International Immunology, Vol. 16, No. 2, pp. 283–293
doi: 10.1093/intimm/dxh039

Analysis of autoreactive T cells associated with murine collagen-induced arthritis using peptide–MHC multimers

Jason C. Huang[1], Mikael Vestberg[2], Alfredo Minguela[3], Rikard Holmdahl[2] and **E. Sally Ward[1]**

[1]Center for Immunology, University of Texas Southwestern Medical Center at Dallas, 6000 Harry Hines Boulevard, Dallas, TX 75390-9093, USA
[2]Section of Medical Inflammation Research, BMC, Lund University, 22184 Lund, Sweden
[3]Seccion de Inmunologia, Hospital Universitario Virgen de la Arrixaca, El Palmar, 30210 Murcia, Spain

Keywords: autoimmunity, MHC, rheumatoid arthritis, T lymphocyte, TCR

Abstract

CD4+ T cells that recognize residues 256–270 of type II collagen (CII) associated with the I-A^q (A^q) molecule play a central role in disease pathogenesis in murine collagen-induced arthritis (CIA). Disease is most efficiently induced by immunization with heterologous CII, which elicits heterologous, e.g. bovine, CII256–270:I-A^q-specific T cells that only poorly cross-react with mouse CII. The self-epitope differs from heterologous CII256–270 by a conservative change of glutamic acid (heterologous) to aspartic acid (mouse) at position 266 which confers a lower affinity for binding to the I-A^q molecule. To date, characterization of the nature of T cell recognition in this model has been hindered by the lack of suitable, labeled multimeric peptide–MHC class II complexes. Here, we describe the biochemical properties of both recombinant bovine CII256–270:I-A^q (bCII256–270:I-A^q) and mouse CII256–270:I-A^q (mCII256–270:I-A^q) complexes, and use these as fluorescently labeled multimers (tetramers) to characterize the specificity of CII-reactive T cells. Our analyses show that an unexpectedly high percentage of bCII256–270:I-A^q-specific T cells are cross-reactive with mCII256–270:I-A^q. Interestingly, one T cell clone which has a relatively high avidity for binding to self-CII256–270:I-A^q shows a marked increase in binding avidity at physiological temperature, indicating that this TCR has unusual thermodynamic properties. Taken together, our analyses suggest that the low affinity of mCII256–270 for I-A^q may lead to a state of ignorance which can be overcome by priming CII-specific T cells with heterologous CII. This has relevance to understanding the mechanism by which CIA is induced and provides an explanation for the low arthritogenicity of mouse CII.

Introduction

Collagen-induced arthritis (CIA) is believed to be a representative model of rheumatoid arthritis in humans (1). Arthritis can be induced in susceptible rodents and primates by injection of type II collagen (CII), and is characterized by both B and T cell responses against CII (2,3). In murine CIA, susceptibility is controlled by the MHC region (4,5) and linked to the MHC class II Ab gene, in the susceptible II-2^q haplotype (4–6). Immunization of DBA/1 mice (which express A^q) with CII induces T cell responses that are primarily directed towards the immunodominant epitope, CII residues 256–270 (core of epitope is 260–267), associated with A^q (7–12). In addition, analogs of this peptide can modulate CIA (11), indicating that CII256–270:A^q-specific T cells are centrally involved in pathogenesis. This peptide binds to the A^q molecule primarily through isoleucine (I260) and phenylalanine (F263) interactions with the P1 and P4 pockets respectively of the A^q molecule (13), whereas the major T cell contact residue is lysine (K264) that can be post-translationally modified (13,14). Interestingly, the CII-specific T cells only poorly cross-react with mouse CII (8), which differs from heterologous (bovine, chick, rat, human) CII by a glutamic acid (heterologous) to aspartic acid (murine) change at position 266. The observation that immunization with mouse CII results in a higher T cell response to heterologous CII (a heteroclitic response) has

Correspondence to: E. S. Ward; E-mail: Sally.Ward@Utsouthwestern.edu

Transmitting editor: M. Feldmann

Received 1 July 2003, *accepted* 28 October 2003

been taken as an indication that the poor cross-reactivity is due to a difference in affinity of the peptide for A^q rather than at the level of T cell recognition (8). However, at high antigen concentrations many of these CII-specific T cells also recognize mouse CII256–270 (mCII256–270) (15). Such potentially autoreactive, mouse CII-specific T cells are of particular interest as they most likely play a central role in the disease process.

For MHC class I-restricted T cells, multimeric MHC molecules have been widely used for the enumeration of antigen specific T cells (16–18). These multimeric peptide–MHC (pMHC) complexes can also be used to characterize the avidities of responding T cells (18–23). However, less data are available for MHC class II- relative to MHC class I-restricted responses and this is particularly so in murine models of autoimmunity (24,25). This is most likely due to the relative instability of I-A complexes (26–28), which are invariably the restricting elements associated with these models. Several laboratories, including our own, have shown that stable peptide–I-A complexes can be made by covalently tethering the antigenic peptide to the I-A_β chain and stabilizing $\alpha\beta$ chain association with acidic/basic zippers (25–29). This approach can be used to generate appropriate multimers/tetramers to detect antigen-specific, autoreactive T cells in both the murine experimental autoimmune encephalomyelitis (EAE) (24) and non-obese diabetic (NOD) models (25). More recently we have shown that analogous bovine CII256–270 (bCII256–270):A^q complexes can be used to detect antigen-specific T cells in the draining lymph nodes of CII-immunized mice (30). To date, however, multimers comprising mCII256–270:A^q complexes, which represent the autoantigen, have not been described. The use of these complexes in the current study has allowed us to directly compare recognition of the heterologous and autologous epitopes by CII-specific T cells.

Analyses of TCR–pMHC interactions using soluble molecules indicate that TCRs bind to cognate ligands with relatively slow on-rates and fast off-rates (31). The low on-rate suggests that some conformational rearrangement is necessary for complex formation. Consistent with the involvement of induced fit, thermodynamic analyses have shown that TCR–pMHC interactions for both MHC class I and class II systems are highly temperature dependent, and are entropically unfavorable (32–34). X-ray crystallographic studies have demonstrated that the TCR–pMHC interface shows poor complementarity (35–38). Together with indications of conformational rearrangements for the TCR, this has led to the conclusion that TCRs are highly 'plastic' (36,39). Plasticity of TCR–pMHC interactions would be predicted to contribute to the cross-reactive nature of T cells (40–44), as conformational adjustments of TCR residues might allow multiple pMHC ligands to be recognized with an appropriate affinity to result in activation of the corresponding T cell (32–34). This cross-reactivity impacts many areas of T cell biology, including the activation of autoreactive T cells by molecular mimicry (43,45–47).

We have recently shown in the model of murine EAE that it is possible to gain some insight into the temperature dependence of the avidities of TCR–pMHC interactions by using appropriate pMHC tetramers to stain T cells at different temperatures (34). This approach can be used, at least in qualitative terms, to assess the relative plasticity of a particular TCR–pMHC interaction, and we have shown correlative data between tetramer binding and thermodynamic analyses using surface plasmon resonance (34). There is a paucity of knowledge concerning the molecular details, including the plasticity, of TCR–pMHC interactions in the CIA model. This is of particular interest as, in contrast to the majority of other inducible models of autoimmunity for which autoantigen is used as immunogen, heterologous CII is much more effective than autologous CII in inducing CIA (1,48). The lower efficacy of mouse CII in inducing arthritis is most likely due to weaker binding of the aspartic acid (D266)-substituted CII peptide to A^q (13). Transgenic expression of CII with glutamic acid at position 266 (E266) leads to higher exposure of CII, but also to partial T cell tolerance (7). Clearly, the degree of T cell recognition of self-CII is central for the disease process. A critical question is whether the poor cross-reactivity of heterologous CII-specific T cells with the immunodominant self-CII peptide is only due to a difference in peptide–MHC binding affinity or is also caused by alteration in the disposition of the T cell contact residues of the peptide. Here, we directly address this issue.

The goal of the current study was to characterize tetrameric bCII260–270:A^q and mCII260–270:A^q complexes, and to use these reagents to carry out an analysis of antigen-specific T cells. We show that these tetramers can be used to stain appropriate hybridomas and responding T cells in antigen-specific, short-term lines. Further, we have used tetramer staining to analyze, in qualitative terms, the thermodynamics of TCR–pMHC interactions in this disease model. Our findings indicate that cross-reactive recognition of mCII256–270 by CII-specific T cells is much higher than anticipated from earlier studies in which antigen-pulsed antigen-presenting cells (APCs) were used to assess autoreactive responses (8,15). This high cross-reactivity has relevance to understanding the mechanism by which heterologous CII acts as a potent arthritogen.

Methods

Mice and cells

DBA/1LacJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free environment in the animal facility at the University of Texas Southwestern Medical Center at Dallas. bCII256–270-specific T cell hybridomas HRC.1, HRC.2, HCQ.4 and HDB.2 have been described previously (14). The 172.10 hybridoma, which is specific for the N-terminal epitope of myelin basic protein (MBP) associated with I-A^u (49), was generously provided by Dr Joan Goverman. A subclone of this hybridoma (34) was used in the current study.

Antigens and antibodies

Bovine CII was purchased from Chondrex (Redmond, WA). The peptides bCII256–270, mCII256–270 and mCII254–274 were synthesized at the Peptide Synthesis Unit of the Howard Hughes Medical Institute, UT Southwestern Medical Center (Dallas, TX). Phycoerythrin (PE)-labeled anti-TCR C_β (H57-597) was purchased from PharMingen (San Diego, CA).

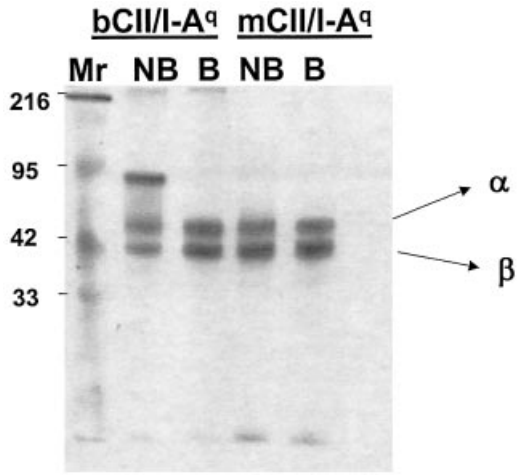

Fig. 1. 12% SDS–PAGE analysis of recombinant peptide:A^q molecules, stained with Coomassie brilliant blue: B, samples were heated to 100°C in 2% SDS sample buffer for 5 min before loading; NB, samples were kept at room temperature in 2% SDS sample buffer for 5 min before loading. Sizes (in kDa) of mol. wt standards are shown on the left margin.

FITC-labeled anti-CD8 (RM2201) and allophycocyanin-labeled anti-CD4 (RM2505) were purchased from Caltag (Burlingame, CA). 7-Aminoactinomycin D and cytochalasin D were purchased from Sigma (St Louis, MO).

Generation of peptide:A^q tetramers

The constructs used for the expression of recombinant A^q molecules were derived from previously described constructs used to generate bCII260–270:A^q complexes (28). The leader sequences of α and β chains were replaced by a signal peptide sequence derived from honey bee melittin. A *Kpn*I site and a flexible linker (GSGSGSS) sequence were inserted between the honey bee melittin signal peptide (50) and the β^q chain sequence. The *Kpn*I site was used for insertion of the corresponding sequences of bCII256–270, mCII256–270 or myelin oligodendrocyte protein (MOG) 79–90 peptide [the latter peptide binds to A^q (51) and was used to generate a control tetramer]. The α and β chains were truncated before the transmembrane region, and sequences encoding acidic and basic zippers were inserted as described (28). In addition, the β chain also encodes a biotinylation signal peptide following the basic zipper. Both α and β chains were tagged with C-terminal polyhistidine tags and cloned into the dual baculovirus expression vector pAcUW51 (PharMingen, San Diego, CA). The recombinant plasmids were co-transfected with Baculogold DNA (PharMingen) into Sf9 cells, and recombinant viruses plaque purified and used to make high-titer virus stocks. HIGH FIVE cells (Invitrogen, Carlsbad, CA) were infected with virus stocks and supernatants from 62- to 64-h cultures harvested. Recombinant protein was purified using Ni-NTA^{2+} affinity columns. Following overnight dialysis against PBS, recombinant CII256–270:A^q complexes were further purified using a second affinity column made by coupling Y-3P to Protein G–Sepharose [Y-3P recognizes A^q in a conformational dependent way (52)]. Recombinant proteins were dialyzed into biotinylation buffer (20 mM Tris–HCl, 50 mM NaCl, pH 8.0) overnight. Biotin ligase (Avidity, Denver, CO) was added to a final concentration of 10 μg/ml and the reaction was carried out at room temperature for 16–20 h. Following biotinylation, free biotin was removed by extensive dialysis. Tetrameric peptide:A^q complexes were generated by adding PE-labeled Extravidin (Sigma, St Louis, MO) to the recombinant protein at a molar ratio of 1:4.

Maintenance of T cell hybridomas

Bovine CII-specific T cell hybridomas (HRC.1, HRC.2, HCQ.4 and HDB.2) and 172.10 hybridoma cells were maintained in complete DMEM: DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (Life Technologies), penicillin/streptomycin (100 U/ml; Life Technologies), non-essential amino acids (0.1 mM; Life Technologies), sodium pyruvate (1 mM; Life Technologies) and 2-mercaptoethanol (55 μM; Life Technologies).

Production of short-term T cell lines

Male DBA/1LacJ mice (8–12 weeks old) were immunized intradermally with 100 μg bovine CII in complete Freund's adjuvant (Sigma) at the tail base. Ten days following immunization, splenocytes and lymph node cells were extracted and expanded *in vitro* with 50 μg/ml bovine CII for 3 days. Following separation with NycoPrep 1.077A (NycoMed Pharma, Oslo, Norway), the cells were cultured in IL-2 (80 U/ml) complete DMEM for 5–7 days. Thereafter, the T cells were re-stimulated with irradiated (2500 rad), syngeneic spleen cells and 50 μg/ml bovine CII for 3 days, and then IL-2 for 5–7 days. A short-term line was established by *in vitro* expansion 4 times before staining with peptide:A^q tetramers and other antibodies. This short-term line was maintained in complete DMEM.

Tetramer staining and flow cytometric analysis

Staining with peptide:A^q tetramers was carried out in the presence of 10 μg/ml anti-CD3ε (145-2C11) at 37°C for 30 min as described (34) unless otherwise indicated. For the analysis of the effect of temperature on staining, TCRs of hybridoma cells were either 'pre-clustered' by incubation with 10 μg/ml anti-CD3ε (145-2C11) at 37°C for 30 min or untreated. Following this, cells were treated with 100 μM cytochalasin D at room temperature for 5 min and then incubated with bCII256–270:A^q tetramers at 12, 25 or 37°C for 1 h. Levels of tetramer staining were normalized for TCR expression using PE-labeled anti-TCR β chain antibody, H57-597, as described (24,34). Multi-color staining of short-term T cell lines was performed in two steps. Cells were pre-incubated with allophycocyanin-labeled anti-CD3ε (145-2C11) for 30 min at 37°C. Following a wash in PBS, cells were incubated with PE-labeled tetrameric bCII-, mCII- or MOG-A^q complexes and PerCP-labeled anti-CD4 at 12°C for 2 h. Stained cells were analyzed with a FACSCalibur (Becton Dickinson) and data analyzed using WinMDI (Scripps Research Institute).

T cell activation assays

T cell hybridomas were stimulated with serially diluted plate-bound anti-CD3ε (145-2C11), bCII256–270:A^q and mCII256–270:A^q as described (28,53). For some assays, T cell hybridomas were incubated with A^q-expressing splenocytes

(from DBA/1 mice) pulsed with bCII256–270 or mCII256–270. In all stimulation assays, cells were stimulated in duplicates for 24 h at 37°C and IL-2 levels in culture supernatants quantitated by IL-2 ELISA (53). To account for differences in TCR expression levels, normalized IL-2 levels were obtained by dividing the optical density at 450 nm (OD_{450}) by the OD_{450} corresponding to responses to immobilized anti-CD3ε (54,55).

Results

Expression and SDS stability of the A^q molecules

Recombinant A^q molecules covalently tethered to mouse or bovine CII256–270 were expressed and purified from baculovirus-infected HIGH FIVE cells in yields similar to those reported for the N-terminal epitope of MBP tethered to A^u (24). We first analyzed the ability of the CII256–270 complexes to form SDS-resistant, compact dimers (56) (Fig. 1). SDS–PAGE analysis comparing the recombinant bCII256–270:A^q and mCII256–270:A^q demonstrates that bCII256–270:A^q complexes form compact dimers, whereas mCII256–270:A^q complexes do not. This is consistent with the observation that substitution of residue 266 by aspartic acid results in a lower affinity of CII256–270 for A^q (13). However, the lack of compact dimer formation does not appear to negatively affect the stability of the recombinant mCII256–270:A^q complexes during storage and this is most likely due to the covalent tethering of the peptide. Similarly, MBP1-11:A^u complexes which do not form SDS-resistant dimers are also stable for many months [(24) and unpublished data], indicating that SDS resistance is not a reliable indicator of the storage stability of recombinant peptide:I-A complexes.

Binding of tetramers to cognate T cells and analysis of responsiveness

Recombinant CII256–270:A^q complexes were site specifically biotinylated and incubated with PE–Extravidin to generate tetramers. A series of T cell hybridomas, specific for the heterologous CII256–270 epitope and with poor cross-reactivity *in vitro* for mouse CII (8), was used for analyzing the tetramers. The tetramers show specific binding to CII256–270:A^q-specific T cell hybridomas and, unexpectedly, this is also seen for mCII256–270:A^q complexes (Fig. 2A). Interestingly, HDB.2 hybridoma cells consistently stain to higher levels with mCII256–270:A^q than bCII256–270:A^q complexes, whereas the reverse is true for HCQ.4 cells (Fig. 2A). Although absolute avidities/affinities cannot be derived from the current analyses, this demonstrates that the relative avidities of the two hybridomas for these complexes are distinct. It also shows that TCR recognition, and not only MHC binding, is affected by the amino acid difference at position 266.

Consistent with the high degree of cross-reactivity of the CII-specific hybridomas, similar percentages of $CD4^+$ T cells are stained with bCII256–270:A^q and mCII256–270:A^q complexes following the generation of short-term CII-specific T cell lines from bovine CII-immunized mice (Fig. 2B). For these analyses, tetramer staining was carried out at 12°C, as we have found that this yields improved staining relative to staining at more physiological temperatures. This is consistent with studies indicating that, in general, TCR–pMHC affinity increases with decreasing temperature (32–34). The data shown in Fig. 2(B) indicate that the level of cross-reactivity of bovine CII for autologous CII may be higher than originally suggested (8,15). As a specificity control in these experiments (Fig. 2B), background levels of staining are seen with complexes made by covalently tethering MOG residues 79–90, which bind tightly to A^q (51), in an analogous construct to that used for the CII peptides.

In a number of different antigen systems, tetramer staining has been used to assess the avidity of TCR–pMHC interactions (18–23). We therefore quantitated the tetramer staining levels following normalization for TCR expression levels and compared these levels with responsiveness ('functional avidity') to bCII256–270 or mCII256–270 peptide-pulsed APCs (Fig. 3). As the TCR expression levels differ for each hybridoma, and are particularly low for HDB.2 cells, it was also necessary to normalize the T cell responses to antigen using anti-CD3ε stimulation (54,55). For bCII256–270:A^q responses, there is a good correlation between the responses of the hybridomas to peptide-pulsed APCs and tetramer staining levels (Fig. 3A and C). In addition, the responses to plate bound bCII256–270:A^q complexes correlate well with binding by the tetrameric complexes (Fig. 3A and E). However, there is a marked difference in responsiveness of the hybridomas to plate-bound mCII256–270:A^q complexes and mCII256–270-pulsed APCs (Fig. 3D and F). Although the hybridomas respond to recombinant complexes in a pattern that correlates well with mCII256–270:A^q tetramer staining levels (Fig 3A and F), they are unresponsive to mCII256–270-pulsed APCs (Fig. 3D). Similar results were obtained for a longer variant of the peptide, mCII254–274 (data not shown). This unresponsiveness to peptide-loaded APCs is consistent with earlier data (8) and suggests that the low affinity of mCII256–270 for A^q which results in poor presentation (13) can be overcome by covalently tethering the peptide to the A_β^q chain. Consistent with the low affinity of the mouse CII peptide for I-A^q, the corresponding complexes do not form compact dimers (Fig. 1). Direct comparisons (on a per nanogram basis) of the stimulatory properties of mCII256–270:A^q versus bCII256–270:A^q complexes therefore cannot be made, as it is possible, for example, that the relative stabilities to immobilization on plastic could vary.

Temperature dependence of the TCR–pMHC interactions

Recent studies have shown that TCR–pMHC interactions are highly temperature dependent, with the affinity increasing with a decrease in temperature (32–34). This temperature dependence has led to the concept that TCR–pMHC interactions occur via an induced fit mechanism involving highly unfavorable entropic forces. We have previously shown that tetramers comprising MBP1–11 covalently tethered to I-A^u bind to higher levels at lower temperatures (12°C) than at 37°C to T cell hybridomas bearing TCRs of known affinity (34). This higher level of binding at lower temperatures was shown to be consistent with analyses using soluble TCRs and pMHC complexes in surface plasmon resonance studies (34). Thus, tetramer staining can provide insight into the temperature dependence of the corresponding TCR–pMHC interaction, which in turn relates to the thermodynamics of complex

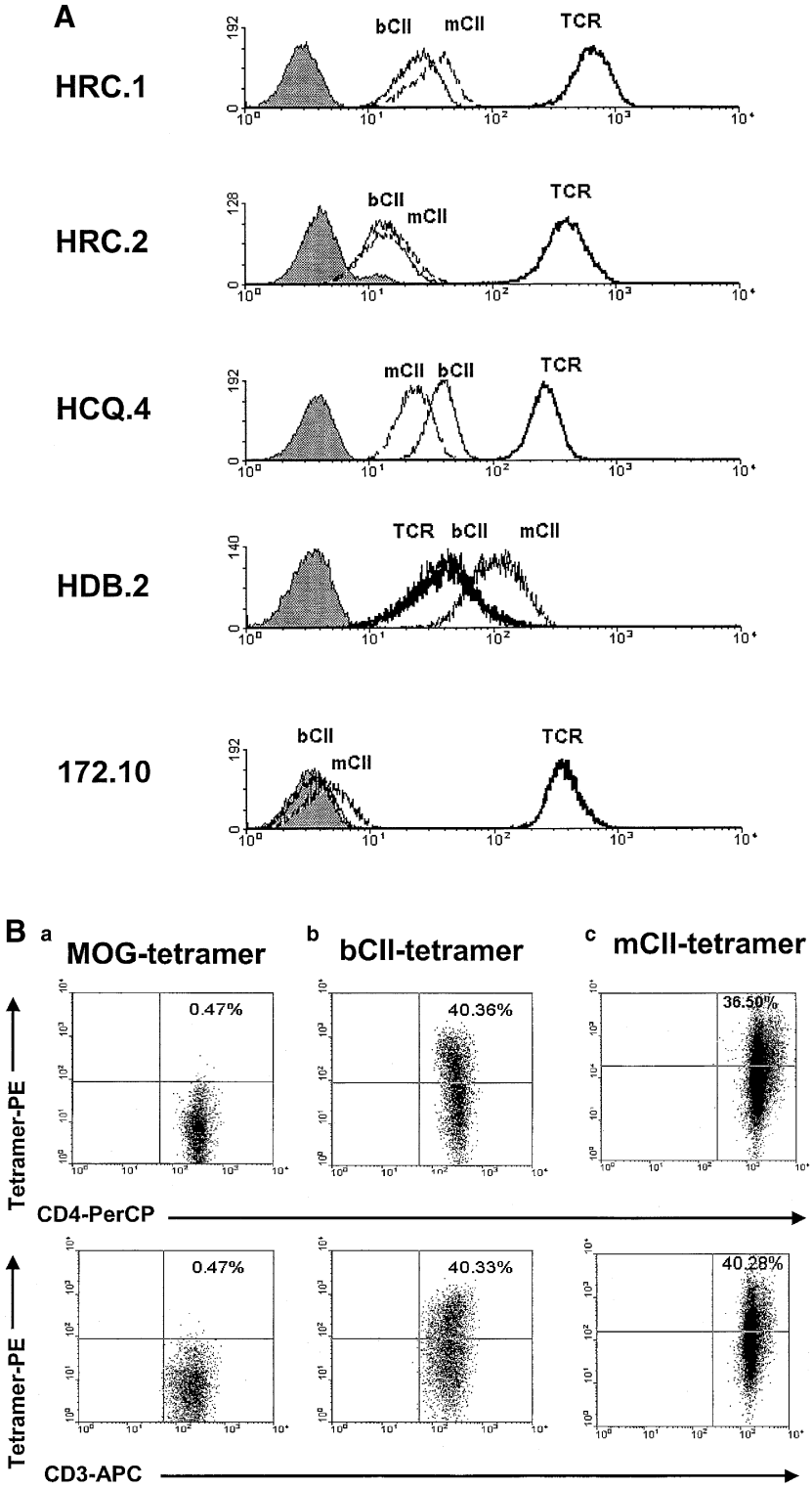

Fig. 2. Staining of the T cell hybridomas and short-term T cell lines with fluorescently labeled CII:A^q tetramers. (A) T cell hybridomas were incubated with Extravidin–PE labeled bCII256–270:A^q ('bCII'), mCII256–270:A^q ('mCII') or PE-labeled H57-597 ('TCR', anti-C_β) at 37°C as described in Methods. Histogram plots for the staining are shown. Filled histograms represent fluorescence levels of T cell hybridomas incubated with Extravidin–PE only. (B) Tetramer staining of *in vitro* stimulated, CII-specific T cells following four rounds of re-stimulation/resting using 50 μg/ml bovine CII or 80 U/ml IL-2. Cells were incubated with allophycocyanin-labeled anti-CD3, PE-labeled bCII, mCII or MOG tetramer, and PerCP-labeled anti-CD4. Percentage of tetramer$^+$ CD4 or CD3 cells is shown in the right upper quadrant. Data are representative of five (A) or two (B) independent experiments.

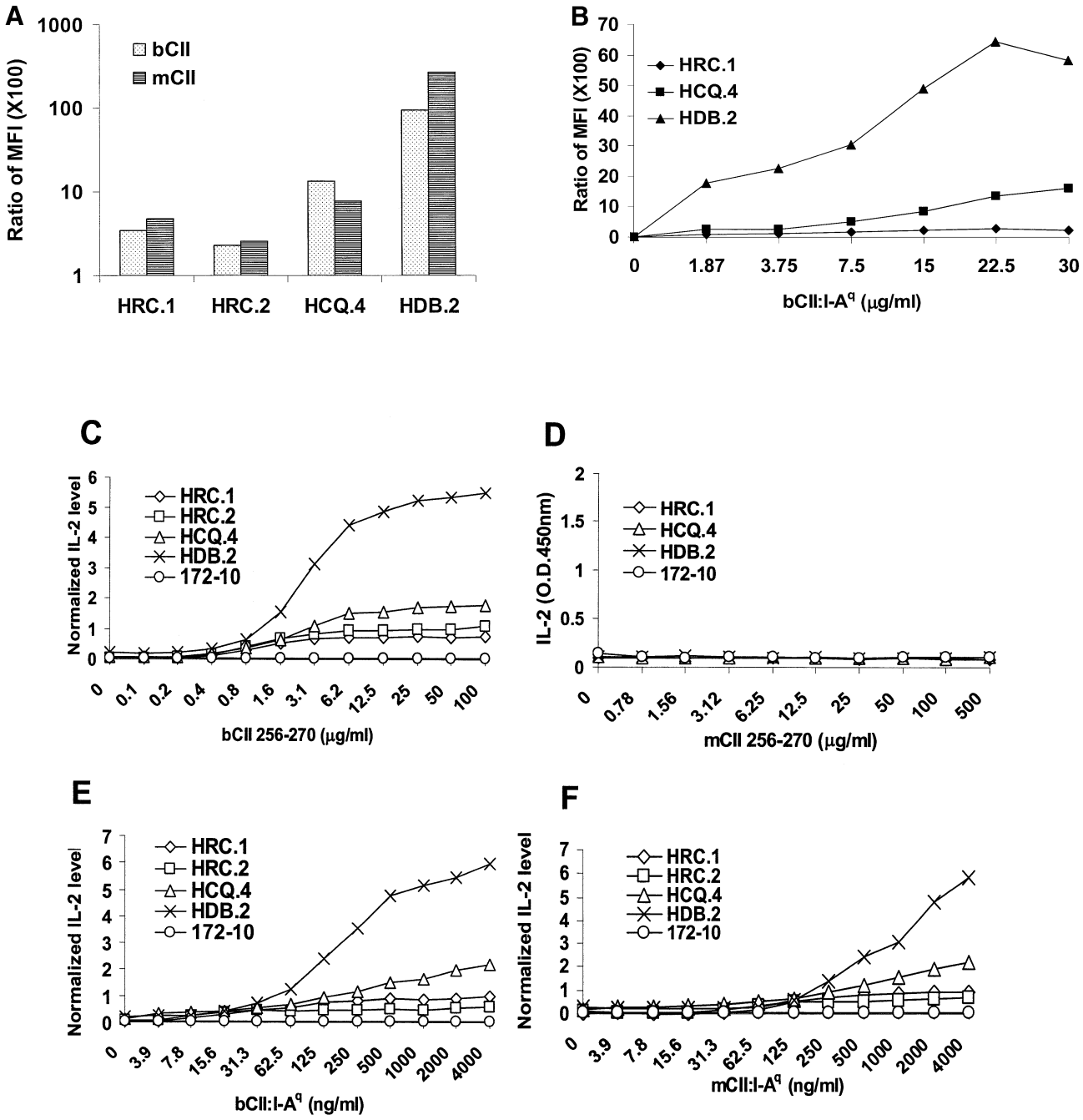

Fig. 3. Correlation between CII256–270:A^q tetramer staining levels and responsiveness of hybridomas to antigen. (A) Normalized tetramer staining levels for the hybridomas to take into account the different TCR expression levels. Cells were stained with Extravidin–PE-labeled CII:A^q tetramers or PE-labeled H57-597 at 37°C for 30 min as described in Methods. Ratios (multiplied by 100) of mean fluorescence intensities for tetramer staining to H57-597 (anti-C_β) staining are shown. (B) T cell hybridomas were incubated with different amounts of bCII256–270:A^q complexes at 37°C for 30 min as in Methods. The *y*-axis represents normalized tetramer staining levels, as in (A). (C) Responsiveness of T cell hybridomas to bCII256–270-pulsed A^q-expressing splenocytes. (D) Responsiveness of T cell hybridomas to mCII256–270-pulsed A^q-expressing splenocytes. (E) Responsiveness of hybridomas to plastic-bound, recombinant bCII256–270:A^q complexes. (F) Responsiveness of hybridomas to plastic-bound, recombinant mCII256–270:A^q complexes. For (C–F), IL-2 levels were quantitated in culture supernatants following 24 h of incubation by ELISA. To account for differences in TCR levels, IL-2 levels in response to antigen were normalized using responses to plate-bound anti-CD3ε, as described previously (with the exception of D) (54,55). Data are representative of four (A and E), two (B) or three (C, D and F) independent experiments.

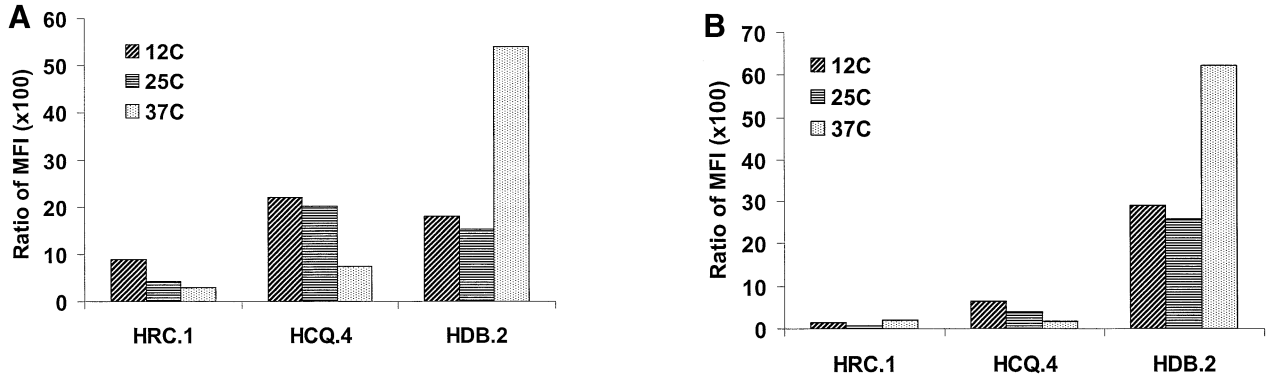

Fig. 4. Temperature effects on staining of the T cell hybridomas with bCII256–270:A^q tetramers. T cell hybridomas were incubated with Extravidin–PE-labeled bCII256–270:A^q or PE-labeled H57-597 (anti-C$_\beta$). (A) With anti-CD3ε pre-clustering or (B) without anti-CD3ε pre-clustering. Ratios (multiplied by 100) of mean fluorescence intensities for tetramer staining to H57-597 (anti-C$_\beta$) staining, i.e. normalized tetramer staining levels are shown. Data are representative of two independent experiments.

formation. We therefore compared the binding of bCII256–270:A^q tetramers at 12, 25 and 37°C to hybridomas HDB.2, HCQ.4, HRC.1 and HRC.2 (Fig. 4). These experiments were initially carried out using a pre-incubation of the cells with the anti-CD3ε antibody, 145-2C11, to induce TCR aggregation on the cell membrane. This 'pre-clustering' was done in an attempt to exclude temperature effects on TCR mobility, which in turn would be expected to affect the tetramer staining levels. Following clustering with anti-CD3ε at 37°C, cells were treated with cytochalasin D to block tetramer-induced internalization and incubated with tetramer at different temperatures (Fig. 4A). The concentration of tetramer used (15 μg/ml) in these analyses was not saturating (Fig. 3B). HRC.1 and HRC.2 behave very similarly, and therefore data only for HRC.1 is shown. The HRC.1 and HCQ.4 hybridomas show greater (normalized) staining at 12 and 25 than at 37°C, whereas the reverse is observed for the HDB.2 hybridoma. Similar results were seen for the HDB.2 hybridoma with mCII256–270:A^q tetramers (data not shown), indicating that this temperature dependence is not a unique property of the HDB.2 TCR interaction with bCII256–270:A^q. A decrease in affinity with temperature in other systems has been interpreted to indicate that the corresponding TCR–pMHC interaction involves induced fit and is entropically unfavorable, i.e. 'plastic' (32–34). Our data suggest that for three of four of the TCR–pMHC interactions analyzed here, this is also the case. In contrast, the HDB.2 TCR–pMHC interaction appears to be thermodynamically distinct.

The tetramer staining levels were also analyzed in the absence of anti-CD3 treatment (Fig. 4B). Several differences in the results become apparent. First, the staining levels of HRC.1 and HCQ.4 relative to those of HDB.2 become much lower, although the pattern of temperature dependence for HCQ.4 is the same. Anti-CD3ε pretreatment reduces TCR (H75-597) staining levels to similar extents for the hybridomas analyzed (data not shown). Thus, it is improbable that this pretreatment results in the differential effects of the staining procedures on the HCQ.4 and HDB.2 hybridomas. Second, at 37°C, HRC.1 cells show slightly higher levels of tetramer staining than at 12 and 25°C. These differences might relate to differences in mobility of the TCRs of distinct hybridomas in the membrane that in turn might affect the temperature dependence of tetramer staining. For this reason, we predict that by pre-clustering the TCR with anti-CD3ε antibody, we obtain a more accurate representation of the nature of the corresponding TCR–pMHC interaction. Nevertheless, the marked increase in staining of the HDB.2 TCR at 37°C is apparent under all of the staining conditions used.

Sequences of the complementarity-determining region (CDRs) of the TCRs

The unusual temperature dependence of the HDB.2 TCR–pMHC interactions prompted us to analyze the sequences of the CDRs of the corresponding TCRs (14). For both TCR α and β chains, the lengths and amino acid composition of CDR1 and CDR2 are similar for the HDB.2 TCR and at least two of the three other TCRs (Fig. 5). However, CDR3α of the HDB.2 TCR is one residue shorter relative to the other three TCRs. It is possible that this may contribute to the apparent decreased flexibility of this TCR, although additional biophysical/structural studies are needed to confirm this. In contrast, CDR3β of HDB.2 is at least two residues longer than that of the other TCRs and also contains two central glycines, suggesting that this region may be more flexible.

Discussion

In the current study we describe the production and characterization of tetrameric pMHC complexes comprising bovine or mouse CII256–270 bound to the MHC class II molecule, A^q. Much evidence supports the idea that CD4$^+$ T cells are involved in pathogenesis in murine CIA (3,57–60). In addition, and of direct relevance to the current analyses, multiple studies indicate that bCII256–270 and mCII256–270 are highly immunodominant following immunization of mice with heterologous CII (7–12). bCII256–270 differs from mCII256–270 by a glutamic to aspartic acid change at position 266 and the epitopes are therefore closely related. Substitution of position 266 with alanine or aspartic acid (to generate the self-peptide) leads to unresponsiveness of all T

TCR α chain	CDR1	CDR2	CDR3
HRC.1 (Vα2.3)	ENSAFDY	SILSVSDK	SANNNNRI
HRC.2 (Vα2.3)	ENSAFDY	SILSVSDK	SANNNNRI
HCQ.4 (Vα4.5)	STTGYPT	KVTTANNK	VRNNNNRI
HDB.2 (Vα2.4)	EDSTFNY	SILSVSDK	SANNNRI

TCR β chain	CDR1	CDR2	CDR3
HRC.1 (Vβ20)	EKGHTA	YFQNQQPLDQIDM	SQNSAETL
HRC.2 (Vβ20)	EKGHTA	YFQNQQPLDQIDM	SPHTGQL
HCQ.4 (Vβ12)	*N.D.	YFRSKSLMEDGGA	SLWGNYAEQ
HDB.2 (Vβ11)	ISGHSA	YFRNQAPIDDSGM	SSTTGGANERL

*N.D.: Not determined

Fig. 5. Comparison of sequences of CDRs for the TCRs of the T cell hybridomas HRC.1, HRC.2, HDB.2 and HCQ.4 (14).

cell hybridomas analyzed (HDB.2, HRC.1, HRC.2 and HCQ.4) by peptide-pulsed APCs (8,9). To date it has not been possible to distinguish whether this effect is due to differences in TCR recognition or peptide–MHC association. This question is of central importance to understand the behavior of CII-specific T cells *in vivo*; if self-CII256–270 (or an equivalent overlapping epitope) is efficiently presented, the T cells would be subjected to tolerance induction, whereas, if not, ignorance would be predicted which could explain the relative inefficiency of inducing arthritis with mouse CII (1,48). By covalently tethering mCII256–270 to A^q in recombinant complexes to generate 'high-density' antigen complexes, we have obtained a clear answer to this question. We demonstrate that inefficient presentation of this peptide rather than poor TCR recognition most likely leads to low arthritogenicity. Indeed, we find that essentially all CII256–270-specific T cells can recognize both heterologous and self-CII256–270 bound to A^q. An alternative explanation for our observations is that the recombinant mCII256–270:A^q complexes are antigenically distinct from complexes formed by mCII256–270-pulsed APCs. Although we cannot exclude this possibility, it is highly improbable that the hybridomas analyzed here would all be equally sensitive to such a potential difference and that these effects would not be seen for bCII256–270:A^q complexes.

Our results provide an explanation for a number of previous observations. The original finding that immunization with mouse CII induces a stronger T cell response to rat CII rather than mouse CII, i.e. a heteroclitic response (8), was correctly interpreted based on earlier findings on T cell recognition of cytochrome *c* (61). The observation that many T cell hybridomas raised against bovine CII cross-react with only very high concentrations of the mouse peptide *in vitro* can be explained by the fact that high peptide concentrations may compensate for low-affinity binding to the MHC molecule. It follows from this observation that T cell recognition, and thereby arthritis pathogenesis, is most likely different between H-2^q mice immunized with mouse CII (48) and mutated mouse collagen (MMC) mice (i.e. mice expressing glutamic acid at position 266 of CII) immunized with rat CII (7). In normal H-2^q mice immunized with mouse CII, the low frequency of arthritis is best explained by T cell ignorance, whereas partial T cell tolerance appears to operate in the MMC mice. A similar situation to that in the MMC mouse probably also occurs in mice humanized for both human CII and DR4, in which T cells are tolerized more efficiently to the aglycosylated, CII259-273 peptide compared with the glycosylated, modified forms of the peptide (62).

Analysis of responding T cells from H-2^q mice immunized with bovine CII indicates that following re-stimulation *in vitro*, a significant proportion (~40%) of T cells are CII256–270:A^q specific. However, a large proportion is not recognized by either of the recombinant bCII256–270:A^q or mCII256–270:A^q complexes. A probable explanation for this is that a high proportion of CII:A^q-specific T cells only recognizes glycosylated variants of the CII256–270 peptide (14), whereas the recombinant bCII256–270:A^q complexes represent only the aglycosylated form of the epitope. In this context, expression of recombinant CII in insect cells results in poor hydroxylation of lysine residues (63) and this post-translational modification is needed for glycosylation of CII256–270 (14). To analyze CII-specific T cells that are directed towards glycoforms of the immunodominant CII256–270 peptide, it will be necessary to make 'empty' A^q that can be subsequently loaded with distinct glycopeptides to analyze glycopeptide-specific cells; however, given the instability of empty I-A or I-A with weakly bound peptides (24), this may not be feasible. Still, however, T cells directed towards aglycosylated forms of the CII peptide play an important regulatory role in the disease as recently demonstrated in experiments showing that expression of only aglycosylated collagen in the skin can protect from arthritis (30) and with vaccination studies using aglycosylated peptides (11). Interestingly, T cell tolerance seems to operate more efficiently to the unmodified form of the peptide and it is possible that position 266 is exposed differently to glycopeptide specific T cells as a subset of such clones is dependent on a glutamic acid at this position for recognition (64).

We have also used the recombinant CII256–270:A^q tetramers to analyze the avidities of the CII-specific hybridomas. One of the TCRs (HDB.2) specific for the aglycosylated CII256–270 peptide apparently has a high avidity for antigen and cross-reacts strongly with mCII256–270:A^q. The high avidity of the HDB.2 TCR for antigen, together with the cross-reactive recognition of autoantigen, might be representative of a class of TCRs that are borne by highly pathogenic T cells. In this context, in both the NOD and EAE models, high-avidity recognition of autoantigen correlates with disease activity (23,65).

The use of bCII256–270:A^q tetramers to analyze the temperature dependence of the TCR–pMHC interactions corresponding to HDB.2, HRC.1, HRC.2 and HCQ.4 hybridomas indicates some interesting differences in behavior. The HRC.1, HRC.2 and HCQ.4 hybridomas all show greater binding of multimer at lower temperature (12 and 25 versus 37°C), consistent with the avidity decrease with temperature that is observed for other TCR–pMHC interactions in distinct antigen recognition systems (32–34). This temperature dependence of affinity/avidity is related to the unfavorable entropic terms that to date have appeared to be a representative feature of TCR–pMHC interactions (32–34) and has provided support for the concept that TCRs are highly plastic (36). However, here we show that the HDB.2 TCR shows a

reverse temperature dependence, i.e. higher avidity at 37 than at 12°C. This suggests that the HDB.2 TCR might interact with cognate ligand in an unusual way that may not involve the same degree of plasticity as that described for other TCRs (32–34). The unusual temperature dependence of this TCR–pMHC interaction has prompted us to carry out a comparative analysis of the CDR sequences of the TCRs of the hybridomas (Fig. 5). There are not marked differences in sequences or length of the CDR1 and CDR2 loops of the HDB.2 TCR relative to at least a subset of the other TCRs, whereas there are differences in CDR3 lengths for both α and β chains of this TCR. For example, the HDB.2 CDR3α is one residue shorter than that of the other three TCRs and it is therefore possible that it is more constrained, which in turn might lead to lower conformational flexibility. In contrast, comparison of the CDR3β of the HDB.2 TCR indicates that it is at least two residues longer than the CDR3β of the other TCRs. In addition to the greater CDR3β length, the presence of two central glycines in the center of CDR3β would suggest that this loop is highly flexible, which would be inconsistent with a less flexible TCR. Taking into consideration the unusual temperature dependence of the HDB.2 TCR–pMHC interaction, analysis of the CDR3 sequences therefore leads to the suggestion that the HDB.2 V_α domain may play a dominant role in the corresponding TCR–pMHC interaction, with the CDR3β loop/V_β domain making more limited contacts with antigen. This would be analogous to the dominance of V_α domain contacts in other TCR–pMHC interactions [(35–38), reviewed in (39)]. However, in the absence of structural/biophysical data, the TCR docking configuration and loop flexibility obviously cannot be predicted with any degree of certainty. Quantitative analysis of the thermodynamics and molecular details of this TCR–pMHC interaction will be an area of future investigation.

In summary, we have used recombinant bCII256–270 and mCII256–270 multimers to characterize the properties of CII-specific T cells associated with murine CIA. Unexpectedly, we have found that these T cells are highly cross-reactive and therefore potentially autoreactive *in vivo*. We have shown that the TCR–pMHC interaction in this model can be accompanied by unusual temperature dependence, suggesting that decreasing affinity with temperature increase is not a universal feature of TCR–pMHC complex formation. Thus, the multimers can be used to evaluate several features of T cell recognition in this system. Future studies will be focused on using them to evaluate the factors, which at the level of the TCR–pMHC interaction, lead to pathogenesis.

Acknowledgements

We thank Silvia Pastor for assistance with flow cytometric analysis and Mihail Firan for assistance with the generation of expression plasmids. This work was supported by an Arthritis Foundation Research Grant (E. S. W), the National Institutes of Health (RO1 AI42949; E. S. W.) and the Swedish Medical Research Council (R. H). A. M. was supported by FIS (FIS-00/5030 and FIS-01/5037).

Abbreviations

APC	antigen-presenting cell
CII	type II collagen
CDR	complementarity-determining region
CIA	collagen-induced arthritis
EAE	experimental autoimmune encephalomyelitis
MBP	myelin basic protein
MMC	mutated mouse collagen
MOG	myelin oligodendrocyte glycoprotein
NOD	non-obese diabetes
PE	phycoerythrin
pMHC	peptide–MHC

References

1 Courtenay, J. S., Dallman, M. J., Dayan, A. D., Martin, A. and Mosedale, B. 1980. Immunisation against heterologous type II collagen induces arthritis in mice. *Nature* 283:666.
2 Stuart, J. M. and Dixon, F. J. 1983. Serum transfer of collagen-induced arthritis in mice. *J. Exp. Med.* 158:378.
3 Seki, N., Sudo, Y., Yoshioka, T., Sugihara, S., Fujitsu, T., Sakuma, S., Ogawa, T., Hamaoka, T., Senoh, H. and Fujiwara, H. 1988. Type II collagen-induced murine arthritis. I. Induction and perpetuation of arthritis require synergy between humoral and cell-mediated immunity. *J. Immunol.* 140:1477.
4 Wooley, P. H., Luthra, H. S., Stuart, J. M. and David, C. S. 1981. Type II collagen-induced arthritis in mice. I. Major histocompatibility complex (I region) linkage and antibody correlates. *J. Exp. Med.* 154:688.
5 Wooley, P. H., Dillon, A. M., Luthra, H. S., Stuart, J. M. and David, C. S. 2001. Genetic control of type II collagen induced arthritis in mice: factors influencing disease susceptibility and evidence for multiple MHC-associated gene control. *Transplant. Proc.* 157:180.
6 Brunsberg, U., Gustafsson, K., Jansson, L., Michaëlsson, E., Åhrlund-Richter, L., Pettersson, S., Mattsson, R. and Holmdahl, R. 1994. Expression of a transgenic class II Ab gene confers susceptibility to collagen-induced arthritis. *Eur. J. Immunol.* 24:1698.
7 Malmström, V., Michaëlsson, E., Burkhardt, H., Mattsson, R., Vuorio, E. and Holmdahl, R. 1996. Systemic versus cartilage-specific expression of a type II collagen-specific T-cell epitope determines the level of tolerance and susceptibility to arthritis. *Proc. Natl Acad. Sci. USA* 93:4480.
8 Michaëlsson, E., Andersson, M., Engström, Å. and Holmdahl, R. 1992. Identification of an immunodominant type-II collagen peptide recognized by T cells in H-2^q mice: self tolerance at the level of determinant selection. *Eur. J. Immunol.* 22:1819.
9 Michaëlsson, E., Broddefalk, J., Engström, Å., Kihlberg, J. and Holmdahl, R. 1996. Antigen processing and presentation of a naturally glycosylated protein elicits major histocompatibility complex class II-restricted, carbohydrate-specific T cells. *Eur. J. Immunol.* 26:1906.
10 Rosloniec, E. F., Whittington, K. B., Brand, D. D., Myers, L. K. and Stuart, J. M. 1996. Identification of MHC class II and TCR binding residues in the type II collagen immunodominant determinant mediating collagen-induced arthritis. *Cell. Immunol.* 172:21.
11 Myers, L. K., Seyer, J. M., Stuart, J. M., Terato, K., David, C. S. and Kang, A. H. 1993. T cell epitopes of type II collagen that regulate murine collagen-induced arthritis. *J. Immunol.* 151:500.
12 Brand, D. D., Myers, L. K., Terato, K., Whittington, K. B., Stuart, J. M., Kang, A. H. and Rosloniec, E. F. 1994. Characterization of the T cell determinants in the induction of autoimmune arthritis by bovine α 1(II)-CB11 in H-2^q mice. *J. Immunol.* 152:3088.
13 Kjellén, P., Brunsberg, U., Broddefalk, J., Hansen, B., Vestberg, M., Ivarsson, I., Engström, Å., Svejgaard, A., Kihlberg, J., Fugger, L. and Holmdahl, R. 1998. The structural basis of MHC control of collagen-induced arthritis; binding of the immunodominant type II collagen 256–270 glycopeptide to H-$2A^q$ and H-$2A^p$ molecules. *Eur. J. Immunol.* 28:755.
14 Corthay, A., Bäcklund, J., Broddefalk, J., Michaëlsson, E., Goldschmidt, T. J., Kihlberg, J. and Holmdahl, R. 1998. Epitope glycosylation plays a critical role for T cell recognition of type II collagen in collagen-induced arthritis. *Eur. J. Immunol.* 28:2580.
15 Rosloniec, E. F., Brand, D. D., Whittington, K. B., Stuart, J. M., Ciubotaru, M. and Ward, E. S. 1995. Vaccination with a

recombinant V_α domain of a TCR prevents the development of collagen-induced arthritis. *J. Immunol.* 155:4504.
16 Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J. and Davis, M. M. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94.
17 Lee, P. P., Yee, C., Savage, P. A., Fong, L., Brockstedt, D., Weber, J. S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P. D., Roederer, M. and Davis, M. M. 1999. Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat. Med.* 5:677.
18 Busch, D. H. and Pamer, E. G. 1999. T cell affinity maturation by selective expansion during infection. *J. Exp. Med.* 189:701.
19 Crawford, F., Kozono, H., White, J., Marrack, P. and Kappler, J. 1998. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity* 8:675.
20 Savage, P. A., Boniface, J. J. and Davis, M. M. 1999. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity* 10:485.
21 Rees, W., Bender, J., Teague, T. K., Kedl, R. M., Crawford, F., Marrack, P. and Kappler, J. 1999. An inverse relationship between T cell receptor affinity and antigen dose during $CD4^+$ T cell responses *in vivo* and *in vitro*. *Proc. Natl Acad. Sci. USA* 96:9781.
22 Gebe, J. A., Falk, B. A., Rock, K. A., Kochik, S. A., Heninger, A. K., Reijonen, H., Kwok, W. W. and Nepom, G. T. 2003. Low-avidity recognition by $CD4^+$ T cells directed to self antigen. *Eur. J. Immunol.* 33:1409.
23 Anderton, S. M., Radu, C. G., Lowrey, P. A., Ward, E. S. and Wraith, D. C. 2001. Negative selection during the peripheral immune response to antigen. *J. Exp. Med.* 193:1.
24 Radu, C. G., Anderton, S. M., Firan, M., Wraith, D. C. and Ward, E. S. 2000. Detection of autoreactive T cells in $H\text{-}2^u$ mice using peptide–MHC multimers. *Int. Immunol.* 12:1553.
25 Liu, C. P., Jiang, K., Wu, C. H., Lee, W. H. and Lin, W. J. 2000. Detection of glutamic acid decarboxylase-activated T cells with $I\text{-}A^{g7}$ tetramers. *Proc. Natl Acad. Sci. USA* 97:14596.
26 Kozono, H., White, J., Clements, J., Marrack, P. and Kappler, J. 1994. Production of soluble MHC class II proteins with covalently bound single peptides. *Nature* 369:151.
27 Scott, C. A., Garcia, K. C., Carbone, F. R., Wilson, I. A. and Teyton, L. 1996. Role of chain pairing for the production of functional soluble IA major histocompatibility complex class II molecules. *J. Exp. Med.* 183:2087.
28 Radu, C. G., Ober, B. T., Colantonio, L., Qadri, A. and Ward, E. S. 1998. Expression and characterization of recombinant soluble peptide: I-A complexes associated with murine experimental autoimmune diseases. *J. Immunol.* 160:5915.
29 Scott, C. A., Peterson, P. A., Teyton, L. and Wilson, I. A. 1998. Crystal structures of two $I\text{-}A^d$–peptide complexes reveal that high affinity can be achieved without large anchor residues. *Immunity* 8:319.
30 Bäcklund, J., Treschow, A., Firan, M., Malmström, V., Issazadeh-Navikas, S., Ward, E. S. and Holmdahl, R. 2002. Reversal of tolerance induced by transplantation of skin expressing the immunodominant T cell epitope of rat type II collagen entitles development of collagen-induced arthritis but not graft rejection. *Eur. J. Immunol.* 32:1773.
31 Davis, M. M., Boniface, J. J., Reich, Z., Lyons, D., Hampl, J., Arden, B. and Chien, Y. 1998. Ligand recognition by $\alpha\beta$ T cell receptors. *Annu. Rev. Immunol.* 16:523.
32 Willcox, B. E., Gao, G. F., Wyer, J. R., Ladbury, J. E., Bell, J. I., Jakobsen, B. K. and van der Merwe, P. A. 1999. TCR binding to peptide–MHC stabilizes a flexible recognition interface. *Immunity* 10:357.
33 Boniface, J. J., Reich, Z., Lyons, D. S. and Davis, M. M. 1999. Thermodynamics of T cell receptor binding to peptide–MHC: evidence for a general mechanism of molecular scanning. *Proc. Natl Acad. Sci. USA* 96:11446.
34 Garcia, K. C., Radu, C., Ho, J., Ober, R. J. and Ward, E. S. 2001. Kinetics and thermodynamics of T cell receptor–autoantigen interactions in murine experimental autoimmune encephalomyelitis. *Proc. Natl Acad. Sci. USA* 98:6818.
35 Garboczi, D. N., Ghosh, P., Utz, U., Fan, Q. R., Biddison, W. E. and Wiley, D. C. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* 384:134.
36 Garcia, K. C., Degano, M., Pease, L. R., Huang, M., Peterson, P. A., Teyton, L. and Wilson, I. A. 1998. Structural basis of plasticity in T cell receptor recognition of a self peptide–MHC antigen. *Science* 279:1166.
37 Ding, Y. H., Baker, B. M., Garboczi, D. N., Biddison, W. E. and Wiley, D. C. 1999. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity* 11:45.
38 Reinherz, E. L., Tan, K., Tang, L., Kern, P., Liu, J., Xiong, Y., Hussey, R. E., Smolyar, A., Hare, B., Zhang, R., Joachimiak, A., Chang, H. C., Wagner, G. and Wang, J. 1999. The crystal structure of a T cell receptor in complex with peptide and MHC class II. *Science* 286:1913.
39 Garcia, K. C., Teyton, L. and Wilson, I. A. 1999. Structural basis of T cell recognition. *Annu. Rev. Immunol.* 17:369.
40 Bhardwaj, V., Kumar, V., Geysen, H. M. and Sercarz, E. E. 1993. Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells. Implications for thymic education and autoimmunity. *J. Immunol.* 151:5000.
41 Reay, P. A., Kantor, R. M. and Davis, M. M. 1994. Use of global amino acid replacements to define the requirements for MHC binding and T cell recognition of moth cytochrome *c* (93–103). *J. Immunol.* 152:3946.
42 Kersh, G. J. and Allen, P. M. 1996. Structural basis for T cell recognition of altered peptide ligands: a single T cell receptor can productively recognize a large continuum of related ligands. *J. Exp. Med.* 184:1259.
43 Wucherpfennig, K. W. and Strominger, J. L. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80:695.
44 Mason, D. 1998. A very high level of cross-reactivity is an essential feature of the T-cell receptor. *Immunol. Today* 19:395.
45 Fujinami, R. S. and Oldstone, M. B. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science* 230:1043.
46 Oldstone, M. B. 1987. Molecular mimicry and autoimmune disease. *Cell* 50:819.
47 Ufret-Vincenty, R. L., Quigley, L., Tresser, N., Pak, S. H., Gado, A., Hausmann, S., Wucherpfennig, K. W. and Brocke, S. 1998. *In vivo* survival of viral antigen-specific T cells that induce experimental autoimmune encephalomyelitis. *J. Exp. Med.* 188:1725.
48 Holmdahl, R., Jansson, L., Larsson, E., Rubin, K. and Klareskog, L. 1986. Homologous type II collagen induces chronic and progressive arthritis in mice. *Arthritis Rheum.* 29:106.
49 Urban, J. L., Kumar, V., Kono, D. H., Gomez, C., Horvath, S. J., Clayton, J., Ando, D. G., Sercarz, E. E. and Hood, L. 1988. Restricted use of T cell receptor V genes in murine autoimmune encephalomyelitis raises possibilities for antibody therapy. *Cell* 54:577.
50 Tessier, D. C., Thomas, D. Y., Khouri, H. E., Laliberte, F. and Vernet, T. 1991. Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. *Gene* 98:177.
51 Abdul-Majid, K. B., Jirholt, J., Stadelmann, C., Stefferl, A., Kjellén, P., Wallström, E., Holmdahl, R., Lassmann, H., Olsson, T. and Harris, R. A. 2000. Screening of several H-2 congenic mouse strains identified $H\text{-}2^q$ mice as highly susceptible to MOG-induced EAE with minimal adjuvant requirement. *J. Neuroimmunol.* 111:23.
52 Janeway, C. A. J., Conrad, P. J., Lerner, E. A., Babich, J., Wettstein, P. and Murphy, D. B. 1984. Monoclonal antibodies specific for Ia glycoproteins raised by immunization with activated T cells: possible role of T cell bound Ia antigens as targets of immunoregulatory T cells. *J. Immunol.* 132:662.
53 Qadri, A., Radu, C. G., Thatte, J., Cianga, P., Ober, B. T., Ober, R. J. and Ward, E. S. 2000. A role for the region encompassing the c″ strand of a TCR V_α domain in T cell activation events. *J. Immunol.* 165:820.
54 Patten, P. A., Rock, E. P., Sonoda, T., Fazekas, D. S., Jorgensen, J. L. and Davis, M. M. 1993. Transfer of putative complementarity-determining region loops of T cell receptor V domains confers

toxin reactivity but not peptide/MHC specificity. *J. Immunol.* 150:2281.
55 Thatte, J., Qadri, A., Radu, C. and Ward, E. S. 1999. Molecular requirements for T cell recognition by a major histocompatibility complex class II-restricted T cell receptor: the involvement of the fourth hypervariable loop of the V_α domain. *J. Exp. Med.* 189:509.
56 Sadegh-Nasseri, S. and Germain, R. N. 1991. A role for peptide in determining MHC class II structure. *Nature* 353:167.
57 Myers, L. K., Tang, B., Rosloniec, E. F., Stuart, J. M., Chiang, T. M. and Kang, A. H. 1998. Characterization of a peptide analog of a determinant of type II collagen that suppresses collagen-induced arthritis. *J. Immunol.* 161:3589.
58 Mauri, C., Williams, R. O., Walmsley, M. and Feldmann, M. 1996. Relationship between T_h1/T_h2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. *Eur. J. Immunol.* 26:1511.
59 Ehinger, M., Vestberg, M., Johansson, Å. C., Johannesson, M., Svensson, A. and Holmdahl, R. 2001. Influence of CD4 or CD8 deficiency on collagen-induced arthritis. *Immunology* 103:291.
60 Corthay, A., Johansson, A., Vestberg, M. and Holmdahl, R. 1999. Collagen-induced arthritis development requires αβ T cells but not γδ T cells: studies with T cell-deficient (TCR mutant) mice. *Int. Immunol.* 11:1065.
61 Schwartz, R. H. 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu. Rev. Immunol.* 3:237.
62 Bäcklund, J., Carlsen, S., Höger, T., Holm, B., Fugger, L., Kihlberg, J., Burkhardt, H. and Holmdahl, R. 2002. Predominant selection of T cells specific for the glycosylated collagen type II epitope (263–270) in humanized transgenic mice and in rheumatoid arthritis. *Proc. Natl Acad. Sci. USA* 99:9960.
63 Myers, L. K., Brand, D. D., Ye, X. J., Cremer, M. A., Rosloniec, E. F., Bodo, M., Myllyharju, J., Helaakoski, T., Nokelainen, M., Pihlajaniemi, T., Kivirikko, K., Yang, C. L., Ala-Kokko, L., Prockop, D. J., Notbohm, H., Fietzek, P., Stuart, J. M. and Kang, A. H. 1998. Characterization of recombinant type II collagen: arthritogenicity and tolerogenicity in DBA/1 mice. *Immunology* 95:631.
64 Bäcklund, J., Treschow, A., Bockermann, R., Holm, B., Issazadeh-Navikas, S., Kihlberg, J. and Holmdahl, R. 2002. The importance of glycosylated type II collagen for T cell tolerance and the pathology in collagen-induced arthritis. *Eur. J. Immunol.* 32:3776.
65 Amrani, A., Verdaguer, J., Serra, P., Tafuro, S., Tan, R. and Santamaria, P. 2000. Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature* 406:739.