

ORIGINAL PAPER

Identification of an immunodominant region of Fel d 1 and characterization of constituent epitopes

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Clinical and Experimental Allergy

Summary

Background Characterization of T cell epitopes restricted by common HLA alleles is a powerful tool in the understanding of the immune responses to allergens and for the identification of potential peptides for future peptide immunotherapy (PIT). One important requirement is the identification and use of peptides that will bind to HLA molecules covering a large proportion of the population.

Objective To identify commonly recognized CD4⁺ T cell epitopes in Fel d 1, restricted through frequently expressed HLA molecules for potential future use in PIT.

Methods HLA matched antigen presenting cells, HLA blocking antibodies, and peptide truncations were used in ELISpot assays to establish HLA-restricted T cell epitopes. Cytokine responses were measured by *ex vivo* and cultured IFN- γ , IL-4, and IL-10 ELISpots.

Results Responses to an immunodominant region of chain 2 were identified in the majority of atopic individuals and epitopes restricted by HLA-DQB1*06 and -DPB1*0401 were characterized in detail. Significantly higher *ex vivo* IL-4 and lower IFN- γ responses were observed to both epitopes in individuals with atopic dermatitis (AD) compared with those without disease. IL-10 responses were significantly lower in those with AD in the individuals with HLA-DPB1*0401.

Conclusions We have identified an immunodominant region of Fel d 1 which is frequently recognized by CD4⁺ T cells from atopic individuals and contains epitopes that are restricted by very common HLA alleles.

Keywords atopic dermatitis, Fel d 1, HLA-DPB1*0401, HLA-DQ B1*06, T cells, T cells epitope

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Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin condition, frequently associated with adaptive immune responses to harmless environmental allergens [1]. The prevalence of AD and other atopic diseases has risen substantially over the last 30 years [2] with 25.6% prevalence at age 6–18 months whilst adult prevalence is believed to be approximately 2–3% [3, 4]. Cat allergy is a common problem in individuals with AD; 19.4% of affected children are sensitized to cat [5].

A number of important studies have proposed regions of Fel d 1 that contain T cell epitopes [6–9] and some have suggested HLA restrictions for these epitopes using computer prediction analyses [9]. Haselden et al. [10] determined the HLA restriction of a number of Fel d 1 epitopes,

including one restricted by both HLA-DRB1*0101 and *1301 and another restricted by HLA DRB1*0405. However only a few epitopes with well-defined HLA restriction have been identified in other allergens [11–15] and few studies have used HLA restricted epitopes to compare T cell responses in individuals with and without atopy or atopic disease [12, 13].

Identification of HLA restricted T cell epitopes is a powerful tool in the understanding of the immune response to that antigen. This allows the direct comparison of antigen specific T cell responses between defined disease groups and controls at the cell specific level [16]. Identification of T cell epitopes may also offer the possibility of future peptide immunotherapy (PIT). Conventional immunotherapy uses repeated subcutaneous injection of increasing doses of whole allergen extracts,

to induce tolerance to the specific allergen [17], however the risk of anaphylactic reaction limits its use [18]. PIT circumvents this problem by using peptides containing T cell epitopes from the allergen which are absent of B cell epitopes and therefore do not initiate anaphylaxis [17]. Trials of PIT for cat allergy in asthmatics have shown the important potential for PIT for treatment of allergy and atopic diseases [18–21]. One potential problem of PIT is the range of MHC alleles in the population and the potential number of T cell epitopes required to make the treatment successful in the majority of individuals.

We sought to identify immunodominant regions of Fel d 1 recognized by specific CD4⁺ T cells with restriction through commonly expressed HLA molecules for potential future use in PIT.

Methods

Subjects

Under the ethical approval of Oxford Clinical Research Committee 26 subjects with moderate to severe AD were recruited through the Department of Dermatology, Churchill Hospital, Oxford, UK. The UK AD diagnostic criteria was used to define all AD patients [22, 23]. In addition, we screened 42 individuals asymptomatic for AD as controls. All donors were HLA typed for HLA-A, B, C, DR, and DQ by Tim Rostron at the MRC Human Immunology unit and DP typing was undertaken by the Tissue Typing Department at the Churchill Hospital, Oxford by PCR-SSP phototyping [22]. Specific IgE to cat dander were measured on all subjects at the Department of Immunology, Churchill hospital, Oxford using Pharmacia Unicap system. All individuals with AD had positive IgE to cat dander and 22 (84.6%) described deterioration in atopic symptoms on close proximity to cats.

Interferon- γ , interleukin-10 and interleukin-4 ELISpots

Ninety-six well multi-screen IP-plates (Millipore, MAIPS4510, Watford, UK) were coated with 50 μ L/well at 15 μ g/mL with IFN- γ capture antibody (monoclonal antibody 1.D1 K, Mabtech, Nacka Strand, Sweden) in sterile phosphate-buffered saline (PBS) pH7.4, IL-4 capture antibody (monoclonal antibody 82.4 Mabtech) or IL-10 capture antibody (monoclonal antibody 9D7 Mabtech) in sterile carbonate buffer pH9.6 and incubated 16 h at 4 °C (adapted from Pala *et al.* [24] and Gabrielsson *et al.* [25]). Plates were washed six times with RPMI supplemented with penicillin, streptomycin and L-glutamine and then blocked with RPMI supplemented with penicillin, streptomycin, L-glutamine and 10% foetal calf serum for 1 h at 37 °C. Cells were added at 1×10^5 /well [peripheral blood mononuclear cells (PBMCs) on IFN- γ ELISpot], 3×10^5 /well (PBMC on IL4 ELISpot), 0.5×10^5 /well (monocyte depleted PBMCs on

IL-10 ELISpot) or 4×10^4 /well (cultured ELISpot). Monocytes were depleted utilizing the difference in plastic adhesion properties between lymphocytes and monocytes [26, 27]. Depletion of monocytes from PBMCs was achieved by incubating them in RPMI supplemented with penicillin, streptomycin and L-glutamine on a polystyrene petri dish for 2 h at 37 °C 5% CO₂. The non-adherent lymphocytes were then removed from the petri dish and counted before use in the IL-10 ELISpot. PBMCs were added in RPMI supplemented with penicillin, streptomycin and L-glutamine to a polystyrene petri dish and was incubated for 2 h at 37 °C 5% CO₂. Cells were stimulated with 10 μ M peptide or 10 μ g/mL (0.56 μ M) recombinant Fel d 1 protein (Indoor Biotechnologies, Cardiff, UK). All peptides were synthesized using standard Fmoc chemistry and purity was established at >90% by high pressure liquid chromatography. Fel d 1 20-mer peptides were frame shifted 10 amino acids to span the sequence and were used to screen for peptide responses (Fig. 1) (swissprot accession number chain one P30438 and chain two P30440). To establish the B1-20 epitope minimum length progressive truncations of the 20-mer from the N-terminus and then C-terminus of the peptide as illustrated in Fig. 3. PHA was used as the positive control at 2 μ g/mL, dengue fever (NS5) peptide as a negative control and RPMI supplemented with penicillin, streptomycin and L-glutamine as a further negative control.

The plate was incubated 40 h at 37 °C before being developed by the addition of 1 μ g/mL of biotin linked anti-IFN- γ anti-IL-4 or anti-IL-10, followed by streptavidin alkaline phosphatase and visualized using alkaline phosphatase substrate kit (Biorad, Hemel Hempstead, UK). Spots were enumerated using an AID ELISpot reader [28]. Background responses were removed (cells plus media only) and results expressed as spot forming units per

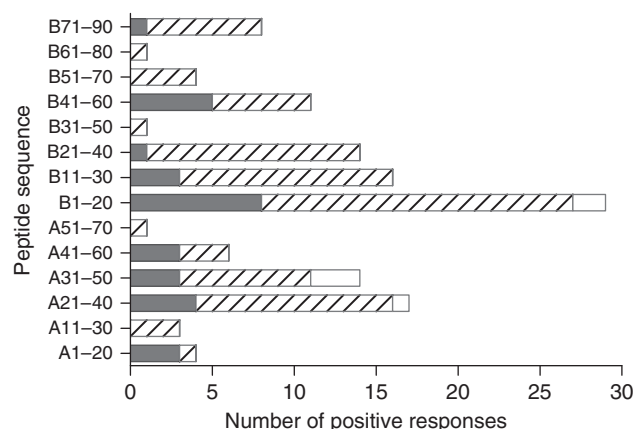


Fig. 1. Frequency of positive responses to overlapping 20-mer peptides of Fel d 1 either *ex vivo* or cultured ELISpots in all individuals ($n = 55$). Positive IFN- γ responses indicated by hatched bars, IL-4 responses by grey bars and both IL-4 and IFN- γ by clear bars. Chain one and two peptides indicated by A and B respectively.

million cells. Positive responses were defined as greater than the 95th percentile for the negative control peptide [29].

Cultured ELISpots

T cell lines for cultured ELISpot were set up from fresh PBMCs at $2-4 \times 10^6/\text{mL}$ incubated with $4 \mu\text{M}$ peptide in RPMI supplemented with penicillin, streptomycin, L-glutamine and 10% human AB serum on 24-well flat bottom culture plate for 10 days at 37°C 5% CO_2 . On day 3 and 7 the cells were supplemented with RPMI supplemented with penicillin, streptomycin, L-glutamine and 10% human AB serum plus 200 IU/mL human IL-2 (Peprotech, London, UK). Day 10 cells were removed from the plate, washed twice in sterile PBS and returned to a clean well overnight in RPMI supplemented with penicillin, streptomycin, L-glutamine and 10% human AB serum to rest before ELISpot assays as described above and elsewhere [28, 30].

B cell line presentation

The Epstein-Barr virus transformed immortalized B cell lines (BCL) matched for individual HLA alleles were used as antigen presentation cells. BCL were incubated with $5 \mu\text{g}$ peptide or media alone for 1 h at 37°C 5% CO_2 and then washed three times with RPMI supplemented with penicillin, streptomycin and L-glutamine. BCL were used in ELISpots at $5 \times 10^3/\text{well}$ and $10 \times 10^3/\text{well}$.

HLA blocking antibodies

HLA-DR (clone L243), HLA-DQ (clone L2) (both grown within the MRC Human Immunology Unit) and HLA-DP (clone B7/21, Leinco Technologies, St Louis, MO, USA) blocking antibodies were used at $10 \mu\text{g}/\text{mL}$ and incubated with the cells for 1 h at 37°C 5% CO_2 on the ELISpot plate before peptide addition [31].

Statistics

Mann-Whitney *U*-test, and one-way ANOVA with *post hoc t*-test and Bonferroni's correction were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Responses to overlapping Fel d 1 peptides

20-mer peptides overlapping by 10 amino acids spanning both chains of Fel d 1 were used to screen subjects with and without AD for T cell epitopes (Fig. 1). The B1-20 peptide (VKMAETCPIFYDVFFAVANG) was the most commonly recognized, with 29/55 (53%) of individuals having a positive response in either *ex vivo* or cultured IL-4 or

IFN- γ ELISpots (Fig. 1). Using CD4^+ or CD8^+ depleted ELISpots, this response was found to be CD4^+ T cell mediated (data not shown). Having documented a commonly recognized region of Fel d 1, we then sought to proceed to the characterization of constituent epitopes.

HLA-DQB1*06-restricted B1-20 epitope

It had observed that many of the individuals who responded to B1-20 expressed HLA-DQB1*06 (13/29) and therefore HLA-DQB1*06-matched target cells were used to present the peptide to T cells. Using cultured B1-20-specific T cells derived from DQB1*06-positive individuals, we observed that the magnitude of response to the peptide-pulsed Epstein-Barr virus transformed B cells (BCL) matched by HLA-DQB1*06 was significantly higher ($P < 0.001$, *post hoc t*-test with Bonferroni's correction) than DQB1*06-negative target cells (Fig. 2a). This assay gives strong evidence of an HLA-DQB1*06 restricted epitope in B1-20.

However in order to confirm these findings, we also used blocking antibodies to the three class II groups with a B1-20-specific T cell line derived from an individual homozygous for DQB1*06. Figure 2b demonstrates that HLA-DQ antibodies were able to significantly block the T cell response to B1-20 peptide ($P < 0.05$, *post hoc t*-test with Bonferroni's correction). HLA-DQB1*06 was a common allele in this cohort with 50% of the subjects with AD and 46% of the controls with the allele.

The minimum epitope was investigated using progressive truncations of the 20-mer from the N-terminus and then C-terminus of the peptide as illustrated in Fig. 3. These truncations were tested in cultured ELISpots to lines raised to the B1-20 20-mer peptide. As demonstrated in Fig. 3 the response was reduced with the removal of the first C-terminal alanine (A), and was totally abrogated by the removal of the valine (V). From the N-terminal the response was reduced with loss of the glutamic acid (E) and completely lost with the removal of the threonine (T). This gives a minimal epitope of ETCPIFYDVFFAVA.

HLA-DQB1*06 is a common allele in both the AD patient group and the control group and 13 individuals with AD and 18 controls were studied for their IL-4, IFN- γ , and IL-10 response to the B1-20 peptide. Figure 4a demonstrates that both individuals with AD and those without made IL-4 and IFN- γ responses to the B1-20 peptide, with greater IL-4 and significantly lower IFN- γ responses ($P < 0.05$, Mann-Whitney *U*-test) in those with AD. The decreased IFN- γ and increased IL-4 response to B1-20 in those with AD was retained in the cultured ELISpot to B1-20 (Fig. 4b), though not statistically significant. There was also a trend towards increased *ex vivo* IL-10 production to the B1-20 peptide in non-atopic individuals (Fig. 4a).

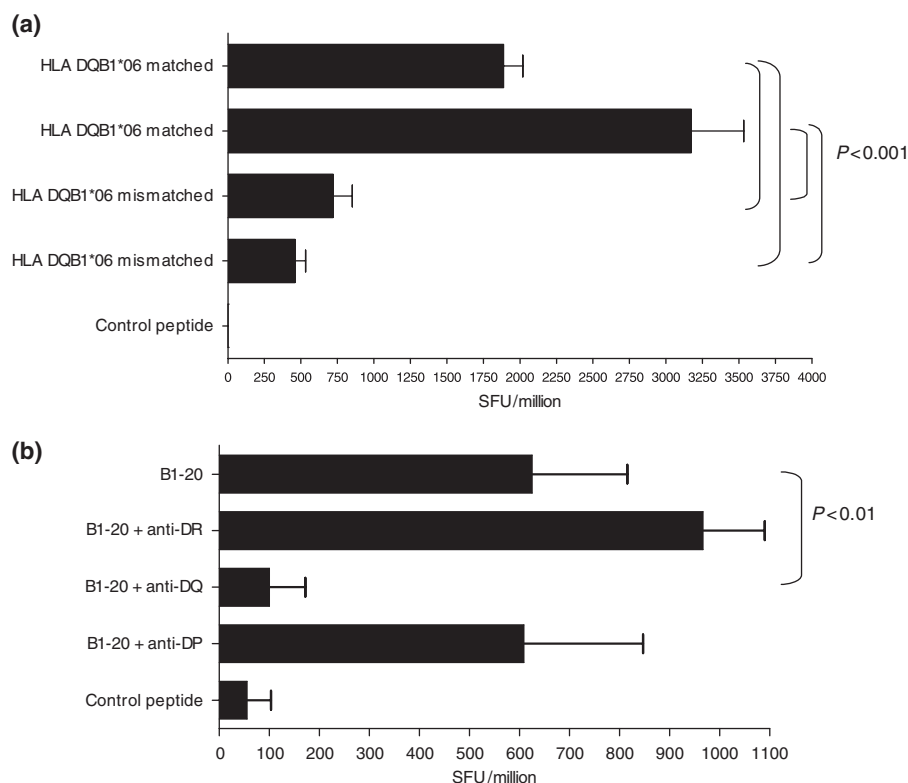


Fig. 2. (a) Establishing the HLA restriction of B1-20 response using multiple peptide-pulsed BCL matched with the responder for limited HLA class II alleles in an IL-4 ELISpot with a B1-20 specific T cell line. (b) Using HLA class II blocking antibodies in an IFN- γ ELISpot on a B1-20 T cell line from an individual DQ6 homozygous. Statistics determined by ANOVA, with *post hoc t*-test and Bonferroni's correction. BCL, B cell lines.

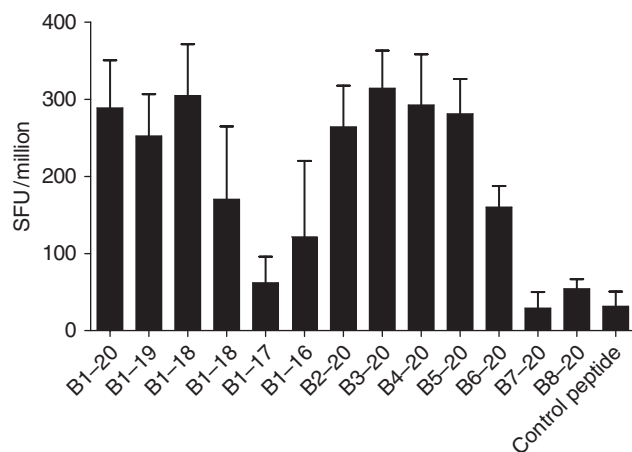


Fig. 3. (a) Establishing the minimum peptide sequence and anchor residues for DQB1*06 B1-20 epitope ($n = 7$).

The majority (82%) of B1-20-specific CD4⁺ T cell lines recognized the Fel d 1 protein, as well as the peptide, indicating that the B1-20 HLA-DQB1*06 epitope was processed and presented from Fel d 1 protein. Overall these data showed that within the B1-20 peptide, there is an HLA-DQB1*06-restricted epitope that is naturally processed and presented for recognition by CD4⁺ T cells.

HLA-DPB1*0401 B1-20 epitope

We noted that HLA-DQB1*06 was not present in all the individuals responding to peptide B1-20, raising the probability of another HLA class II restricted epitope in the B1-20 20-mer peptide. Of the 29 individuals who initially respond to B1-20, 16 were not DQB1*06-positive and there was no common HLA DR or DQ molecule between them, but the majority did share HLA-DPB1*0401 (13/16).

Figure 5a demonstrates the magnitude of responses initiated by particular HLA class II matched BCL in response to B1-20-specific T cells grown from an HLA-DPB1*0401-positive, but HLA-DQB1*06-negative individual. The responses to both the HLA-DPB1*0401 matched BCL was significantly higher ($P < 0.001$, *post hoc t*-test with Bonferroni's correction) than the non-matched BCL. This assay gives strong evidence of an HLA-DPB1*0401 restricted epitope in B1-20.

HLA blocking antibodies to the three class II groups were utilized to confirm HLA restriction. Figure 5b demonstrates that HLA-DP antibodies were able to significantly block the T cell response to B1-20 peptide in two individuals ($P < 0.05$, *post hoc t*-test with Bonferroni's correction), one individual was homozygous at this loci, thus confirming the HLA restriction of the epitope as HLA-DPB1*0401.

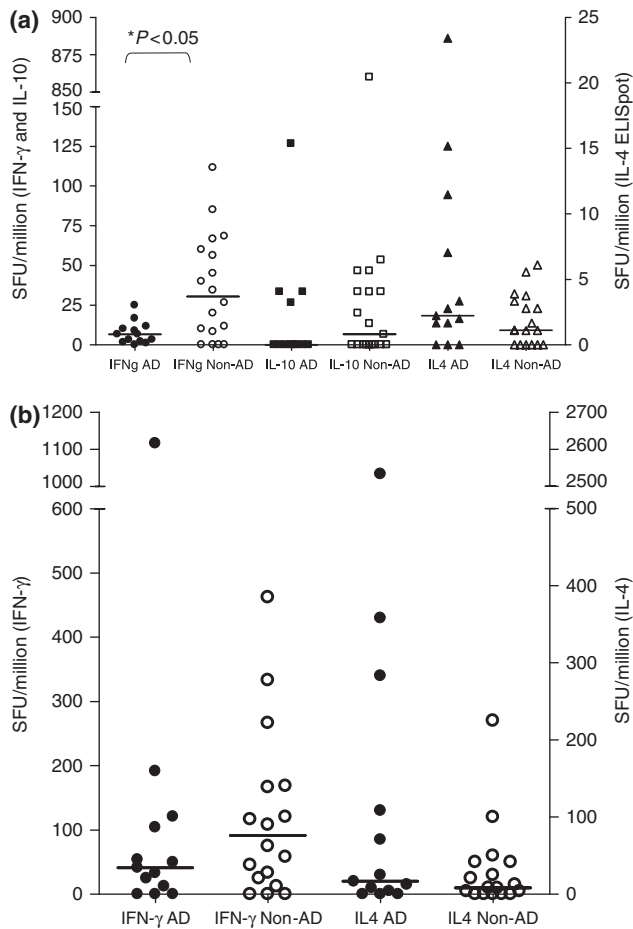


Fig. 4. The frequency of cytokine producing cells in HLA-DQB1*06 individuals in response to the B1-20 peptide measured by (a) IFN- γ IL-10 and IL-4 *ex vivo* ELISpot (b) cultured IFN- γ and IL-4 ELISpot. Bars indicate median. $*P < 0.05$ determined by Mann-Whitney *U*-test.

The minimal epitope was investigated and the terminal residues required for binding to the HLA molecule established using progressive truncations of the 20-mer from the N-terminus and then C-terminus of the peptide as illustrated in Fig. 6. These truncations were tested in cultured ELISpots using lines grown to the B1-20 20-mer peptide. As demonstrated in Fig. 6 the response was reduced with the removal of the first C-terminal alanine (A), and was totally abrogated by the removal of the valine (V). From the N-terminal the response was completely lost with the removal of the threonine (T). This gives a minimal epitope of TCPIFYDVFFAVA, but the epitope AETCPIFYDVFFAVA may be preferential (Fig. 6).

The frequency of HLA-DPB1*0401 was high with 16/22 (73%) of the patients and 15/25 (60%) controls having this allele. The individuals with HLA-DPB1*0401 were studied further for their *ex vivo* and cultured ELISpot response to the B1-20 peptide.

Figure 7 illustrates the IL-4 and IFN- γ responses to B1-20 peptide in the DPB1*0401 individuals. The trend of the data followed the pattern of responses shown to the

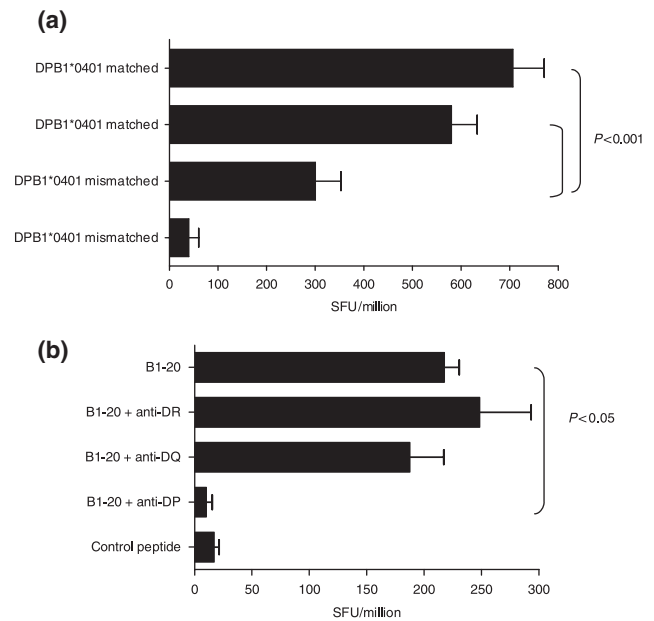


Fig. 5. (a) Establishing the HLA restriction of the second B1-20 response using multiple peptide-pulsed BCL HLA matched with the responder in an IL-4 ELISpot with a B1-20 T cell line. (b) Using HLA class II blocking antibodies in an IFN- γ ELISpot on a B1-20 T cell line from an individual DPB1*0401 homozygous. Statistics determined by ANOVA, with *post hoc* *t*-test and Bonferroni's correction. BCL, B cell lines.

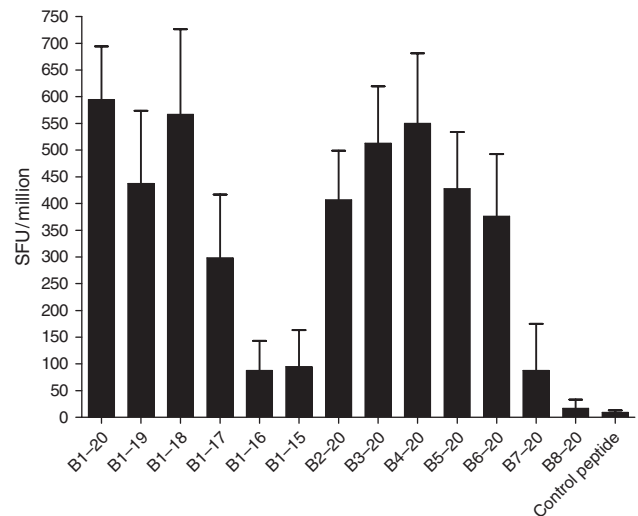


Fig. 6. (a) Establishing the minimum peptide sequence and terminal residues required for binding to DPB1*0401 of the B1-20 epitope ($n = 6$).

DQB1*06 epitope. *Ex vivo* IFN- γ responses (Fig. 7a) were significantly higher in the non AD group ($P < 0.05$, Mann-Whitney *U* test) and a trend remained in the cultured ELISpot (Fig. 7b). There was a trend towards higher IL-4 responses in the AD group, though again these differences did not reach statistical significance (Fig. 7a and b). There was significantly higher *ex vivo* IL-10 response to B1-20 peptide ($P < 0.05$, Mann-Whitney *U*-test, Fig. 7a) in the individuals without AD compared

with those with AD, compatible with the HLA-DQB1*06 data above and with those of Reefer et al. [9]. The recognition of Fel d 1 protein by the majority of B1-20 peptide specific-CD4 T cell lines indicates that this epitope is processed and presented from Fel d 1 protein.

Discussion

This paper describes the identification of two HLA class II epitopes within the immunodominant 20-mer B1-20 region, restricted by HLA-DQB1*06 and DPB1*0401. The optimal epitopes for both HLA were very similar, ETCPIFYDVFFAVA for HLA-DQB1*06 and AETCPIFYDVFFAVA for HLA-DPB1*0401. Cytokine response to both the HLA-DQB1*06 and HLA-DPB1*0401 epitopes demonstrate significantly higher IFN- γ responses and a trend towards

lower IL-4 responses in individuals without AD compared with those with AD.

HLA-DQB1*06 was a common HLA class II allele and was in comparable frequency in both the individuals with AD and the control group. It is also a common allele in the Black (45.39%), Caucasian (39.47%), and Oriental (36.29%) populations and is in linkage disequilibrium with DRB1*15 alleles [32]. The role of DQB1*06 in disease is not well documented in the literature, but there is suggestion of possible association with multiple sclerosis, though due to linkage disequilibrium the precise role of DQ6 is still unclear [33]. HLA-DQB1*0602 has been found to be reduced in frequency in individuals with insulin-dependent diabetes mellitus. The high stability and wide binding repertoire of this molecule is thought to play a major role in protection from insulin-dependent diabetes mellitus [34].

Subtyping of DQB*06 alleles is difficult by PCR-SSP, due to primer mixes having shared epitopes for other DQ alleles. However, there is strong linkage disequilibrium between HLA-DRB1*150101 and DQB1*0602, with these alleles almost always occurring together in the Caucasian European population [35]. The B1-20 epitope has a phenylalanine at position 1 and alanine at position 9 which correspond well with amino acids critical to binding in the corresponding HLA-DQB1*0602 pockets, while the amino acids at the other three pockets are not inhibitory to binding [36, 37].

HLA-DPB1*0401 is the most common class II allele worldwide, found in all ethnic groups [38], with a frequency in the Caucasian population of 40% [39]. HLA-DP molecules and restricted epitopes have not been studied to the same extent as HLA-DR or DQ. Initially HLA-DP was thought to be less important in the immune response as incompatibility at these loci did not appear to contribute to graft vs. host disease (GvHD) post transplantation [40, 41]. However, it is now known that a single HLA-DP mismatch can result in GvHD T cell responses after bone marrow transplant [42, 43]. This evidence and the identification of HLA-DP restricted epitopes in conditions such as HIV and melanoma confirms that HLA-DP molecules perform an important role in the immune recognition and response to antigens [44–46].

The HLA-DPB1*0401 epitope described in this paper corresponds well with predicted motifs for both HLA-DPB1*0401 and *0402 [47]. HLA-DPB1*0201 is also very similar in sequence to both HLA-DP4 alleles and is predicted to bind a similar repertoire of peptides [47]. It is possible therefore that this epitope is presented by other common alleles beyond DPB1*0401. The prevalence in all racial groups means that a significant number of individuals can be compared using this allele, where it is estimated six HLA-DR alleles would be required to cover the same percentage of individuals [47]. HLA-DPB1*0401 is not in linkage disequilibrium with either DR or DQ

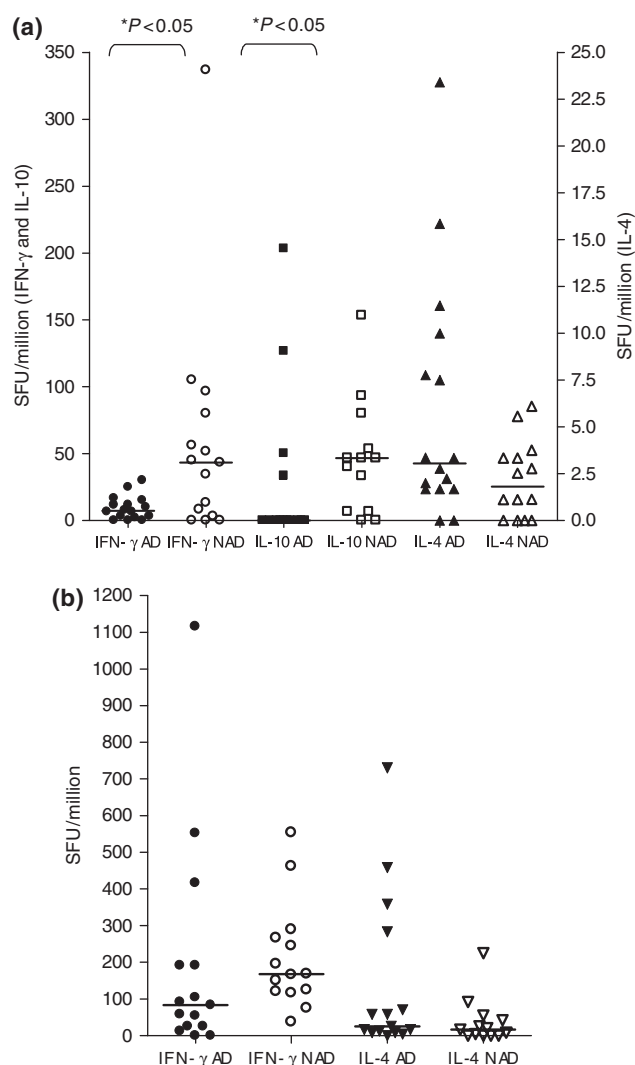


Fig. 7. The frequency of cytokine producing cells in HLA-DPB1*0401 individuals in response to the B1-20 peptide measured by (a) IFN- γ , IL-10 and IL-4 *ex vivo* ELISpot (b) cultured IFN- γ and IL-4 ELISpot. Bars indicate median. * $P < 0.05$ determined by Mann-Whitney *U*-test.

alleles and it would therefore be present in individuals with a range of HLA-DR alleles [48]. HLA class II tetramers have been successfully made for HLA-DPB1*0401 [49] and thus the HLA-DPB1*0401/Fel d 1 epitope identified in this paper offers the potential to accurately establish frequency and phenotype of these T cells. It also offers the potential to monitor alterations in antigen specific T cell frequency and phenotype with changes in disease severity or treatment.

The identification of a peptide that binds two very common HLA alleles (HLA-DQB1*06 and HLA-DPB1*0401) and may bind two others (HLA-DPB1*0402 and HLA-DPB1*0201) offers important potential for PIT. While HLA-DPB1*0401 is very common with ~40% prevalence in Caucasian population, HLA-DPB1*0402 is not as well represented (13.3%) [39] but together the two alleles represent a gene frequency of 60% in South America, 80% in North America, 60% in India, and 25% in Africa [50]. Furthermore, IgE epitopes have not been documented within the B1-20 peptide region [51].

HLA-DQB1*06 and HLA-DPB1*0401 bind very similar Fel d 1 epitopes raising interesting questions about the binding of class II molecules and their promiscuity. There are documented examples of peptides that can bind multiple HLA class molecules [15, 46], due to the similarities in binding pockets between some class II molecules and the open ended nature of the class II groove. A number of publications have demonstrated similarities in binding motifs between different alleles in the HLA-DR class and the ability of the same peptide to bind a range of these alleles [52–54]. There is growing evidence of the ability of peptides to bind different molecules across the three HLA class II allele groups [46]. Indeed the CLIP peptide generated during class II loading, can bind all class II molecules.

Ex vivo IFN- γ responses to both the HLA-DQB1*06 and HLA-DPB1*0401 epitope were significantly lower in the individuals with AD and there was a trend to a higher IL-4 responses compared with individuals without AD. This is consistent with the model in which atopic disease is, at least in part, driven by type 2 cytokine responses [55, 56]. IL-10 responses to HLA-DPB1*0401-B1-20 epitope were significantly higher in individuals without AD and followed a comparable trend in HLA-DQB1*06 individuals. This is consistent with the findings of Reefer et al. [9], though in this paper the restriction of the B1-20 epitope was attributed to HLA-DR7. Overall our data support a model in which both atopics and non-atopics have circulating allergen-specific CD4⁺ T cells, but in those with disease the cells circulate with a different frequency and phenotype than in those without [57, 58].

The B1-20 peptide has been described by two papers as containing T cell epitopes [9, 59] and it was suggested to contain multiple HLA-DR ligands, as T cells from individuals with diverse HLA-DR types responded to this peptide [9]. Taken together with our data showing pre-

sentation through common DQ and DP alleles, this argues that the B1-20 region of chain 2 should be considered for use within future peptide immunotherapeutic approaches.

In summary we have documented an immunodominant region of Fel d 1 that is recognized by CD4⁺ T cells from the majority of individuals with AD. We have proceeded to characterize two epitopes restricted through HLA-DQB1*06 and HLA-DPB1*0401, which together are expressed by the majority of atopic individuals. We hope these findings will be of value to those investigating the role of T cells in the pathogenesis of atopic disease and the potential future use of PIT.

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