EXTENDED REPORT

T cell responses to a non-glycosylated epitope predominate in type II collagen-immunised HLA-DRB1*0101 transgenic mice

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Aim: To study collagen-induced arthritis in human leucocyte antigen (HLA)-DR1 transgenic mice lacking endogenous major histocompatibility complex class II molecules (MHC-II) and to determine T cell specificity against the arthritogenic CII_{259–273} epitope of type II collagen either unmodified or post-translationally glycosylated at Lys₂₆₄.

Methods: Arthritis was induced by immunisation with human type II collagen in complete Freund's adjuvant and measured by footpad swelling, clinical score and histology. T cell responses were assessed by proliferation of spleen and lymph node cells and in antigen presentation assays, using T cell hybridomas specific for the glycosylated and non-glycosylated CII_{259–273} epitope.

Results: The incidence of arthritis was 50% in DR1-transgenic mice lacking endogenous MHC-II molecules. Recall T cell responses in draining lymph nodes and spleen were consistently greater against the non-glycosylated epitope than to the glycosylated CII_{259–273}. Most of the T cell hybridomas generated from CII-immunised mice recognised the non-glycosylated CII epitope and this form of the epitope was also presented with 100-fold higher efficiency and 1 h faster kinetics by both macrophages and dendritic cells.

Conclusion: This study shows that T cell responses to the non-glycosylated epitope of heterologous (human) CII are dominant in HLA-DR1 transgenic mice lacking MHC-II, which could contribute to the pathogenicity of autoimmune arthritis.

The contribution of human leucocyte antigen (HLA)-DRB1 alleles to the pathogenesis of rheumatoid arthritis (RA) is estimated as 40%.¹ In particular, the expression of HLA-DR4 (HLA-DRB1*0401) and HLA-DR1 (HLA-DRB1*0101) has been shown to predispose to RA.^{2–5} HLA-DRB1*0401 is associated with severe erosive RA, whereas expression of HLA-DRB1*0101 is associated with less severe non-erosive disease.^{5–7}

The primary structure of the peptide-binding pocket of mouse major histocompatibility complex class II (MHC-II) A^q molecules has been shown to be very similar to that of HLA-DRB1*0401 and HLA-DRB1*0101⁸ and expression of A^q confers susceptibility to collagen-induced arthritis (CIA).^{9–10} Mice expressing non-susceptible MHC haplotypes (ie, not H-2^q or H-2^{t11}) that express HLA-DRB1*0401 or HLA-DRB1*0101 also acquire susceptibility to CIA^{12–14} but it is not known whether the endogenous mouse MHC class molecules contribute in anyway to the induction or severity of arthritis.

T cells from type II collagen (CII)-immunised HLA-DRB1*0401 and HLA-DRB1*0101 mice predominantly recognise the same immunodominant CII_{259–273} epitope.^{11–13 15 16} In HLA-DRB1*0401 CD4 double transgenic mice lacking MHC-II, the T cells recognised many different forms of the peptides including the glycosylation of the Lys₂₆₄ residue within the CII_{259–273} epitope.^{17–19} Interestingly, when these mice expressed humanised CII the response to the glycosylated structure was relatively stronger indicating a less well-developed tolerance to post-translationally modified CII epitopes.^{14 17}

METHODS

Antigens

Human CII was purchased from MD BioSciences (Zürich, Switzerland). The glycosylated peptide (GIAGFKGEQGPGET; K = GalHyL₂₆₄) corresponding to epitope CII_{259–273} was synthesised.²⁰ The non-glycosylated peptide pCII_{259–273} was

purchased from GenScript Corp (Piscataway, New Jersey, USA).

Animals

Transgenic (tg) mice carried full-length genomic constructs for HLA-DRA1*0101 and HLA-DRB1*0101²¹ and were crossed for more than six generations to C57BL/6 A^b null mice.²² Breeding founders were genotyped by PCR for the presence of the HLA transgenes and HLA-DR expression was monitored by flow cytometry.^{23 24} The proportion of T cells, B cells, macrophages and dendritic cells as well as the level of expression of MHC II molecules (HLA-DR1 compared with A^b) and T cell receptor (TCR) Vβ family distribution were comparable between HLA-DR1-tg mice and C57BL/6, the background mouse strain (data not shown). The work was approved by the ethics committee of the Newcastle University.

Collagen-induced arthritis

CIA was induced by immunisation at the base of the tail with 100 µg human CII in complete Freund's adjuvant (CFA, Chondrex, Washington, USA). The same dose of CII in incomplete Freund's adjuvant (IFA) was given 3 weeks later. The severity of CIA was monitored as the thickness of the hind paws using an Oditest dial caliper gauge (reading error of 0.01 mm, Kroeplin GmbH, Schüchtern, Germany). Clinical scoring was performed using an established scoring system: 0 = normal joints, 1 = 1 or 2 swollen joints, 2 = >2 swollen

Abbreviations: APC, antigen presenting cells; CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; DC, dendritic cells; FITC, fluorescein isothiocyanate; HLA, human leucocyte antigen; IFA, incomplete Freund's adjuvant; MHC-II, major histocompatibility complex class-II; PBS, phosphate-buffered saline; PE, phycoerythrin; RA, rheumatoid arthritis; TCR, T cell receptor; TG, transgenic

Figure 1 Clinical scoring of collagen-induced arthritis (CIA). CIA was induced by single immunisation with type II collagen (CII)/ complete Freund's adjuvant (CFA; filled squares) or followed by a second immunisation with CII/IFA 3 weeks later (filled circles). Mice received phosphate-buffered saline/CFA as a negative control (empty squares). Hind paw thickness (A), clinical score (B) and incidence of arthritis (C) were measured. Data shown as mean and SEM of 12 mice per group. PBS, phosphate-buffered saline.

joints and 3 = extreme swelling of the entire paw and/or ankylosis.¹²

Cells

Culture media ingredients were from Sigma Chemical Co (Dorset, UK). Cells were grown in culture medium (RPMI 1640 medium containing 3 mM L-glutamine, 50 μ M 2-mercaptoethanol, 10% fetal bovine serum and 30 μ g/ml gentamycin). T cell hybridomas were generated by polyethylene-glycol fusion of BW5147 (TCR α β) cells with T cell lines from CII-immunised mice after six rounds of restimulation. Macrophages (purity ~95% based on CD11b expression) were grown from femoral bone marrow cells in culture medium containing L929 supernatant as a source of macrophage colony stimulating factor (M-CSF).²³ Macrophages were activated with 10 U/ml recombinant IFN γ (R&D Systems, Abingdon, UK) for 24 h.²³ Bone marrow dendritic cells (DC, purity ~92% based on CD11c expression) were grown in culture medium containing 20 ng/ml recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF, BioSource International, Nivelles, Belgium). DC were purified using anti-CD11c-coated magnetic microbeads (Miltenyi Biotec, Bisley, UK) and maturation was induced by 0.2 μ g/ml lipopolysaccharide for 24 h.²⁴

Proliferation assays

CII-specific T cell proliferation responses were measured in popliteal lymph nodes removed 7 days after immunisation with 50 μ g CII emulsified 1:1 in TiterMax adjuvant (Sigma Chemical Co) or phosphate-buffered saline (PBS)/TiterMax. Responses of spleen cells were measured in mice with CIA. Cells (2×10^5 /well) were mixed with antigens or the polyclonal T cell mitogen

concanavalin A (5.0 μ g/ml) and incubated for 4 days, including 14.8 kBq of [3 H]-thymidine for the past 18 h and radioactivity was measured as described below.

Antigen presentation assays

Adherent macrophages at 10^5 /well or DC at 10^4 /well were pulsed with a dilution series of CII or synthetic peptides, fixed with 1.0% paraformaldehyde, and T cell hybridoma HCII-9.1 ((CD3+ TCR α β + V β 14+) specific for the non-glycosylated peptide) or HCII-9.2 ((CD3+ TCR α β + V β 8.1/8.2+) specific for the glycosylated peptide) were added (5×10^4 /well) and incubated for 24 h at 37°C.²³ Responses to synthetic peptides were used as a positive control for T cell hybridomas. The interleukin (IL)2 content of hybridoma supernatants was measured as proliferation of the cytotoxic T cell line 2 (CTL-2, 3×10^4 /well, American Type Culture Collection ATCC, TIB 214, Rockville, Maryland, USA) incubated for 18 h in the presence of 14.8 kBq of [3 H]thymidine (TRA310, specific activity 307 MBq/mg; Amersham International plc, Didcot, UK). Cells were harvested on glass fibre membranes and radioactivity was quantitated using a direct beta counter (Matrix 9600, Packard Instrument Company, Meriden, Connecticut, USA). To measure non-specific, polyclonal responses of T cell hybridomas, a dilution series of rat anti-CD3 antibodies (clone KT3, generous gift of Dr CG Brooks, Newcastle University) were coated on 96 well plates, and T cell hybridoma were added and assayed as above.

Histology

Arthritic and non-arthritic paws were removed and fixed in 7% formaldehyde pH 7.4, decalcified in 14% EDTA pH 7.4, and embedded in paraffin wax. Serial sections (5 μ m) of the joints were stained with H&E and examined as blinded samples at the Department of Cellular Pathology, Royal Victoria Infirmary, Newcastle upon Tyne, UK.

Flow cytometry

Cells were incubated for 30 min at 4°C in Hank's balanced salt solution containing 2% fetal bovine serum, 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid with purified anti-mouse CD16/CD32 (Fc Block, BD-PharMingen, Oxford, UK) followed by incubation for 30 min at 4°C with either of the following mAb fluorescent conjugates (BD-PharMingen) specific for: TCR α β /phycoerythrin (PE), HLA-DR/fluorescein isothiocyanate (FITC) or HLA-DR/PE, CD11c/FITC, CD11b/FITC, CD19/PE, CD4/FITC, CD8/FITC, I-A b /FITC or rat IgG2a/PE plus IgG2b/FITC as isotype controls. The TCR V β family distribution in the spleen was studied using a panel of FITC-conjugated mAb to mouse TCR V β chains (BD-PharMingen).²⁵ Cells were analysed with by flow cytometry (Becton Dickinson FACScan, Mount View, California, USA) and 10 000 events were collected and analysed for each sample using CellQuestPro software.

Statistical analysis

Statistical differences were analysed by two-way analysis of variance and two-tailed paired *t* tests. Analysis of variance Bonferroni post-tests were used to compare replicate means. The dose of CII that induced 50% T cell hybridoma responses (Effective dose₅₀, ED₅₀) was calculated according to the sigmoidal or bottom-to-top curve fitting models (Prism 4.03 and GraphPad StatMate 1.00, GraphPad Software Inc, San Diego, USA).

RESULTS

Development of collagen-induced arthritis

We induced CIA in HLA-DR1-tg mice lacking MHC-II by a single subcutaneous immunisation with 100 μ g CII emulsified

A CII/CFA immunised

B Untreated mice
Fore paw

Hind paw

Histology

Figure 2 Appearance of collagen-induced arthritis (CIA). Mice with CIA (A) but not untreated mice (B) displayed extensive rubor and oedema around metacarpophalangeal and interphalangeal joints (top two panels) and metatarsophalangeal and interphalangeal joints (middle two panels). H&E-stained sections are shown in the bottom two panels, highlighting synovial hyperplasia and inflammation (1), cartilage destruction (2), and bone resorption with pannus formation (3). Magnification $\times 400$.

in CFA. In some experiments CII/CFA immunisation was followed by boosting with CII/IFA. Arthritis was observed 30 days after immunisation independently of the immunisation protocol, evidenced by the increase in hind paw thickness and clinical score compared with mice immunised with PBS/CFA (fig 1A,B). The severity of arthritis peaked 45 days after immunisation (fig 1A,B) corresponding to the maximum incidence of 50% (fig 1C).

Macroscopically both hind and fore paws developed oedema and redness with pronounced loss of function compared with PBS/CFA-immunised mice (fig 2). Histological examination revealed cartilage loss, synovial inflammation and bone

destruction of all small joints inspected from representative hind-paws of three mice with CIA compared with control mice (fig 2). The data showed that mice were susceptible to CIA with clinical and histological features characteristic of RA.

Specificity of T cell responses

We studied the effect of CII₂₅₉₋₂₇₃ glycosylation on T cell specificity after CII/CFA immunisation. Sixty days after CII/CFA immunisation, CII-specific spleen T cell responses were detected only in arthritic mice (fig 3A,B). These T cell responses showed a bias towards specificity for the non-glycosylated, as compared with the glycosylated, CII₂₅₉₋₂₇₃ epitope ($p < 0.001$;

Figure 3 Type II collagen (CII)-specific T cell responses. Proliferation responses of spleen cells of mice immunised with CII/CFA with arthritis (A) or without arthritis (B) to CII (squares), non-glycosylated (circles) or glycosylated (diamonds) peptides were measured (C). Responses of popliteal lymph node cells of mice foot-pad immunised with CII/TiterMax (filled bars), or PBS/TiterMax (empty bars) are shown as mean + SD of three independent experiments and "*" indicates a significant difference between responses to the glycosylated and non-glycosylated epitope ($p < 0.001$). (D) The proportion of CII-specific T cell hybridomas generated from six CII-immunised mice that recognised CII and the glycosylated or non-glycosylated peptides. Glyc pept, glycosylated peptide; Non-glyc pept, non-glycosylated peptide.

fig 3A). Mice were also foot-pad immunised with CII/TiterMax before popliteal lymph node assays 7 days later. T cell proliferation was again biased to recognition of the non-glycosylated epitope in addition to a response to intact CII ($p < 0.001$; fig 3C).

Specificity of T cell hybridoma responses

We generated a panel of 44 CII-specific T cell hybridomas from six mice and assayed responses to the glycosylated and non-glycosylated CII₂₅₉₋₂₇₃ in an antigen presentation assay. Most CII-specific T cell hybridomas (42/44, 95.5%) recognised the non-glycosylated CII₂₅₉₋₂₇₃ epitope, and only two T cell hybridomas recognised the glycosylated epitope (fig 3D). Altogether, our data showed that immunisation of HLA-DR1-tg mice resulted in a predominant response to the non-glycosylated CII₂₅₉₋₂₇₃ epitope.

We addressed whether differences in processing and presentation of CII in professional antigen presenting cells (APCs) accounted for the observed expansion of CD4 T cells specific for the non-glycosylated CII₂₅₉₋₂₇₃ epitope. We assayed antigen presentation of the glycosylated and non-glycosylated CII₂₅₉₋₂₇₃ epitope from soluble CII by DC and macrophages using two T cell hybridomas with similar TCR affinities judged from dose-response titrations to synthetic peptides and anti-CD3 antibodies (fig 4), and levels of TCR expression by flow cytometry (data not shown). The requirement for proteolytic processing of soluble CII for presentation of the glycosylated and

non-glycosylated CII₂₅₉₋₂₇₃ was reported previously.²³ Presentation of the glycosylated, as compared with the non-glycosylated epitope, was characterised by significantly lower efficiency in both macrophages (100-fold difference) and DC (200-fold difference; fig 5A,B). Similarly, significantly delayed kinetics (by approximately 1 h) of antigen presentation was characteristic of the glycosylated, as compared with the non-glycosylated epitope, in macrophages and in DC (fig 5C,D). The data showed that CII was more efficiently processed by professional APCs for presentation of the non-glycosylated, compared with the glycosylated epitope. This apparent immunodominance of the non-glycosylated CII₂₅₉₋₂₇₃ in antigen presentation experiments in vitro is consistent with expansion of polyclonal T cells against this form of the epitope in HLA-DR1-tg mice in vivo, shown in fig 3.

DISCUSSION

We studied the development of CIA after CII immunisation in HLA-DR1-tg mice lacking MHC-II. In this study, a high severity of CIA was observed with the incidence rate of approximately 50%. By contrast, HLA-DR1-tg(H2^I) mice and HLA-DR4-tg mice lacking MHC-II were reported to be highly susceptible to CIA with 88–100% incidence rate.^{12–14} Previous population studies in patients with RA showed that different HLA-DRB1 alleles influence the severity of RA with HLA-DRB1*0401 being associated with severe, seropositive, erosive RA, whereas DRB1*0101 with less severe, seronegative, non-erosive

disease.⁵⁻⁷ Further studies will shed light on the reasons for the reduced incidence rate (but not the severity) of CIA in HLA-DR1-tg mice lacking MHC-II.

CII-specific T cells in HLA-DR4-tg mice were shown to recognise predominantly the non-glycosylated CII₂₅₉₋₂₇₃, but the response was skewed towards recognition of the glycosylated form when the human CII was transgenically expressed.¹⁷⁻¹⁹ We studied T cell responses in draining lymph nodes and spleen of HLA-DR1-tg mice after CII immunisation and showed a bias towards T cell responses specific for the non-glycosylated in preference to the glycosylated CII₂₅₉₋₂₇₃ epitope. This conclusion was confirmed by producing a panel of CII-specific T cell hybridomas, most of which recognised the non-glycosylated epitope. We addressed whether the APC type determined the predominance of T cells specific for the non-glycosylated CII₂₅₉₋₂₇₃ by studying antigen presentation of both forms of CII₂₅₉₋₂₇₃ to T cells by macrophages and DC, which have been previously shown to be the major APC involved in CII presentation.^{23, 26, 27} Presentation of the glycosylated CII₂₅₉₋₂₇₃ was characterised by more than 100-fold lower efficiency and occurred with about 1 h slower kinetics, as compared with the non-glycosylated epitope independent of the APC type. Hence, high efficiency of antigen presentation of the non-glycosylated CII₂₅₉₋₂₇₃ could account for the predominant specificity of peripheral T cells against the non-glycosylated epitope. Low efficiency of antigen presentation of the glycosylated CII₂₅₉₋₂₇₃ could potentially reflect its lower affinity for HLA-DR1, as glycosylation was shown previously to decrease CII peptide affinity for Aⁱ molecules.²⁸

According to our unpublished data, adoptive transfer of the T cell hybridoma HCII-9.1 specific for the non-glycosylated CII₂₅₉₋₂₇₃ epitope, but not the HCII-9.2 specific for the glycosylated epitope, induced mild synovial inflammation in small interphalangeal joints. Synovitis in small joints is reported to be a reliable marker for subsequent x ray changes in the same joints in RA,²⁹ suggesting potential arthritogenicity of the non-glycosylated form of CII₂₅₉₋₂₇₃ in HLA-DR1-tg mice. By contrast, arthritogenic T cell responses in HLA-DR4CD4-tg

Figure 5 Antigen presentation. Macrophages (A) or DC (B) were pulsed with CII for 5 h. Macrophages (C) or DC (D) were pulsed with CII for different periods of time. Responses of T cell hybridoma HCII-9.1 (closed symbols, non-glycosylated epitope) or HCII-9.2 (open symbols, glycosylated epitope) are shown as mean cpm (SD) of a representative of three experiments. Curve fitting was performed for EC₅₀ calculations. Dose-response EC₅₀ values of the glycosylated and non-glycosylated epitope in macrophages were 172.7 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$, respectively, and in DC 2.5 $\mu\text{g/ml}$ and 0.01 $\mu\text{g/ml}$, respectively. Kinetics EC₅₀ values of the glycosylated and non-glycosylated epitope in macrophages were 4.9 h and 3.6 h, respectively, and in DC by EC₅₀ 2.4 h and 1.5 h (1.0-1.9, 95% CI), respectively.

mice lacking MHC-II had higher responses towards the glycosylated CII₂₅₉₋₂₇₃ epitope,¹⁷ possibly due to the differential processing of CII in HLA-DR1-tg and HLA-DR4/CD4-tg mice. Our data suggest that post-translational glycosylation of Lys₂₆₄ has different consequences for CII-specific T cell responses restricted by different HLA-DRB1 alleles that confer susceptibility to RA. We propose that HLA-DR1 biases response towards the non-glycosylated epitope, as shown in the present study. It remains to be determined whether the response will be skewed towards recognition of the glycosylated peptide, as observed in the DR4 mice, in a tolerising situation when human CII is expressed.

Our finding is consistent with the presence of T cells specific for both the glycosylated and non-glycosylated CII₂₅₉₋₂₇₃ in peripheral blood of patients with RA.^{17, 30} Coexpression of both HLA-DR1 and HLA-DR4 has been shown to increase the risk of developing RA,³ which we propose could be due to the additive effect of two HLA-DR susceptibility alleles presenting essentially different forms of the CII₂₅₉₋₂₇₃ epitope. Our study suggests a pathophysiological role for HLA-DR1-restricted T cell responses against a non-glycosylated CII epitope in murine CIA, and shows that HLA-DR1-tg mice lacking MHC-II may be a useful model to study the specificity of T cell responses in RA.

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