

Enhancement of Antigen-Specific IFN- γ Production from CD8⁺ T Cells by a Single Amino Acid-Substituted Peptide Derived from Bovine α_{s1} -Casein

Mamoru Totsuka, Masahiro Kakehi, Masako Kohyama, Satoshi Hachimura,
Tatsuhiko Hisatsune, and Shuichi Kaminogawa

Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Modulation of CD8⁺ T-cell responses specific for an exogenous antigen by epitope variants would be advantageous to develop a novel means of antigen-specific immune regulation. We have analyzed CD8⁺ T-cell responses to single amino acid-substituted variants of a peptide corresponding to residues 142–149 (p142-149; LAYFYPEL) of α_{s1} -casein, a major milk allergen, which is a dominant determinant restricted by H-2K^b. An analog peptide L142I with a substitution of Ile for Leu at the nonanchor N-terminal residue induced more IFN- γ secretion than p142-149 from specific CD8⁺ T cells. Furthermore, L142I could prime CD8⁺ T cells more efficiently *in vivo*, and these L142I-primed cells secreted more IFN- γ than p142-149 in *vitro*. These findings are mainly explained by the greater ability of L142I to form stable K^b-peptide complexes. These findings indicate that appropriate analog peptides may be useful as efficient inducers of CD8⁺ T cells which recognize the parent peptide and secrete IFN- γ , a potent inhibitor of Th2-dependent events, including IgE production. © 1998 Academic Press

Key Words: analog peptides; α_{s1} -casein; CD8⁺ T cells; IFN- γ ; peptide-based immunotherapy.

INTRODUCTION

Most CD8⁺ T cells recognize endogenous peptide antigens presented by MHC class I molecules and show cytotoxic activity. Therefore, CD8⁺ T cells have important roles in tumor immunity (1) or in suppressing infections by viruses (2) and microbial pathogens (3). However, it has been shown that exogenous antigens also can be presented by MHC class I molecules (4) and generate CD8⁺ T-cell responses (5, 6). These CD8⁺ T cells have been shown to play an important role in regulation of immune responses (7–10). Thus, modulation of CD8⁺ T-cell responses would be effective in treatment of some kinds of immune diseases.

Many attempts have been made to analyze and modify T-cell responses by using analog peptides which

have an amino acid substitution at their T-cell receptor (TCR)-contacting residues or residues anchoring in MHC molecules (11). Analog peptides can generate qualitatively different T-cell responses compared to those induced by the wild-type peptide, such as cytokine production without proliferation (12), anergy (13, 14), or modulation of cytokine production patterns (15, 16). Moreover, some analog peptides are able to induce quantitatively different T-cell responses. It has been shown that the immunogenicity of antigenic peptides correlated well with both their affinities for MHC class I molecules (17) and the stability of the peptide/MHC class I molecule complexes (18). Thus, appropriate modification of an antigenic peptide to achieve a greater ability to bind to MHC class I molecules and/or to achieve more stable peptide/MHC class I molecule complexes may result in stronger CD8⁺ T-cell responses. In fact, many approaches to improve the immunogenicity of epitopes from tumor-specific antigens or viruses have been done by replacing anchor or non-anchor residues (19–22).

Analog peptides which strongly induce CD8⁺ T cells specific for an exogenous antigen to produce interferon (IFN)- γ would be useful for peptide-based therapy for Th2-biased immune diseases such as allergies, because IFN- γ is a potent inhibitor of Th2-dependent events, including IgE production (23, 24). Moreover, these peptides would modulate the immune responses to the antigen without the risk of accidental activation of CD4⁺ Th2 cells. In the present study, we prepared a panel of 90 analog peptides each with a single amino acid substitution based on the sequence of an octamer peptide corresponding to residues 142–149 (p142-149) of bovine α_{s1} -casein, a major milk allergen. p142-149 is recognized in a H-2K^b-restricted manner by CD8⁺ T-cell clones specific for α_{s1} -casein (10, 25, 26). Considering that p142-149 has a sequence in exact accordance with a K^b-binding motif reported by Falk *et al.* (27), substitutions at its anchor residues are not likely to increase its ability to bind to K^b molecules. Thus, the substitutions were introduced at nonanchor residues of

the peptide. We screened this panel of peptides for strong inducers of IFN- γ production in assays of CD8⁺ T-cell clones. We observed that an analog peptide increased IFN- γ secretion from not only the CD8⁺ T-cell clones but also polyclonal p142-149-specific CD8⁺ T cells. In addition, the analog peptide could strongly prime CD8⁺ T cells *in vivo*, and these primed cells produced IFN- γ upon stimulation with the wild-type peptide.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (6 weeks old) were purchased from Clea Japan Inc. (Tokyo, Japan). BALB.B mice were kindly provided by Dr. M. Takada (Kochi Medical School, Nangoku, Japan).

Ags and Abs

An octamer peptide corresponding to residues 142–149 of bovine α_{s1} -casein (p142-149, LAYFYPEL) was synthesized by means of a peptide synthesizer (430A; Applied Biosystems, Foster City, CA) and purified by HPLC. An analog of p142-149 with a substitution of Ile for ¹⁴²Leu, designated L142I, was purchased from Bio Synthesis (Lewisville, TX). The other analog peptides (Fig. 1) were purchased from Chiron Mimotopes Pty LTD (Clayton, Victoria, Australia).

Anti-IFN- γ mAb XMG1.2 (28) was kindly provided by Dr. J. S. Abrams (DNAX, Palo Alto, CA). Anti-IFN- γ mAb R4-6A2 (29) was obtained from the American Type Culture Collection (Rockville, MD). The cells producing these mAbs were grown in ascites, and the mAbs were purified using MAb Trap G II (Pharmacia Biotech, Uppsala, Sweden) and concentrated by ammonium persulfate precipitation. Anti-K^b mAb E122.46 (30) was purchased from Seikagaku Corp. (Tokyo, Japan).

Cell Lines

α_{s1} -Casein-specific CD8⁺ T-cell clones 5F1, Uc (25), and 6B1 (10) were derived from C57BL/6 mice immunized with α_{s1} -casein in CFA and have been shown to respond to trypsin-digested α_{s1} -casein (t-CN) in the context of K^b molecules (6). Each of these CD8⁺ T-cell clones expresses a different TCR V β gene (V β 11, V β 2, and V β 3 for 5F1, 6B1 and Uc, respectively; Ref. 26). These clones were maintained in conditioned RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μ g/ml streptomycin, in the presence of 10% FCS and 20 U/ml IL-2, and were stimulated every 10 to 14 days with irradiated syngeneic splenocytes and 5 μ g/ml t-CN. The mouse mutant lymphoma cell

line RMA-S (31) was kindly provided by Dr. T. Mizuochi (National Institute of Infectious Diseases, Tokyo, Japan), and these cells were maintained in conditioned RPMI 1640 medium containing 10% FCS.

Separation of CD8⁺ T Cells from Ag-Primed Lymph Node Cells

C57BL/6 mice were each immunized with 10 μ g of a peptide emulsified in complete Freund's adjuvant H37Ra (CFA H37Ra; Difco Laboratories, Detroit, MI) in the footpads and at the base of tail. Seven days after immunization, the inguinal and popliteal lymph node cells were prepared, and CD8⁺ T cells were purified using anti-CD8-mAb-coupled magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, the lymph node cells (1×10^8) were treated with 100 μ l of microbeads for 15 min at 4°C, and CD8⁺ T cells were concentrated using MACS separation columns (Miltenyi Biotec GmbH).

Assessment of the Quantity of Cytokine Secretion

Freshly isolated T cells (4×10^5 /well) or cloned T cells (4×10^4 /well) were plated into 96-well flat-bottom plates with irradiated syngeneic splenocytes (2×10^5 /well) in the presence or absence of various concentrations of antigen in 200 μ l of RPMI supplemented with 10% FCS. Supernatants were collected after 48 or 72 h. The quantity of IFN- γ in the culture supernatant was assessed by two-site ELISA as previously reported by Abrams *et al.* (32). To detect IFN- γ , microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with R4-6A2 and treated with biotinylated XMG1.2, followed by incubation with alkaline phosphatase-conjugated streptavidin and disodium *p*-nitrophenylphosphate for color development. The OD was measured at a test wavelength of 405 nm and at a reference wavelength of 490 nm.

Peptide Binding to H-2K^b on RMA-S Cells

Binding of peptides to H-2K^b molecules was assessed using RMA-S cells. This T lymphoma cell line expresses thermolabile, empty MHC class I (K^b) molecules because of lack of expression of the TAP-2 peptide transporter, which transports peptides from the cytoplasm to the ER where MHC class I molecules bind peptides (31). The level of expression of MHC class I molecules on the surface of RMA-S cells increases when they are incubated at 22–28°C (33) or when they are incubated with peptides that bind to the class I molecule (34). Relative levels of peptide binding to K^b on the surface of RMA-S cells were examined by basi-

cally the same methods as described previously (35). RMA-S cells were incubated in RPMI 1640 medium supplemented with 10% FCS at 25°C for 16–18 h to allow the cells to express empty K^b on the cell surface. The cells were washed twice with RPMI 1640 medium, resuspended in RPMI 1640 medium containing 1% FCS in the presence or absence of serial dilutions of peptides, and left at 25°C for 1 h. Then the cells were incubated at 37°C for 3 h. The cells were washed with RPMI 1640 medium containing 10% FCS and resuspended in HBSS (Gibco BRL, Gaithersburg, MD). Biotin-conjugated anti-mouse H-2K^b mAb (E121.46) was added to the cells at the appropriate concentration and the cells were incubated for 30 min on ice. The cells were washed and incubated for 20 min on ice with an appropriate dilution of streptavidin-PE (Gibco BRL). The cells were washed and fluorescence intensity was measured by FACSsort (Becton–Dickinson, Mountain View, CA).

Relative dissociation rates of peptide/K^b complexes on the surface of RMA-S cells were estimated by the method described previously (36). RMA-S cells were incubated in RPMI 1640 medium supplemented with 10% FCS at 25°C for 16–18 h and for a further 1 h at 25°C in the presence of each peptide at 10 μ M. The cells were then washed three times with RPMI 1640 medium and placed again at 25°C. This time point was taken as time zero. At this time point and at 2, 4, 6, 7, 8, and 9 h, aliquots of the cells were transferred to a 37°C incubator. At 10 h, each group of cells was collected and stained for K^b–peptide complexes with anti-K^b mAb as described above. Cells loaded with peptides and incubated at 25°C for 10 h were also examined.

RESULTS

Efficient Induction of IFN- γ Secretion from α_{s1} -Casein-Specific CD8⁺ T-Cell Clones by Variants of the Epitope Peptide

In our previous report, we showed that CD8⁺ T cells purified from lymph node cells of C57BL/6 mice immunized with bovine α_{s1} -casein secreted IFN- γ in response to t-CN (6). The minimal determinant recognized by the purified CD8⁺ T cells was found to be an octamer peptide corresponding to p142-149 (26). Three α_{s1} -casein specific CD8⁺ T-cell clones, 5F1, 6B1, and Uc, were established from lymph node cells derived from α_{s1} -casein-immunized C57BL/6 mice as described previously (10, 25). These clones exhibited IFN- γ secretion in response to t-CN (10, 25) or p142-149 (26). According to the H-2K^b binding motif reported by Falk *et al.* (27), anchor residues of p142-149 were considered to be residues ¹⁴⁴Tyr, ¹⁴⁶Tyr, and ¹⁴⁹Leu. To find variants of p142-149 with the potential to augment the

CD8⁺ T-cell responses, we designed 90 analog peptides with single amino-acid substitutions at nonanchor residues, ¹⁴²Leu, ¹⁴³Ala, ¹⁴⁵Phe, ¹⁴⁷Pro, and ¹⁴⁸Glu of p142-149 (Fig. 1) and IFN- γ secretion by CD8⁺ T-cell clones treated with these peptides was examined.

A summary of the levels of IFN- γ produced by the three CD8⁺ T-cell clones in response to each of the 90 analog peptides at 10 μ M is shown in Fig. 1. We found that two of the analog peptides induced significantly greater amounts of IFN- γ production than did p142-149. The peptide L142I, with a substitution of Ile for residue ¹⁴²Leu, exhibited strong induction of IFN- γ secretion from all three clones. L142I induced two to five times greater amounts of IFN- γ secretion than p142-149. Another peptide, F145H, with a substitution of His for ¹⁴⁵Phe, also induced about a threefold greater amount of IFN- γ secretion from 5F1 and Uc than p142-149. However, no IFN- γ secretion from 6B1 was detected upon stimulation with F145H.

Subsequently, we focused on L142I and examined the levels of IFN- γ secretion from CD8⁺ T-cell clones in response to various concentrations of p142-149 or L142I (Fig. 2). The amount of secreted IFN- γ increased in proportion to the antigen dosage. IFN- γ secretion induced by L142I constantly exceeded that induced by p142-149 at any peptide concentration from 10⁻⁸ to 10⁻⁴ M in the case of both 5F1 and Uc. There were considerable differences in the maximum levels of IFN- γ produced by Uc cells in response to L142I and p142-149. This suggests that L142I possesses the potential to elicit IFN- γ secretion from Uc more strongly than p142-149 even when present in an amount in excess of that required for saturating the K^b molecules.

The cytolytic activity of the T-cell clones when stimulated with L142I was greater than that when stimulated with p142-149, although the difference was not so significant (data not shown). Therefore, the enhanced production of IFN- γ induced by L142I does not appear to be due to less effective lysis of antigen-presenting cells as suggested by a previous report showing that the poorer target of cytotoxicity was the better inducer of IFN- γ production from a CTL and vice versa (37). These CD8⁺ T-cell clones showed a proliferative response when stimulated with L142I greater than that elicited by p142-149 (data not shown). These observations suggest that L142I is not likely to selectively enhance activation of the signaling pathway leading to IFN- γ production alone. IL-2 production was not observed in the culture supernatants 24 h after stimulation with p142-149 (26) or L142I (data not shown).

K^b-Molecule Binding Capacity of p142-149 and L142I

To assess the mechanisms underlying the enhanced levels of IFN- γ secretion induced by L142I, the abilities of L142I and p142-149 to bind to MHC class I (K^b)

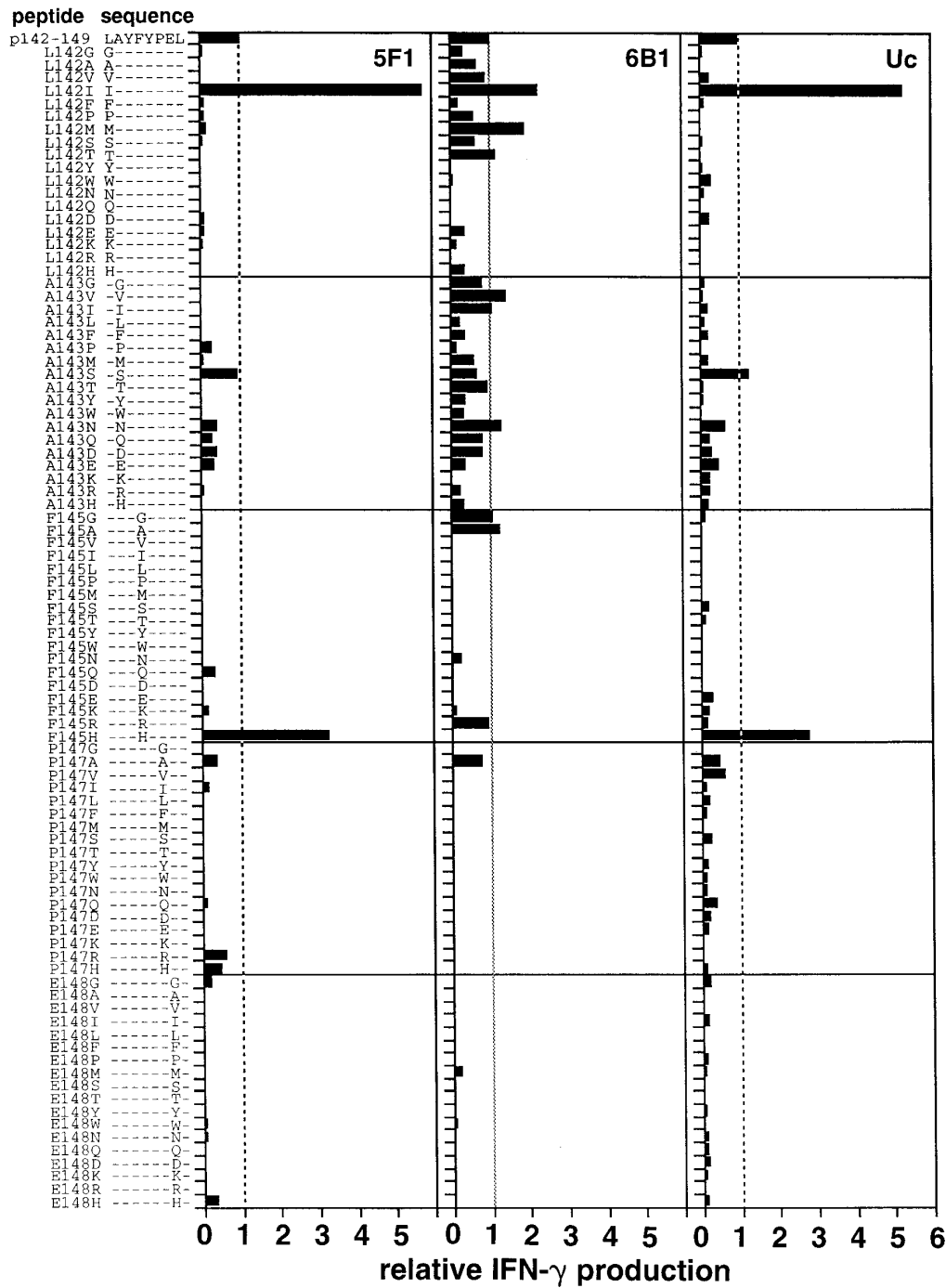


FIG. 1. IFN- γ secretion from α_{s1} -casein-specific CD8⁺ T-cell clones induced by single amino acid-substituted analogs of an epitope peptide corresponding to residues 142–149 of bovine α_{s1} -casein (p142-149). α_{s1} -Casein-specific CD8⁺ T-cell clones 5F1, 6B1, and Uc (4×10^4 /well) were cultured with irradiated syngeneic splenocytes (2×10^5 /well) in the presence or absence of each peptide at 10 μ M. Supernatants were collected 48 h later and the quantity of IFN- γ in each culture supernatant was assessed by two-site ELISA. Relative values were calculated as below: (relative IFN- γ production) = [(IFN- γ production induced by the relevant peptide) – (IFN- γ production in the absence of peptide)]/[(IFN- γ production induced by p142-149) – (IFN- γ production in the absence of peptide)].

molecules were examined. For this analysis, we used RMA-S cells and examined the ability of each of the two peptides to stabilize expression of H-2K^b on the cell surface. RMA-S cells are defective in TAP function and cannot load MHC class I molecules with peptides derived from the cytoplasm, resulting in expression of

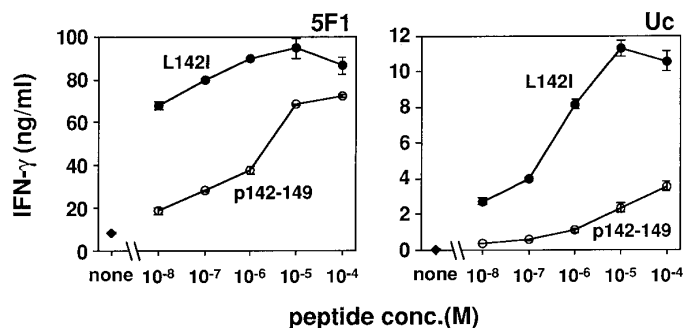


FIG. 2. IFN- γ secretion from α_{s1} -casein-specific CD8⁺ T-cell clones 5F1 and Uc induced by various concentrations of p142-149 or L142I. CD8⁺ T-cell clones 5F1 and Uc (4×10^4 /well) were stimulated with various concentrations of p142-149 or L142I in the presence of irradiated syngeneic splenocytes (2×10^5 /well) for 48 h. Culture supernatants were collected and the quantity of IFN- γ present was assessed by two-site ELISA.

empty K^b molecules on the cell surface which are stable at 22–28°C but not at 37°C (33). When RMA-S cells that have been grown at 25°C to induce expression of high levels of empty K^b molecules are incubated with peptides capable of binding to K^b molecules, the peptide/K^b complexes can stably exist on the cell surface even after the cells have been transferred to 37°C (34). Therefore, the finding of a larger amount of K^b molecules on the surface of cells incubated with a certain peptide would suggest its higher affinity for K^b molecules. K^b molecules were detected by means of biotin-conjugated anti-mouse H-2K^b mAb followed by treatment with streptavidin-PE and the fluorescence intensity was measured by flow cytometry. It was assumed that the fluorescence intensity was directly proportional to the number of peptide/MHC complexes on the cell surface. At the same time, a cytokine production assay was carried out using peptide-pulsed RMA-S cells as antigen-presenting cells (APC) to further study the relationship between the amounts of secreted IFN- γ and the number of peptide/MHC complexes expressed on APC.

The results are shown in Fig. 3. L142I treatment generated stronger fluorescence intensity than treatment with p142-149 at concentrations in the range of 10^{-9} to 10^{-4} M (Fig. 3A). L142I induced the same level of K^b molecule expression as that induced by about a 100-fold higher concentration of p142-149, suggesting that L142I possesses about 100 times higher affinity for K^b molecules than p142-149. In accordance with the binding assay results, the amount of IFN- γ secretion induced by L142I presented on RMA-S cells exceeded that induced by p142-149 at all concentrations examined (Fig. 3B).

It has been reported that the intensity of T-cell responses induced by a peptide in complex with a MHC class I molecule depends on the stability of the com-

plexes formed between the peptide and the MHC class I molecules (18) as well as its affinity for MHC class I molecules (17). To assess the ability of L142I and p142-149 to form stable complexes with K^b molecules, we analyzed the time-dependent decrease in number of peptide/K^b complexes on the surface of RMA-S cells at 37°C by flow cytometry. As shown in Fig. 4, L142I/K^b complexes on the cell surface were more stable than the p142-149/K^b complexes. The half-lives of the p142-149/K^b complex and the L142I/K^b complex were 3.3 h and 8 h, respectively.

These results revealed that one of the reasons for the increase in IFN- γ production induced by L142I was its higher capacity to form a stable complex with K^b molecules as well as its higher affinity for K^b molecules. In addition, the amino-acid substitution in L142I may have affected its interaction with TCR, especially in the case of Uc cells, resulting in signals of greater intensity being transduced through the TCR, considering that Uc cells secreted a larger amount of IFN- γ upon stimulation with L142I at 10^{-4} M than that occurring upon stimulation with p142-149 at 10^{-4} M while almost the same numbers of peptide/K^b complexes were expressed on the RMA-S cells (Fig. 3). Although there has been a report that the enhanced IFN- γ secretion from a CD4⁺ T-cell clone induced by an analog peptide was due to increased IL-12 production from APC displaying the analog peptide upon interaction with the T cells (38), addition of anti-IL-12 antibodies to the culture of Uc cells in the presence of irradiated splenocytes as APC had no effect on IFN- γ secretion induced by p142-149 or L142I (data not shown).

IFN- γ Secretion from p142-149-Primed Polyclonal CD8⁺ T Cells in Response to L142I

Since L142I enhanced IFN- γ secretion from all three p142-149-specific CD8⁺ T-cell clones, L142I was expected to show the same effect on p142-149-primed polyclonal CD8⁺ T cells. Thus, we examined the levels of IFN- γ secretion from CD8⁺ T cells purified from among lymph node cells isolated from p142-149-immunized mice. Polyclonal CD8⁺ T cells derived from C57BL/6 mice immunized with 10 μ g of p142-149 were stimulated with p142-149 or L142I in the presence of APC for 72 h, and then the culture supernatants were collected to measure the amount of secreted IFN- γ . As shown in Fig. 5A, stimulation with L142I resulted in production of a greater amount of IFN- γ than that observed following stimulation with p142-149. Production of IL-4 and IL-10 was not observed (data not shown). These results clearly indicate that L142I could enhance not only IFN- γ secretion by particular p142-149-specific CD8⁺ T-cell clones but also that by polyclonal CD8⁺ T cells. Probably, this enhancement was mainly attributable to a larger number of L142I/K^b complexes on APC compared to the number of p142-

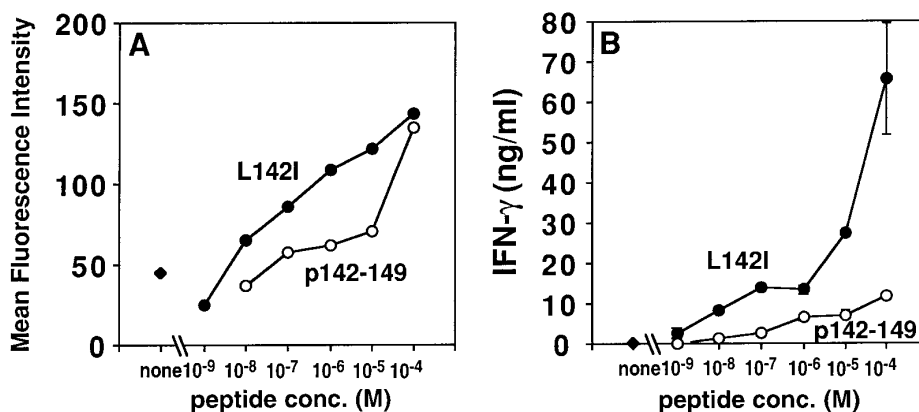


FIG. 3. Relative binding of p142-149 and L142I to K^b molecules on the surface of RMA-S cells (A) and IFN- γ secretion from Uc cells upon stimulation with these peptides displayed on RMA-S cells (B). RMA-S cells were incubated at 25°C for 16–18 h to allow empty K^b molecules to be expressed on the cell surface. The cells were pulsed with serial dilutions of p142-149 or L142I for an hour at 25°C. (A) Peptide-loaded RMA-S cells (half of the pulsed sample) were transferred to a 37°C incubator, left for 3 h, and stained with biotin-conjugated anti-mouse H-2K ^{b} followed by streptavidin-PE. Fluorescence intensity was measured by FACSsort. (B) Peptide-loaded RMA-S cells (the remaining half of the pulsed sample) were cultured at 5×10^4 /well with Uc cells at 4×10^4 /well for 48 h, and then the culture supernatants were collected and the quantity of IFN- γ present was assessed by two-site ELISA.

149/ K^b complexes formed, resulting in more efficient interaction with TCR. As expected, CD8 $^+$ T cell-depleted lymph node cells from p142-149-immunized mice secreted no detectable IFN- γ when stimulated with L142I or p142-149 (data not shown).

It is possible that the enhanced IFN- γ secretion induced by L142I is only relevant for CD8 $^+$ T cells de-

rived from mice with a tendency to display Th1-type responses such as C57BL/6 mice. To examine this possibility, we performed the same experiments described above using BALB.B mice, which have the same genetic background as that of BALB/c mice having a tendency to develop Th2 type responses and which have the same H-2 locus as that of C57BL/6 mice. As shown in Fig. 5B, L142I induced higher levels of IFN- γ than did p142-149 as was the case using C57BL/6 mice. Again, production of IL-4 and IL-10 was not observed (data not shown). These results clearly demonstrate that the ability of L142I to increase IFN- γ production is relevant for not only CD8 $^+$ T cells derived from “Th1” mice, but also those from “Th2” mice.

In Vivo Priming with L142I Resulted in Augmentation of IFN- γ Secretion from CD8 $^+$ T Cells upon Stimulation with p142-149 in Vitro

Our *in vitro* results led us to examine whether priming with L142I *in vivo* would result in more efficient induction of p142-149-responding CD8 $^+$ T cells than priming with p142-149. As shown in Fig. 6, L142I-primed CD8 $^+$ T cells from either C57BL/6 or BALB.B mice could recognize p142-149, and, surprisingly, secreted more IFN- γ than p142-149-primed CD8 $^+$ T cells in response to subsequent stimulation with p142-149. Secretion of IFN- γ from CD8 $^+$ T-cell-depleted lymph node cells derived from L142I-immunized mice was not detected (data not shown). IL-4 and IL-10 were not detected in any of the samples (data not shown). These results demonstrate that L142I could prime CD8 $^+$ T cells for responsiveness to p142-149, and probably for

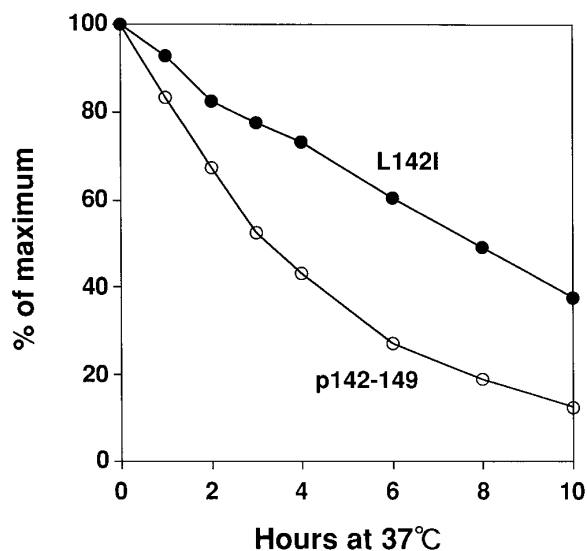


FIG. 4. Dissociation curves of p142-149 or L142I/ K^b complexes at the surface of RMA-S cells. RMA-S cells were incubated at 25°C for 16–18 h, pulsed with p142-149 or L142I at 10 μ M, and left at 25°C for an hour. The cells were washed three times and placed again at 25°C. Aliquots of the cells were transferred to a 37°C incubator in order. After 10 h, each aliquot was collected and the peptide/ K^b complexes on the cell surface were stained with biotin-conjugated anti-mouse H-2K ^{b} followed by avidin-PE. Fluorescence intensity was measured by FACSsort.

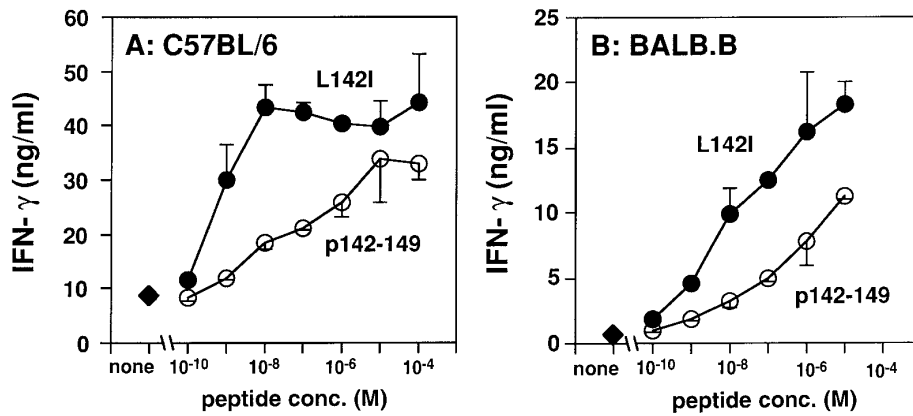


FIG. 5. IFN- γ secretion from p142-149-primed bulk CD8⁺ T cells in response to p142-149 or L142I *in vitro*. C57BL/6 (A) or BALB.B (B) mice were each immunized with 10 μ g of p142-149 emulsified in CFA H37Ra. After 7–10 days, lymph nodes were isolated and CD8⁺ T cells were concentrated by means of anti-CD8-mAb-coupled magnetic microbeads. The purified CD8⁺ T cells (4×10^5 /well) were stimulated *in vitro* with the indicated concentration of p142-149 or L142I in the presence of irradiated syngeneic splenocytes (2×10^5 /well) for 72 h. Culture supernatants were collected and the quantity of IFN- γ present was assessed by two-site ELISA. The level of IFN- γ secretion from CD8⁺ T cell-depleted lymph node cells was below the detection limit of 0.4 ng/ml.

responsiveness to α_{s1} -casein as well, more efficiently than p142-149 *in vivo*.

DISCUSSION

In the present study, we showed that an analog peptide L142I having a single amino-acid substitution at a non-anchor residue of the octamer peptide p142-149, which is recognized by α_{s1} -casein-specific CD8⁺ T cells, significantly enhanced IFN- γ secretion from CD8⁺ T cells. Furthermore, compared to p142-149, the analog peptide could prime specific CD8⁺ T cells more efficiently *in vivo*, and these primed cells produced more IFN- γ upon sub-

sequent stimulation with p142-149 than those primed with p142-149. These results indicate that appropriately substituted peptides can prime CD8⁺ T cells recognizing the wild-type peptide more efficiently than the wild-type peptide itself *in vivo*. Such an analog peptide would be useful as an efficient peptide vaccine to induce specific CD8⁺ T-cell responses.

Previous attempts to enhance CD8⁺ T-cell responses by stimulation with variant antigenic peptides have focused predominantly on cytotoxicity against tumor cells (21, 22) or virus-infected cells (19, 20). In contrast to these studies, we focused on cytokine secretion by

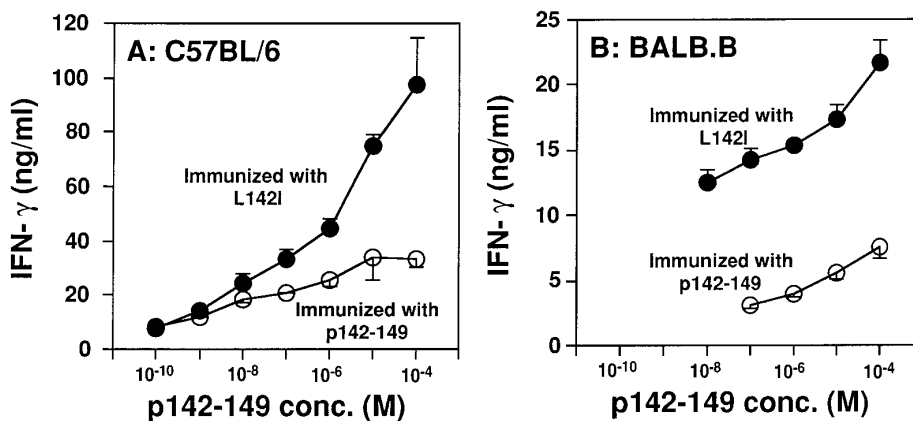


FIG. 6. IFN- γ secretion from p142-149- or L142I-primed bulk CD8⁺ T cells in response to p142-149. C57BL/6 (A) or BALB.B (B) mice were each immunized with 10 μ g of p142-149 or L142I emulsified in CFA H37Ra. After 7–10 days, lymph nodes were isolated and CD8⁺ T cells were concentrated by means of anti-CD8-mAb-coupled magnetic microbeads. The purified CD8⁺ T cells were stimulated with p142-149 *in vitro* and the amount of secreted IFN- γ was measured as described in the legend to Fig. 5. The level of IFN- γ secretion from p142-149- or L142I-primed CD8⁺ T cells in the absence of p142-149 was 8.7 ± 0.6 and 7.5 ± 0.5 ng/ml, respectively, for (A) and <0.4 and 4.9 ± 0.1 ng/ml, respectively, for (B). The level of IFN- γ secretion by CD8⁺ T cell-depleted lymph node cells derived from either p142-149- or L142I-immunized mice in the presence of 100 μ M p142-149 was below the detection limit.

responding CD8⁺ T cells specific for an exogenous soluble antigen, and we have demonstrated that a particular analog peptide can augment IFN- γ production by these CD8⁺ T cells. Since such CD8⁺ T cells have been shown to play an important role in immune regulation (8–10), our results imply that an analog peptide capable of enhancing cytokine production by CD8⁺ T cells would be useful in developing a novel strategy for peptide-based immune regulation.

As shown in Fig. 3A, L142I compared with p142-149 could form the same number of complexes with K^b molecules at a lower peptide concentration, suggesting that L142I has a higher affinity for K^b molecules than p142-149. Furthermore, the L142I/K^b complexes were more stable than the p142-149/K^b complexes (Fig. 4). Therefore, at the same peptide concentration, the number of L142I/K^b complexes displayed on APC surpasses that of p142-149/K^b complexes. Thus, mainly because L142I has an enhanced ability to be displayed on APC, as a result of the substitution of Ile for ¹⁴²Leu in p142-149, and because this substitution has no negative effect on the interaction with TCR, the levels of IFN- γ production by the CD8⁺ T-cell clones (Figs. 1–3) and purified CD8⁺ T cells derived from p142-149-immunized mice (Fig. 5) were increased upon stimulation with L142I. A larger number of L142I/K^b complexes could engage more TCRs, resulting in an increase in the signals leading to IFN- γ production in each T cell and/or an increase in the number of activated T cells secreting IFN- γ . Likewise, more efficient priming of CD8⁺ T cells capable of responding to p142-149 as a result of immunization with L142I compared to p142-149 can be considered to be mainly due to L142I being more efficiently displayed on APC.

When the CD8⁺ T cell clone Uc was stimulated with p142-149 or L142I at 100 μ M, using RMA-S cells as APC, the Uc cells secreted a much larger amount of IFN- γ in response to L142I compared to p142-149 despite there being almost equal numbers of peptide/K^b complexes on the RMA-S cells (Fig. 3). These findings and the difference in maximum levels of IFN- γ secretion from Uc cells induced by the two peptides (Fig. 2) suggest that the substitution of Ile for ¹⁴²Leu affects the interaction with the TCR of Uc cells as well as the interaction with K^b molecules, resulting in an increase in the signals to enhance IFN- γ production. This may be responsible in part for the increased IFN- γ secretion by CD8⁺ T cells derived from p142-149-immunized mice. One possible explanation for these observations could be the conformational change of TCR engaged with L142I/K^b complexes (conformational model (39)). The subtle conformational changes of the L142I/K^b complexes would generate structural alterations of engaged TCRs on Uc cells, leading to augmentation of signal transduction. It is also possible to assume that a conformational change in the L142I/K^b complexes results in increased binding of CD8 molecules

to the complexes and brings about qualitatively and/or quantitatively different signals through the TCR and CD8 molecules, since it has been reported that a CD8 molecule beside a TCR binds to the same MHC class I molecule and prolongs the duration of the interaction between the TCR and the peptide/MHC complex (40, 41). Alternatively, according to the kinetic model (42–44), the increased affinity of L142I/K^b complexes for TCRs of Uc cells would result in more efficient recruitment of intracellular protein kinases such as ZAP-70 or Lck which associate with TCR or CD8 molecules and play important roles in signaling through the TCR complexes.

The three-dimensional structures of complexes formed between K^b molecules and a single type of peptide have been determined for several peptides by crystallographic analyses (45–49). One of these peptides, vesicular stomatitis virus nucleoprotein [52–59] (VSV-8, RGYVYQGL), has a sequence with the same anchor residues of Tyr at position 3 (P3), Tyr at P5, and Leu at P8 as those of p142-149. The side chains of these anchor residues are accommodated in so-called pockets on the peptide-binding groove of the K^b molecule, confirming their importance for binding to K^b molecules (46, 47). In this complex, a side chain of the peptide N-terminal Arg residue extends vertically toward the solvent, which seems to be a feature common to peptides in complex with K^b molecules (46, 47, 49). This implies that the N-terminal residue ¹⁴²Leu of p142-149 has the same conformation in complex with K^b molecules, which is consistent with our results suggesting that the side chain of the residue at this position is recognized by TCR. In contrast, the difference in efficiency of binding to K^b molecules between p142-149 and L142I indicates that the side chain of the N-terminal residue of a peptide can influence binding to K^b molecules despite its predicted orientation toward TCR. Comparable with this, it has been reported that, in the case of a peptide from ovalbumin (ovalbumin [257–264], OVA-8; SIINFEKL), a substitution of Asp for Glu at position 6 where the side chain was shown to be solvent exposed and not directly involved in binding to K^b (49) can considerably decrease the binding efficiency (50). The difference in K^b binding efficiency of p142-149 and L142I may be due to a difference in the interaction between the wall of the peptide-binding groove of the K^b molecule and the part near the base of the side-chain moiety of Leu or Ile at the N-terminus, since almost the entire surface of the short side chain of the Ser residue at the N-terminus of OVA-8, which pointed toward TCR, was in contact with the K^b molecule (49). Alternatively, conformational changes within the peptide induced by the substitution (48) might possibly exert a significant influence on its binding to K^b molecules.

The increased ability of a single substituted peptide to form a stable peptide/MHC class I complex has been

reported for the complex of a human class I molecule HLA-A*0201 and a variant of a 9-mer peptide derived from human immunodeficiency virus (19). In that report, a substitution at the N-terminal residue of the peptide resulted in a binding affinity similar to that of the wild-type peptide, but the stability of the HLA-A*0201-peptide complex was significantly increased. These and our findings imply that a side chain of the N-terminal residue of a MHC class I binding peptide may in general play an important role in stabilizing the peptide-MHC class I complex.

Recent studies have shown that, in certain immune diseases, either the Th1-type or the Th2-type immune response is dominant; for example, multiple sclerosis and rheumatoid arthritis are Th1-dominant diseases, whereas allergies are Th2-dominant diseases (51). It has been reported that IFN- γ selectively inhibits Th2-type responses (23), and that CD8⁺ T cells secreting IFN- γ selectively inhibit IgE production *in vivo* (24). We have demonstrated that efficient induction of IFN- γ production by L142I is relevant not only for mice with a tendency to display Th1-type responses but also mice prone to display Th2-type responses (Figs. 5 and 6). Therefore, such a peptide capable of increasing IFN- γ production from CD8⁺ T cells may be useful for ameliorating Th2-biased immune diseases such as allergies. Moreover, short peptides capable of inducing CD8⁺ T-cell responses have a low possibility of being recognized by CD4⁺ T cells, because of the distinct MHC-molecule requirements. Thus, such peptides could be used without the risk of accidental activation of CD4⁺ T cells relevant to the disease. In this study, we have demonstrated that, using a single amino-acid substituted analog of an epitope peptide, it is possible to enhance responses of exogenous antigen-specific CD8⁺ T cells and to induce such cells efficiently *in vivo* without priming CD4⁺ T cells. Our observations may have important implications for controlling the Th1-Th2 balance via antigen-specific CD8⁺ T cells.

REFERENCES

- Boon, T., Cerottini, J. C., Van den Eynde, B., van der Bruggen, P., and Van Pel, A., Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.* **12**, 337–365, 1994.
- Biron, C. A., Cytokines in the generation of immune responses to, and resolution of, virus infection. *Curr. Opin. Immunol.* **6**, 530–538, 1994.
- Li, S., Rodrigues, M., Rodriguez, D., Rodriguez, J. R., Esteban, M., Palese, P., Nussenzweig, R. S., and Zavala, F., Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8⁺ T-cell-mediated protective immunity against malaria. *Proc. Natl. Acad. Sci. USA* **90**, 5214–5218, 1993.
- Kovacsovics, B. M., and Rock, K. L., A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**, 243–246, 1995.
- Raychaudhuri, S., Tonks, M., Carbone, F., Ryskamp, T., Morrow, W. J., and Hanna, N., Induction of antigen-specific class I-restricted cytotoxic T cells by soluble proteins *in vivo*. *Proc. Natl. Acad. Sci. USA* **89**, 8308–8312, 1992.
- Hisatsune, T., Nishijima, K., Kohyama, M., Kato, H., and Kaminogawa, S., CD8⁺ T cells specific to the exogenous antigen: Mode of antigen recognition and possible implication in immunosuppression. *J. Immunol.* **154**, 88–96, 1995.
- Noble, A., Staynov, D. Z., Diaz, S. D., Lee, T. H., and Kemeny, D. M., Elimination of IgE regulatory rat CD8⁺ T cells *in vivo* increases the co-ordinate expression of Th2 cytokines IL-4, IL-5 and IL-10. *Immunology* **80**, 326–329, 1993.
- Kemeny, D. M., Noble, A., Holmes, B. J., and Diaz, S. D., Immune regulation: A new role for the CD8⁺ T cell. *Immunol. Today* **15**, 107–110, 1994.
- Nishijima, K., Hisatsune, T., Kato, H., Shiho, O., and Kaminogawa, S., α_{s1} -casein-specific CD8⁺ T cell clone that inhibits its own interferon- γ production by producing interleukin-10. *Biosci. Biotechnol. Biochem.* **59**, 2274–2276, 1995.
- Nishijima, K., Hisatsune, T., Minai, Y., Kohyama, M., and Kaminogawa, S., Anti-IL-10 antibody enhances the proliferation of CD8⁺ T cell clones: Autoregulatory role of murine IL-10 in CD8⁺ T cells. *Cell. Immunol.* **154**, 193–201, 1994.
- Sloan-Lancaster, J., and Allen, P. M., Altered peptide ligand-induced partial T cell activation: Molecular mechanisms and role in T cell biology. *Annu. Rev. Immunol.* **14**, 1–27, 1996.
- Evavold, B. D., and Allen, P. M., Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science* **252**, 1308–1310, 1991.
- Sloan-Lancaster, J., Evavold, B. D., and Allen, P. M., Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* **363**, 156–159, 1993.
- Sloan-Lancaster, J., Evavold, B. D., and Allen, P. M., Th2 cell clonal anergy as a consequence of partial activation. *J. Exp. Med.* **180**, 1195–1205, 1994.
- Windhagen, A., Scholz, C., Höllsberg, P., Fukaura, H., Sette, A., and Hafler, D. A., Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity* **2**, 373–380, 1995.
- Kohyama, M., Kakehi, M., Totsuka, M., Hachimura, S., Hisatsune, T., and Kaminogawa, S., Selective induction of CD8⁺ T cell functions by single substituted analogs of an antigenic peptide: Distinct signals for IL-10 production. *FEBS Lett.* **423**, 138–142, 1998.
- Sette, A., Vitiello, A., Reherman, B., Fowler, P., Naytersina, R., Kast, W. M., Melief, C. J., Oseroff, C., Yuan, L., Ruppert, J., Sidney, J., del Guercio, M., Southwood, S., Kubo, R. T., Chesnut, R. W., Grey, H. M., and Chisari, F. V., The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. *J. Immunol.* **153**, 5586–5592, 1994.
- van der Burg, S. H., Visseren, M. J., Brandt, R. M., Kast, W. M., and Melief, C. J., Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J. Immunol.* **156**, 3308–3314, 1996.
- Pogue, R. R., Eron, J., Frelinger, J. A., and Matsui, M., Amino-terminal alteration of the HLA-A*0201-restricted human immunodeficiency virus pol peptide increases complex stability and *in vitro* immunogenicity. *Proc. Natl. Acad. Sci. USA* **92**, 8166–8170, 1995.
- Lipford, G. B., Bauer, S., Wagner, H., and Heeg, K., Peptide engineering allows cytotoxic T-cell vaccination against human papilloma virus tumour antigen, E6. *Immunology* **84**, 298–303, 1995.

21. Parkhurst, M. R., Salgaller, M. L., Southwood, S., Robbins, P. F., Sette, A., Rosenberg, S. A., and Kawakami, Y., Improved induction of melanoma-reactive CTL with peptides from the melanoma antigen gp100 modified at HLA-A*0201-binding residues. *J. Immunol.* **157**, 2539–2548, 1996.
22. Bakker, A. B., van der Burg, S. H., Huijbens, R. J., Drijfhout, J. W., Melief, C. J., Adema, G. J., and Figdor, C. G., Analogues of CTL epitopes with improved MHC class-I binding capacity elicit anti-melanoma CTL recognizing the wild-type epitope. *Int. J. Cancer* **70**, 302–309, 1997.
23. Mosmann, T. R., and Coffman, R. L., TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**, 145–173, 1989.
24. McMenamin, C., and Holt, P. G., The natural immune response to inhaled soluble protein antigens involves major histocompatibility complex (MHC) class I-restricted CD8⁺ T cell-mediated but MHC class II-restricted CD4⁺ T cell-dependent immune deviation resulting in selective suppression of immunoglobulin E production. *J. Exp. Med.* **178**, 889–899, 1993.
25. Nishijima, K., Kohyama, M., Hisatsune, T., Kato, H., Kakehi, M., and Kaminogawa, S., Enhancing effect of interleukin-4 on the secretion of interferon- γ by α_{s1} -casein-specific CD8⁺ T cells. *Biosci. Biotechnol. Biochem.* **61**, 1156–1162, 1997.
26. Nishijima, K., Hisatsune, T., Kato, H., Kohyama, M., Kakehi, M., Hachimura, S., and Kaminogawa, S., Oral administration of antigen does not influence the proliferation and IFN- γ production of responsive CD8⁺ T cells but enables to establish T cell clones with different lymphokine production profile. *Cytotechnology* **25**, 89–100, 1997.
27. Falk, K., Rotzschke, O., Stevanovic, S., Jung, G., and Rammensee, H. G., Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**, 290–296, 1991.
28. Cherwinski, H. M., Schumacher, J. H., Brown, K. D., and Mosmann, T. R., Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* **166**, 1229–1244, 1987.
29. Spitalny, G. L., and Havell, E. A., Monoclonal antibody to murine γ -IFN inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. *J. Exp. Med.* **159**, 1560–1565, 1984.
30. Kawai, M., Obata, Y., Hamasima, N., Takahashi, T., Uenaka, A., Monden, M., Mori, T., Shiku, H., and Nakayama, E., Differential involvement of CD4⁺ cells in mediating skin graft rejection against different amounts of transgenic H-2K^b antigen. *J. Exp. Med.* **173**, 261–264, 1991.
31. Kärre, K., Ljunggren, H. G., Piontek, G., and Kiessling, R., Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* **319**, 675–678, 1986.
32. Abrams, J. S., Roncarolo, M. G., Yssel, H., Andersson, U., Gleich, G. J., and Silver, J. E., Strategies of anti-cytokine monoclonal antibody development: Immunoassay of IL-10 and IL-5 in clinical samples. *Immunol. Rev.* **127**, 5–24, 1992.
33. Ljunggren, H. G., Stam, N. J., Öhlén, C., Neefjes, J. J., Höglund, P., Heemels, M. T., Bastin, J., Schumacher, T. N. M., Townsend, A., Kärre, K., and Ploegh, H. L., Empty MHC class I molecules come out in the cold. *Nature* **346**, 476–480, 1990.
34. Townsend, A., Öhlén, C., Bastin, J., Ljunggren, H. G., Foster, L., and Kärre, K., Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* **340**, 443–448, 1989.
35. Takamiya, Y., Schönbach, C., Nokihara, K., Yamaguchi, M., Ferrone, S., Kano, K., Egawa, K., and Takiguchi, M., HLA-B*3501-peptide interactions: Role of anchor residues of peptides in their binding to HLA-B*3501 molecules. *Int. Immunol.* **6**, 255–261, 1994.
36. Sigal, L. J., Goebel, P., and Wylie, D. E., D^b-binding peptides from influenza virus: Effect of non-anchor residues on stability and immunodominance. *Mol. Immunol.* **32**, 623–632, 1995.
37. Klein, J. R., Pasternack, M. S., and Bevan, M. J., Activation requirements for antigen- and mitogen-induced interferon- γ release from cytotoxic T lymphocytes. *J. Immunol.* **134**, 2456–2461, 1985.
38. Matsuoka, T., Kohrog, H., Ando, M., Nishimura, Y., and Matsushita, S., Altered TCR ligands affect antigen-presenting cell responses: Up-regulation of IL-12 by an analogue peptide. *J. Immunol.* **157**, 4837–4843, 1996.
39. Janeway, C. J., Ligands for the T-cell receptor: Hard times for avidity models. *Immunol. Today* **16**, 223–225, 1995.
40. Luescher, I. F., Vivier, E., Laver, A., Mahiou, J., Godeau, F., Malissen, B., and Romero, P., CD8 modulation of T-cell antigen receptor–ligand interactions on living cytotoxic T lymphocytes. *Nature* **373**, 353–356, 1995.
41. Malissen, B., Two faces are better than one. *Nature* **384**, 518–519, 1996.
42. McKeithan, T. W., Kinetic proofreading in T-cell receptor signal transduction. *Proc. Natl. Acad. Sci. USA* **92**, 5042–5046, 1995.
43. McConnell, H. M., Wada, H. G., Arimilli, S., Fok, K. S., and Nag, B., Stimulation of T cells by antigen-presenting cells is kinetically controlled by antigenic peptide binding to major histocompatibility complex class II molecules. *Proc. Natl. Acad. Sci. USA* **92**, 2750–2754, 1995.
44. Rabinowitz, J. D., Beeson, C., Lyons, D. S., Davis, M. M., and McConnell, H. M., Kinetic discrimination in T-cell activation. *Proc. Natl. Acad. Sci. USA* **93**, 1401–1405, 1996.
45. Silver, M. L., Guo, H. C., Strominger, J. L., and Wiley, D. C., Atomic structure of a human MHC molecule presenting an influenza virus peptide. *Nature* **360**, 367–369, 1992.
46. Matsumura, M., Fremont, D. H., Peterson, P. A., and Wilson, I. A., Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science* **257**, 927–934, 1992.
47. Zhang, W., Young, A. C. M., Imarai, M., Nathenson, S. G., and Sacchettini, J. C., Crystal structure of the major histocompatibility class I H-2K^b molecule containing a single viral peptide: Implications for peptide binding and T cell receptor recognition. *Proc. Natl. Acad. Sci. USA* **89**, 8403–8407, 1992.
48. Madden, D. R., Garboczi, D. N., and Wiley, D. C., The antigenic identity of peptide–MHC complexes: A comparison of the conformations of five viral peptides presented by HLA-A2. *Cell* **75**, 693–708, 1993.
49. Fremont, D. H., Stura, E. A., Matsumura, M., Peterson, P. A., and Wilson, I. A., Crystal structure of an H-2K^b-ovalbumin peptide complex reveals the interplay of primary and secondary anchor positions in the major histocompatibility complex binding groove. *Proc. Natl. Acad. Sci. USA* **92**, 2479–2483, 1995.
50. Chen, W., Khilko, S., Fecondo, J., Margulies, D. H., and McCluskey, J., Determinant selection of major histocompatibility complex class I-restricted antigenic peptides is explained by class I-peptide affinity and is strongly influenced by nondominant anchor residues. *J. Exp. Med.* **180**, 1471–1483, 1994.
51. Mosmann, T. R., and Sad, S., The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* **17**, 138–146, 1996.