

The response of bovine beta-lactoglobulin-specific T-cell clones to single amino acid substitution of T-cell core epitope

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Cow's milk is one of the most common food allergens in the first year of life, with approximately 2.5% of infants experiencing an allergic reaction to it. Beta-lactoglobulin (BLG) is one of the major allergens in cow's milk. Previously, we reported that four of six T-cell clones (TCC) which were established from cow's milk allergy patients recognized BLGp97-117 as the core sequence and also recognized BLG in association with the human leucocyte antigen (HLA)-DRB1*0405 allele. Using two of these four TCCs, we evaluated the T-cell response to BLG peptides with single amino acid substitution or deletion and identified BLGp102-112 as the minimum essential region in BLGp97-117. In the alanine-scan assay, the proliferative responses of TCCs to pE108A disappeared, and the proliferative responses of TCCs to pC106A decreased. In the analog peptide proliferation assay, pY102S had retained some T-cell response to the two TCCs. Collecting these results, we propose a motif for the interaction between the HLA-DRB1*0405 allele and antigen peptide, and suggest that BLGp105-108 are important residues to retain the TCR/BLG-peptide/HLA complex. pY102A and pY102S are partial agonists for the T-cell receptor. These peptides might be considered as candidate peptides for the modification of the T-cell response to BLG in cow's milk allergy.

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Cow's milk allergy is one of the most common food allergies in young children, with approximately 2% to 2.5% of all infants experiencing allergic reactions to it. The majority of children out-grow their allergy to cow's milk before the age of 3 yr, but 15% of these infants with IgE-mediated cow's milk allergy retain their sensitivity into the second decade of life (1, 2).

Beta-lactoglobulin (BLG) is the major whey protein of the milk of the ruminants. It is

relatively resistant to acidic pH and to proteolytic enzymes, leaving its structure relatively unchanged during digestion and possibly allowing the passage of intact protein into the circulation (3). Moreover, it is not present in human milk and is considered to be a major allergen of cow's milk (3). IgE-binding regions of BLG have been identified. Selo et al. showed that the major IgE-epitopes of BLG appeared to be fragments p41-60, p102-124, and p149-162 (4).

The isotype switching of antigen-specific B cells to IgE is controlled by T cells that play a key role in the initiation of allergic symptoms, as well as in tolerance induction. Antigenic peptide-made

Abbreviations: BLG, beta-lactoglobulin; IFN-gamma, interferon gamma; PBMcs, peripheral blood mononuclear cells.

Table 1. Patients' profile and characterization of T-cell clones

	Sex	Age (yr)	IgE (IU/ml)	CAP-RAST		Antigen presenting HLA†	Recognition site of BLG peptide†
				Milk	BLG		
YA	Female	1	1136.8	94	55.4	DRB1*0405	p97-117 (TDYKKYLLFCMENSAEPEQSL)
HA	Female	2	249	2.3	1.5	DRB1*0405	p97-117 (TDYKKYLLFCMENSAEPEQSL)

BLG, beta-lactoglobulin; RAST, radioallergosorbent test.

†Previously reported data (6).

complexes with HLA class II molecules are displayed on the surface of antigen-presenting cells for recognition by CD4⁺ T cells.

We have previously reported that the BLG peptide (BLGp97-117) was presented by HLA-DRB1*0405 using BLG-specific T-cell lines and clones (TCCs) obtained from patients with cow's milk allergy (5, 6). Four of six TCCs recognized the BLG peptide (BLGp97-117) presented by HLA-DRB1*0405, and suggested that HLA-DRB1*0405 is an immunoregulatory gene product for T-cell responses to BLG (6). We hypothesize that single amino acid substitutions on an allergen peptide carrying the T-cell epitope might modify the T-cell response to allergen. In this study, we identified the minimal core epitope of TCCs specific to BLG using BLG-derived peptides, and the analog peptides which modified the T-cell responses. We proposed a motif for the interaction between the HLA-DRB1*0405 allele and antigen peptide.

Material and methods

Subjects

As shown in Table 1, we obtained BLG-specific TCCs from two patients (YA and HA) with cow's milk allergy. Allergic symptoms such as eczema, urticaria, and wheezing occurred in less than 1 h after cow's milk ingestion in these patients.

Both of the TCC recognized BLGp97-117 as the core sequence and recognized BLG in association with the HLA-DRB1*0405 allele (6).

TCCs specific to BLG Culture

The TCCs were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 10% pooled AB, heat-inactivated normal human male plasma in 24-well flat-bottomed cultured plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA). The plates were incubated at 37°C in 5% CO₂ humidified air. After 7–10 days, irradiated

(30 Gy) autologous peripheral blood mononuclear cells (PBMCs) (1.5×10^5 /well) pulsed with a BLG-derived peptide mixture (1 µM each for 5h), human rIL-2 (50 U/ml) (Genzyme, Cambridge, MA, USA), and human rIL-4 (10 U/ml) (Biosource International, Camarillo, CA, USA) were added to the culture wells and the TCCs were maintained for another 7 days.

Intracellular IFN-gamma and IL-4 staining of BLG-specific TCCs

The TCCs specific to BLG were cultured at a density of 2×10^6 cells/ml in an RPMI 1640 medium for 4 h at 37°C. During the 4-h incubation, the cells were stimulated with a combination of 25 ng/ml of phorbol 12-myristate 13-acetate (SIGMA, St Louis, MO, USA) and 2 µg/ml of ionomycin (SIGMA) in the presence of 10 ng/ml of Brefeldin-A (SIGMA). Then the cells were directly stained with a PC-5-conjugated anti-CD4 monoclonal antibody (Coulter-Immunotech, Marseille, France) for 15 min at room temperature, and fixed with a Fluorescence-activated cell sorting (FACS) Lysing Solution (Becton Dickinson, Mountain View, CA) for 10 min. After washing, they were pre-incubated with a FACS Permeabilizing Solution (Becton Dickinson) for 10 min and after washing again, they were incubated with FASTIMMUNE interferon (IFN)-gamma FITC/IL-4 PE (Becton Dickinson) for 30 min at room temperature. Flow cytometric analysis was performed using a FACS Calibur.

Synthesis of BLG-derived peptides

BLG-truncated peptides for searching the core sequences of BLG p97-117 (TDYKKYLLFCMENSAEPEQSL), BLG-derived peptides for analyzing alanine-scan mutant peptides, and BLG-derived analog peptides of 11 amino acid residues were synthesized using the multiple method based on the Fmoc strategy (5). The substitution of a single amino acid residue was done for each peptide. All peptides were purified by C18 reverse-phase HPLC (Gilson, Middleton, WI, USA).

Proliferative responses of the TCCs using BLG-derived peptides

The proliferative responses of the TCCs using BLG-derived peptides were assayed as described previously (5). Briefly, the TCCs (3×10^4 /well) were cultured in 96-well flat-bottomed culture plates in the presence of BLG-derived analog peptides (1 μ M), a soluble BLG peptide mixture (1 μ M each), BLG crude protein (25 or 2.5 μ g/ml) and 30 Gy-irradiated autologous PBMC (1.5×10^5 /well) for 72 h. Before the final 16-h period, 1 μ Ci per well of [^{3} H]TdR was added, and the incorporated radioactivity was measured by liquid scintillation counting (7).

Production of IL-4, IFN-gamma and IL-10 in supernatants of TCCs using BLG-derived peptides

The TCCs (3×10^4 cells/well in 96-well flat-bottomed culture plates) were cultured in the presence of a soluble BLG peptide mixture (1 μ M) and irradiated autologous PBMC (1.5×10^5 /well) for 56 hr. Culture supernatants of the TCCs were collected and stored in aliquots at -80°C until the determination of lymphokine concentrations. Enzyme-linked immunosorbent assay kits for detecting human IL-4 (Biosource International), IFN-gamma (Ohtsuka, Tokyo, Japan) and IL-10 (Biosource International) were used for quantification of the lymphokines in the supernatants, according to the manufacturers' instructions.

Binding images between BLGp102-112 and HLA-DRB1*0405 molecules

The binding images between the BLGp102-112 and HLA-DRB1*0405 molecules were displayed with RASMOL, which is the standard molecular graphics program (8).

Results

Intracellular IFN-gamma and IL-4 staining of BLG-specific TCCs

To characterize the TCCs (YA4, HA5.7), intracellular IFN-gamma and IL-4 were analyzed. YA4 showed an intracellular IL-4 single positive cell pattern and HA5.7 showed an IFN-gamma single positive cell pattern (data not shown). These results suggested that YA4 had the Th2 phenotype and HA5.7 had the Th1 phenotype.

Proliferative responses of the TCCs using BLG-truncated peptides

Previously, we reported that the TCCs recognized BLGp97-117 as the core sequence. To

define the minimum essential region in BLGp97-117, BLGp97-117 (TDYKKYLLFCMENSAE-PEQSL)-truncated peptides in the N and C termini were designed. The proliferative responses of the TCCs using these truncated peptides were compared (Fig. 1). Although BLGp102-112 (YLLFCMENSAE) induced proliferative responses of TCCs, BLGp103-112 and BLGp102-111 induced no proliferative responses in YA4 and HA5.7. Therefore, both YA4 and HA5.7 recognized BLG p102-112 (YLLFCMENSAE) as the minimal epitope. Proliferative responses and cytokine production of the TCCs using BLG-derived alanine-scan mutant peptides.

To predict the TCR/BLGp102-112/HLA complex structure, BLGp97-117-derived alanine-scan mutant peptides were designed. The proliferative responses of TCCs to pE108A disappeared, and the proliferative responses of TCCs to pC106A decreased (Fig. 2a). The cytokine production patterns are shown in Fig. 2b. In YA4, which had the Th2 phenotype, IL-4 production increased in proportion to its proliferative reaction. In HA5.7, which had the Th1 phenotype, IFN-gamma production increased in proportion to its proliferative reaction. IL-10 production increased according to its proliferative reaction in YA4 and HA5.7 (Fig. 2c).

Proliferative responses and cytokine production of the TCCs using BLG-derived analog peptides

Single amino acid substitution peptides of BLGp102-112 were designed. The proliferative responses of TCCs to these peptides were investigated. pY102S had retained some T-cell responses to both YA4 and HA5.7 (Fig. 3a). pY102S, pL103V and p104V had retained some T-cell responses to HA5.7. IFN-gamma and IL-4 production increased in proportion to their proliferative reaction, as observed in the case of the BLG alanine scan peptide (data not shown). BLG crude induced the IL-10 production of the clones of both YA4 and HA5.7 (Fig. 3b). pY102S showed some IL-10 production of YA4, not HA5.7. pY102S, pL103V, and p104V showed no IL-10 production of HA5.7.

Binding images between BLGp102-112 and HLA-DRB1*0405 molecules

We analyzed that the binding images between the BLGp102-112 and HLA-DRB1*0405 molecules based on the structural modeling. Amino acid residues of L104 and M107 on the BLGp102-112 could be fitted in the pocket of HLA-DRB1*0405 molecules, and F105 and E108 on

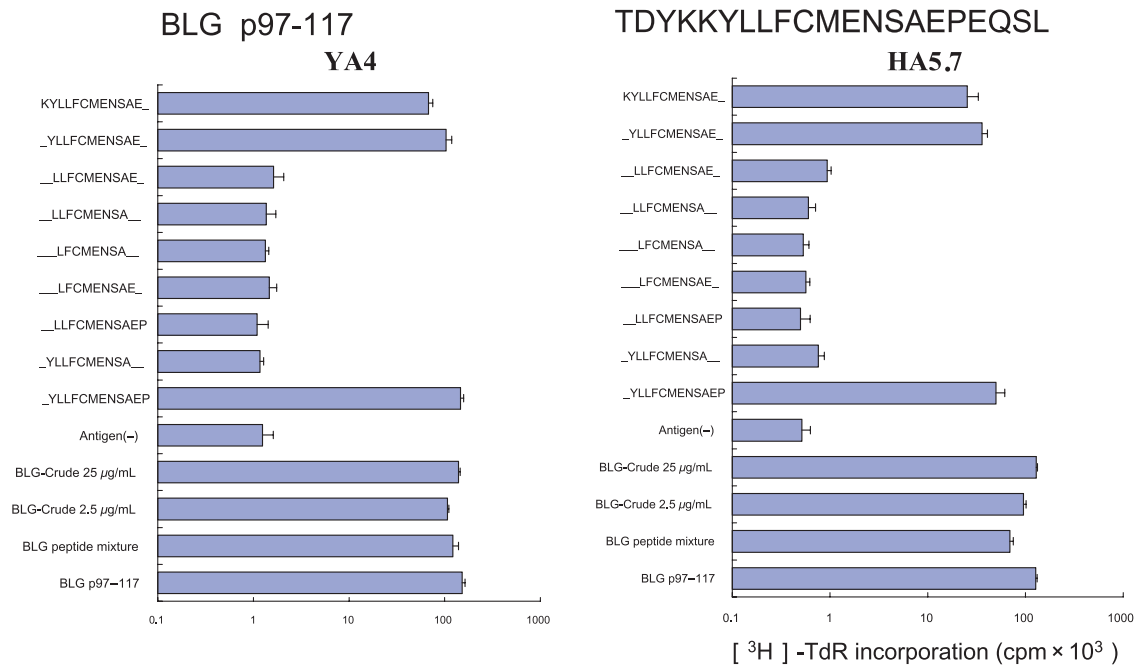


Fig. 1. T-cell clone responses to truncated beta-lactoglobulin (BLG) peptides. Peptides truncated from the N- or C-terminal were designed to define the minimal T-cell activation-inducing epitope. The proliferative responses of the TCCs using these truncated peptides are shown. Both TCCs (YA4 and HA5.7) recognized minimal peptide BLGp102-112 (YLLFCMENSAE).

the BLGp102-112 could be presented on the surface of HLA-DRB1*0405 molecules.

CDR3 loops of TCCs (HA5.7) were demonstrated to be 'QKV' in a previous study (6). This sequence is composed of an amide-bearing residue, a positively charged residue, 'K', and a hydrophobic residue, 'Q'. The negatively charged residue, E108, interacted electrically with the 'K' of the CDR3 loops. The hydrophobic residue, F105, and the 'V' of the CDR3 loops interacted with each other (Fig. 4).

Discussion

YA had an immediate type milk allergy and the food challenge test was positive. Her main symptom was urticaria and a skin rash after milk ingestion. HA had immediate and non-immediate type milk allergy. She had atopic dermatitis, a symptom which worsens after milk ingestion. The YA4 clone had a Th2 phenotype and the HA5.7 clone had a Th1 phenotype. In these cases, the clone phenotype was suitable for each clinical symptom. Two TCC (YA4 and HA5.7) needed a minimum of peptide BLGp102-112 (YLLFCMENSAE) presented by HLA-DRB1*0405 to proliferate. By using BLG-derived alanine-scan mutant peptides, the

proliferative responses of the TCCs to pE108A disappeared, and those to pC106A decreased. Cytokine secretions of TCCs decreased in proportion to their proliferative responses. Considering the binding images between the BLGp102-112 and HLA-DRB1*0405 molecules based on the structural design reported above, E108 on the BLGp102-112 is the key residue in the proliferation of TCCs.

The major safety concern regarding food allergen immunotherapy has been addressed by engineering 'hypoallergenic' forms of major allergenic food proteins. These mutated ('engineered') major food proteins have lost their ability to bind to IgE but have retained the ability to interact with T cells (9). In previous literature, the tolerogenic peptide size is around 20 amino acids (10–14). These tolerogenic peptide sizes are in accord with the fact that peptides with 12 to 20 amino acids presented with HLA complex class II molecules on the surface of antigen-presenting cells are recognized by T cells (15). The presence of T-cell epitopes is essential for tolerogenic peptides as immunomodulation is mainly induced by T cells (16). In our cases, the analog peptides that change amino acids pY102A and pY102S have some T-cell response to TCCs. pY102A induced some IL-10 production in YA4

T-cell clone responses to peptides of single amino acid substitution

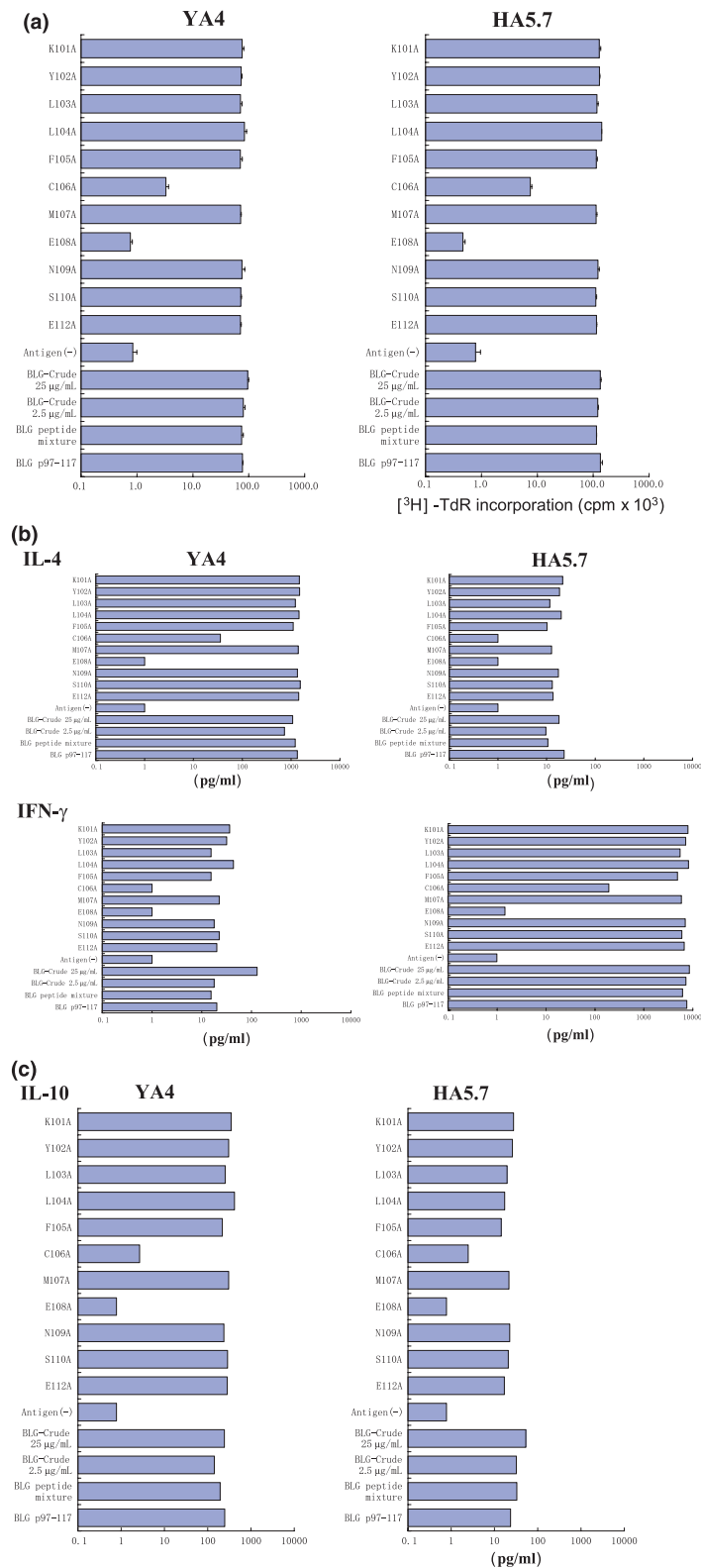


Fig. 2. T-cell clone (TCC) responses to beta-lactoglobulin (BLG) alanine-scan mutant peptides. BLGp97-117-derived alanine-scan mutant peptides were designed. Each polypeptide has 21 amino acid residues from number 97 to 117 and contains one amino acid substitution for alanine. The BLG peptide mixture is a mixture of synthetic peptides composed of 12-21 residues overlapping each other by 11 residues (5). BLG crude is a BLG protein (25 or 2.5 µg/ml). (a) The proliferative responses of the TCCs against BLGp97-117-derived alanine-scan mutant peptides were shown. (b) IL-4 and IFN-γ production of the TCCs against BLGp97-117-derived alanine-scan mutant peptides was shown. (c) IL-10 production of the TCCs against BLGp97-117-derived alanine-scan mutant peptides was shown.

(Fig. 3b). It has been reported that IL-10 induced T-cell anergy and played an important role in the induction and maintenance of antigen-specific T-cell tolerance (17). These results show that

pY102A and pY102S might have potential as immunoregulatory peptides.

In this study, alanine-scan was performed because it lacks the side chain of the native

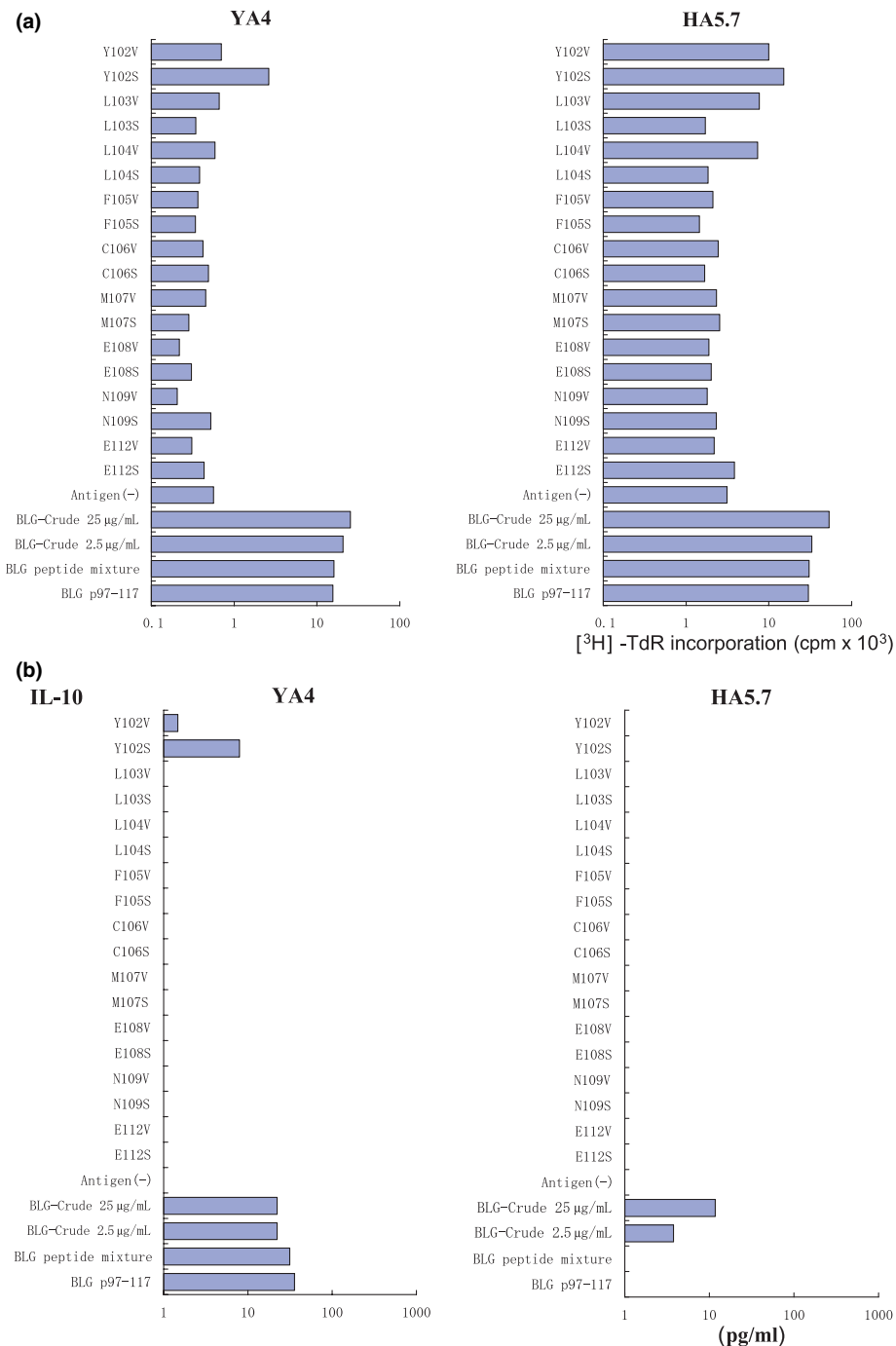


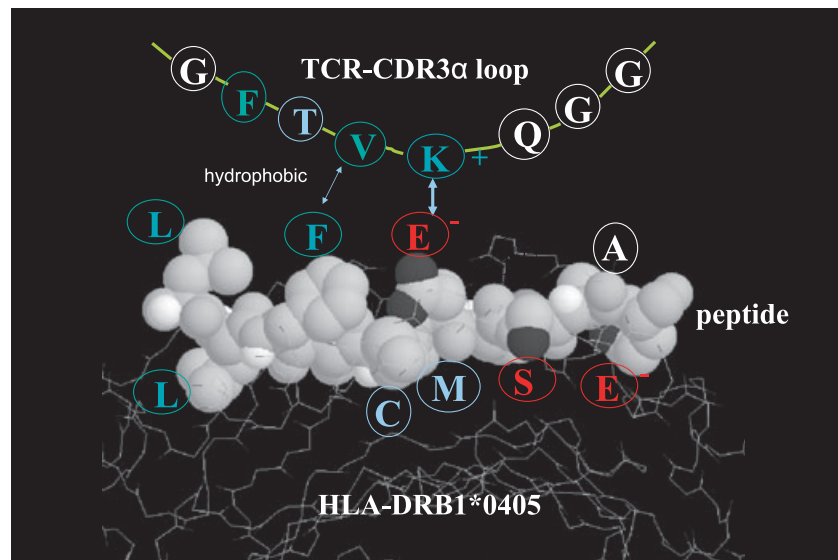
Fig. 3. T-cell clone (TCC) responses to beta-lactoglobulin (BLG) analog peptides. Single amino acid substitution peptides of BLGp102-112 were designed. Each polypeptide has 11 amino acid residues from number 102 to 112 and contains one amino acid substitution, as indicated in Fig. 3. (a) Proliferative responses of the TCCs to single amino acid-substituted peptides of BLG p102-112 are shown. (b) IL-10 production of the TCCs to single amino acid-substituted peptides of BLG p102-112 is shown.

amino acid beyond the β carbon and thus can assess the impact of its absence on binding affinity. It does not alter significantly the main-chain conformation and does not impose extreme electrostatic or steric effects.

In conclusion, this study has identified the T-cell minimal core epitope, predicted the TCR/

BLG-peptide/HLA complex structure, and searched for the immunomodulated peptides. The p105-108 substitutions are important to retain the TCR/BLG-peptide/HLA complex structure. Our data suggest that peptides with a mutated core epitope might be considered as candidate peptides for the modification

Fig. 4. Binding images between BLGp102-112 and HLA-DRB1*0405 molecules based on structural modeling. The CDR3 loops of the TCCs (HA5.7) were demonstrated to be 'QKV'. The negatively charged residue pE108 and the positively charged residue 'K' of the CDR3 loops interacted. The hydrophobic residue pF105 and 'V' of the CDR3 loops interacted with each other.



of the T-cell response to BLG in cow's milk allergy.

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References

1. SAARINEN KM, JUNTUNEN-BACKMAN K, JARVENPAA AL, et al. Supplementary feeding in maternity hospitals and the risk of cow's milk allergy: A prospective study of 6209 infants. *J Allergy Clin Immunol* 1999; 104: 457–61.
2. SAMPSON HA. Food allergy. Part 1: immunopathogenesis and clinical disorders. *J Allergy Clin Immunol* 1999; 103: 717–28.
3. WAL J-M. Cow's milk allergens. *Allergy* 1998; 53: 1013–22.
4. SELO I, CLEMENT G, BERNARD H, et al. Allergy to bovine beta-lactoglobulin: specificity of human IgE to tryptic peptides. *Clin Exp Allergy* 1999; 29: 1055–63.
5. INOUE R, MATSUSHITA S, KANEKO H, et al. Identification of beta-lactoglobulin-derived peptides and class II HLA molecules recognized by T cells from patients with milk allergy. *Clin Exp Allergy* 2001; 31: 1126–34.
6. SAKAGUCHI H, INOUE R, KANEKO H, et al. Interaction among human leucocyte antigen-peptide-T cell receptor complexes in cow's milk allergy: the significance of human leucocyte antigen and T cell receptor-complementarity determining region 3 loops. *Clin Exp Allergy* 2002; 32: 762–70.
7. KONDO N, AGATA H, FUKUTOMI O, MOTOYOSHI F, ORII T. Lymphocyte responses to food antigens in patients with atopic dermatitis who are sensitive to foods. *J Allergy Clin Immunol* 1990; 86: 253–60.
8. SAYLE RA, MILNER-WHITE EJ, RASMOL: biomolecular graphics for all. *Trends Biochem Sci* 1995; 20: 374.
9. FERREIRA F, EBNER C, KRAMER B, et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998; 12: 231–42.
10. BOUSQUET J, BECKER WM, HEJJAOUI A, et al. Differences in clinical and immunologic reactivity of patients allergic to grass pollens and to multiple-pollen species. II. Efficacy of a double-blind, placebo-controlled, specific immunotherapy with standardized extracts. *J Allergy Clin Immunol* 1991; 88: 43–53.
11. PECQUET S, BOVETTO L, MAYNARD F, FRITSCHÉ R. Peptides obtained by tryptic hydrolysis of bovine beta-lactoglobulin induce specific oral tolerance in mice. *J Allergy Clin Immunol* 2000; 105: 514–21.
12. FRITSCHÉ R, PAHUD JJ, PECQUET S, PFEIFER A. Induction of systemic immunologic tolerance to beta-lactoglobulin by oral administration of a whey protein hydrolysate. *J Allergy Clin Immunol* 1997; 100: 266–73.
13. FERGUSON TA, PETERS T JR, REED R, PESCE AJ, MICHAEL JG. Immunoregulatory properties of antigenic fragments from bovine serum albumin. *Cell Immunol* 1983; 78: 1–12.
14. LITWIN A, PESCE AJ, FISCHER T, MICHAEL M, MICHAEL JG. Regulation of the human immune response to ragweed pollen by immunotherapy. A controlled trial comparing the effect of immunosuppressive peptic fragments of short ragweed with standard treatment. *Clin Exp Allergy* 1991; 21: 457–65.
15. RUDENSKY AYU, PRESTON-HURLBURT P, HONG SC, BARLOW A, JANEWAY CA JR. Sequence analysis of peptides bound to MHC class II molecules. *Nature* 1991; 353: 622–7.
16. HOYNE G, CALLOW M, KUO M, THOMAS W. Inhibition of T-cell responses by feeding peptides containing major and cryptic epitopes: studies with the Der p1 allergen. *Immunology* 1994; 83: 190–5.
17. GROUX H, BIGLER M, DE VRIES JE, RONCAROLO MG. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* 1996; 184: 19–29.