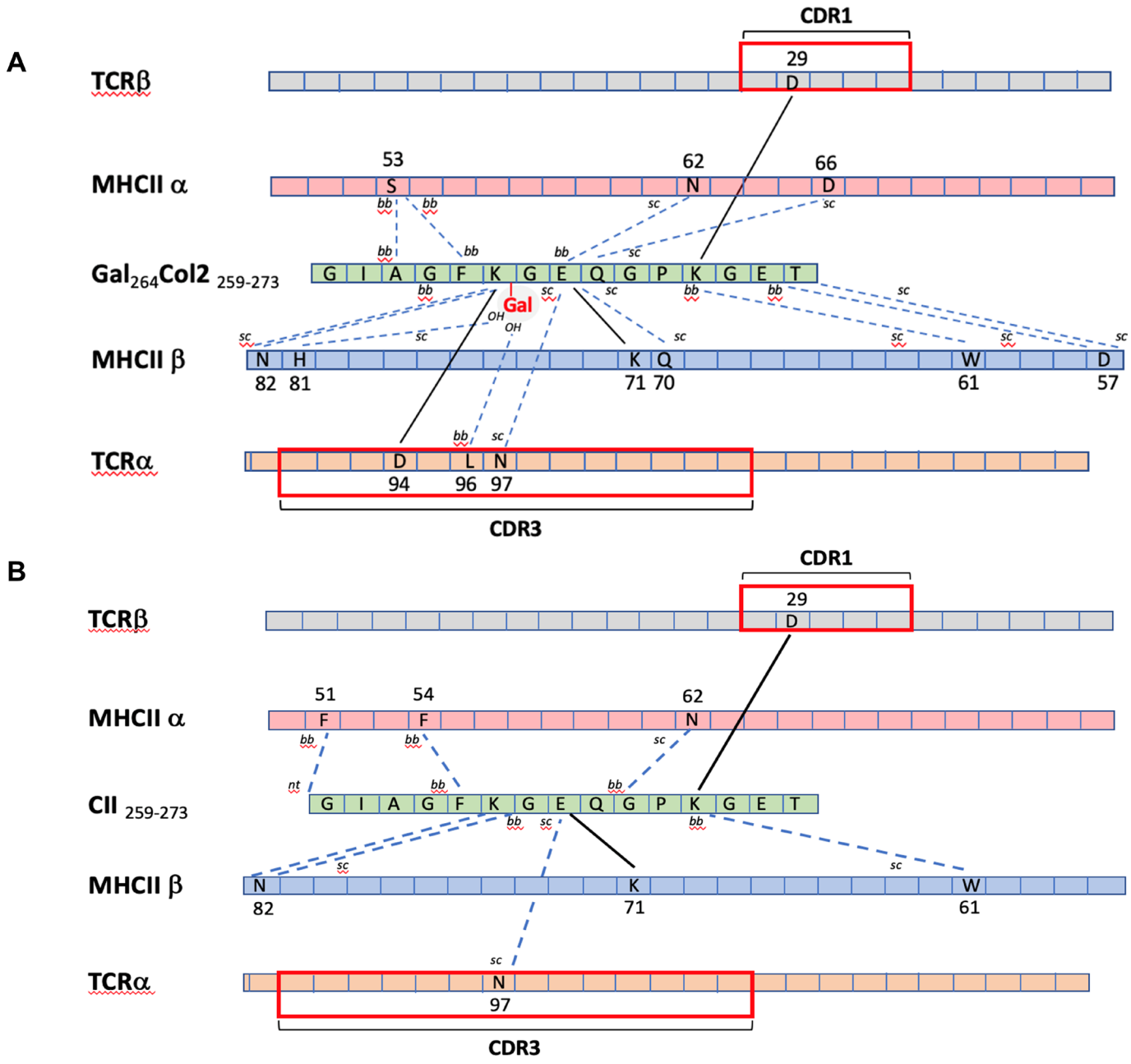

Figure 7 Comparative overview of intermolecular bonds formed in the trimolecular complexes of TCR#16 and DRB1*04:01 (MHCII) associated with either (A) gal$_{264}$Col2 or (B) nCol2. Amino acids are shown in one letter code and their positions in the respective protein sequences indicated by number. Bold lines indicate salt bridges and dashed lines indicate hydrogen bonds. ai, aromatic interaction; bb, backbone; CDR, complementarity determining region; MHC, major histocompatibility complex; sc, side chain, TCR, T-cell receptor.

Col2 variant revealed a major difference in the propagation of TCR dynamics from the V-regions to key allosteric sites in the Cα and Cβ region pertaining particularly to the so-called CαAB loop.[33] This domain was previously described for its role in the allosteric regulation of TCR signalling as evidenced by fluorescence-based conformational changes on MHCII/peptide perception and signalling impairment by mutational analysis.[37] Another region exhibiting reinforced molecular fluctuations on TCR recognition of the DRB1*04:01/gal$_{264}$Col2 in our studies is in immediate proximity to the CβFG loop that has been critically incriminated in T-cell activation and thymic selection.[38 39]

Thus, our dynamic modelling studies provide evidence for allosteric changes initiated by CDR3α-region interaction with a single galactose residue in the DRB1*04:01-bound gal$_{264}$Col2 peptide, which propagates to result in increased conformational flexibility at distant sites in the constant TCR regions contacting the CD3 signalling complex. Whereas it remains enigmatic how these allosteric changes are transmitted across the cell membrane, our complementary functional T-cell studies provide experimental support for translation into a reinforced TCR signal in response to challenge by gal$_{264}$Col2-bound versus nCol2-bound DRB1*04:01complexes. Moreover, the data obtained by analysis of RA T cells and the prototypic TCR #16 might reflect mechanisms of central tolerance by thymic medullary epithelial cells that do not express galactosylated Col2 and accordingly execute clonal deletion exclusively via presentation of unmodified Col2 determinants.[29] Thus, T cells rescued from thymic selection due to weak TCR recognition of DRB1*04:01/

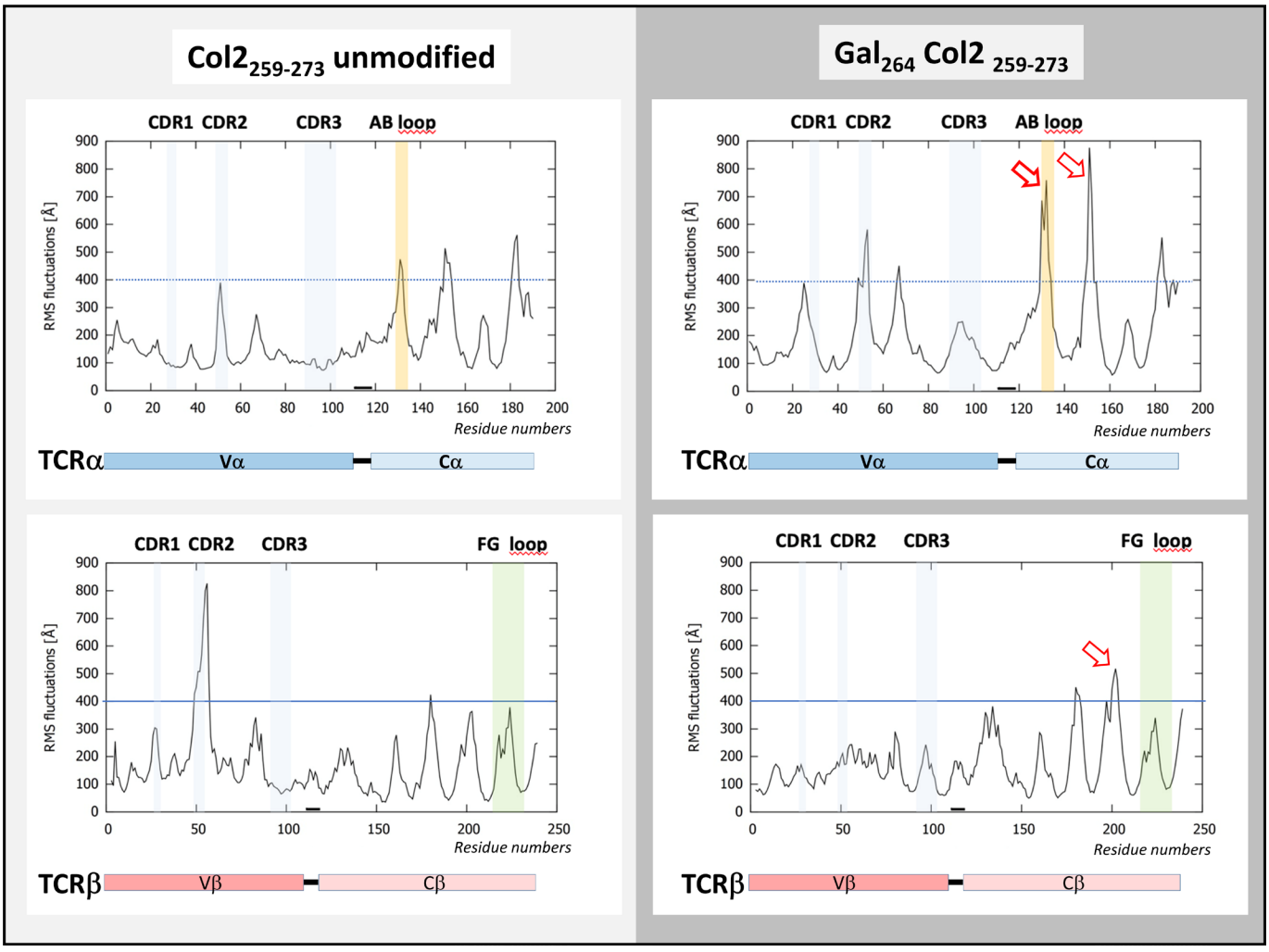

Figure 8 Molecular dynamics simulations of trimolecular complexes consisting of TCR#16 and DRB1*04:01 associated with either the galactosylated (gal264) (left) or unmodified $Col2_{259-273}$ peptide (right). Depicted are the root mean squares of fluctuation values for the variable and constant region residues of TCRα and TCRβ within the trimolecular complex over a simulation period of 1000 ns. In complex with the unmodified Col2 peptide, a peak of molecular mobility is detectable carboxyterminal of the TCRβ-CDR2 domain and to a minor degree in TCRα-CDR2 in complex with the gal264Col2 peptide. Most notable is the increase of molecular fluctuations in the constant region of TCR#16 (arrows), especially in the α-chain, also affecting the CαAB loop and clearly much more pronounced in complex with gal264 compared with the unmodified Col2 peptide. CDR, complementarity-determining region; RMS, root mean square; TCR, T-cell receptor.

nCol2 complexes and the resulting low TCR signal intensities could subsequently become activated on MHCII presentation of physiologically galactosylated Col2 in peripheral tissues[39] via reinforced signalling dynamics initiated by the recognition of posttranslational Col2 peptide modifications. However, the outcome of the activation, which can result in either arthritogenic or tolerogenic T cell responses, would remain context dependent. An obvious objection to our findings on the lack of a contribution of citrulline residues to MHCII binding in the two crystallised control complexes containing citrullinated epitopes of RA T cell responses and our focus on TCR recognition of Col2 peptides is how our proposed concept could be linked to citrulline-specific immunity and its strong association with DRB1*04:01 in RA. However, it has been shown that Col2 can be citrullinated in vivo, both in mice and in humans with RA.[22] Moreover, the autoantibody response to citrullinated Col2 is prominent in B cell recognition of Col2. Accordingly, it is easily conceivable that B cells specific for citrullinated Col2 can present the non-citrullinated 259–273 peptide to T cells and vice versa.[40] Such T cells could help to activate B cells specific for citrullinated epitopes, potentially breaking tolerance and allowing pathogenic epitope spreading of the anticitrullinated protein antibody response target, joint cartilage.[40]

Although we have provided indirect evidence for the proposed scenario, further studies are clearly needed to provide additional experimental support and to answer the question of whether this hypothesis might also apply to citrullinated antigens, such as the CILP-derived peptide cocrystallised with DRB1*04:01 in this study.

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Competing interests NS, BX, SW, RH and HB are listed as inventors on Patent EP2020072287 (https://www.onscope.com/ipowner/en/ip/ptwo/EP2020072287.html). SW, N-ND, RH and HB are lasted as inventors on Patent EP2020072280 (https://www.onscope.com/ipowner/en/ip/ptwo/EP2020072280.html). The owner of both patents is Fraunhofer-Gesellschaft zur Förderung der Angewandten Forschung E.V. (Germany). SW is listed on these patents under her maiden name of Sylvia Cienciala.

Patient consent for publication Not applicable.

Ethics approval All blood sample donors gave prior written consent for study inclusion. The current study has been approved by the ethical approval committee of the University Hospital Frankfurt, Germany.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. All data relevant to the study are included in the article or uploaded as supplementary information. The crystallographic coordinates and structure factors elucidated in this study have been deposited in the Protein Data Bank with the accession codes listed in online supplemental table S1 (7NZE, 7NZF, 7NZH, 7O00).[41–44]

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