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Selective induction of CD8$^+$ T cell functions by single substituted analogs of an antigenic peptide: distinct signals for IL-10 production

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Abstract The CD8$^+$ T cell clone 5F1 produces interleukin 10 (IL-10) and interferon γ (IFN-γ) in response to stimulation with a peptide corresponding to region 142–149 of bovine α_{s1}-casein (p142–149). Ninety analog peptides derived from p142–149 with single amino acid substitutions of putative T cell receptor contact residues were prepared to examine whether production of IL-10 and IFN-γ by 5F1 can be altered by stimulation with these peptides. We found that some peptides triggered only IL-10 production whereas others induced production of IFN-γ alone or both of these cytokines. Peptides inducing IFN-γ production triggered both cytotoxicity and a proliferative response, whereas peptides inducing production of IL-10 but not IFN-γ triggered neither of these responses. Our results clearly demonstrate that the signaling pathway required for IL-10 production in CD8$^+$ T cells differs from that required for IFN-γ production. The distinct cellular signals for IL-10 production appear to be independent of those for cytotoxicity and the proliferative response of CD8$^+$ T cells.

Key words: Altered peptide ligand; CD8$^+$ T lymphocyte; Interleukin 10; Interferon γ; Cytotoxicity

1. Introduction

CD8$^+$ T cells mostly recognize endogenously synthesized antigens presented by MHC class I molecules; however, some CD8$^+$ T cells can be triggered by exogenous antigens [1–5]. These exogenous antigen-specific CD8$^+$ T cells are considered to participate in regulation of the immune system via soluble factors. It is accepted that CD4$^+$ T cells are classified into two subsets each with a particular cytokine production pattern: Th1 cells producing IL-2, IFN-γ, and lymphotoxin, and Th2 cells producing IL-4, IL-5, IL-6, IL-10, and IL-13 [6]. In contrast to CD4$^+$ T cells, the exogenous antigen-specific CD8$^+$ T cells produce IL-10 or IL-4 in addition to Th1-type cytokines [5]. We have previously described the finding that bovine α_{s1}-casein, a major milk allergen, can induce specific CD8$^+$ T cells that produce IL-10 in addition to Th1-type cytokines [7–11]. Our studies of artificial stimulation of the responding CD8$^+$ T cells, such as with anti-CD3 antibody, or dibutyryl cAMP (Bt_2cAMP) plus a calcium ionophore, have provided evidence suggesting that separate cellular signals are required for IL-10 or Th1-type cytokine production [12].

Recent studies showed that T cell activation is not a simple on-off-type event; rather, qualitative changes in T cell responses can be induced by amino acid substitutions in antigenic peptides. Some analog peptides having substitutions of amino acid residues involved in interaction with the T cell receptor (TCR) can elicit T cell responses qualitatively different from those produced by the original peptide. These peptides are called altered peptide ligands [13]. For example, some analog peptides induce cytokine production without proliferation [14], cytotoxicity without proliferation and cytokine production [15,16], alteration of the pattern of cytokine production [17], or anergy [18]. In this study, we demonstrate the selective induction of IL-10 production or IFN-γ production in an α_{s1}-casein-specific CD8$^+$ T cell clone, using a panel of 90 analogs of a peptide corresponding to region 142–149 of bovine α_{s1}-casein (p142–149) each with a single amino acid substitution at a TCR contact residue.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan).

2.2. Antigens

Bovine α_{s1}-casein was purified from fresh raw skim milk, and was digested with trypsin (Sigma, St. Louis, MO, USA) as previously described [19]. p142–149 was synthesized by means of a peptide synthesizer (430A; Applied Biosystems, Foster City, CA, USA), and purified by reversed-phase HPLC. Single amino acid substituted peptide analogs of p142–149 (Table 1) were obtained from Chiron Mimotopes Pty Ltd. (Clayton, Vic., Australia).

2.3. CD8$^+$ T cell clone 5F1

An α_{s1}-casein-specific CD8$^+$ T cell clone, 5F1, was used in this study [11]. This clone was maintained in a conditioned RPMI 1640 medium which was supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% T cell growth factor (TCGF) [7]. These cells were stimulated with irradiated syngeneic spleen cells as antigen-presenting cells (APC) in the presence of trypsin-digested α_{s1}-casein at 10 μg/ml every 10 days.

2.4. Enzyme-linked immunosorbent assay (ELISA)

T cells (4×10^4/well) were plated into 96-well plates with irradiated spleen cells (2×10^5/well) and each peptide in 200 μl of medium. The culture supernatants were collected 48 h later. The levels of IFN-γ and IL-10 in the culture supernatants were assessed by two-site ELISA as described previously [11]. In brief, to detect IFN-γ, plates (MaxiSorp; NUNC, Roskilde, Denmark) were coated with anti-IFN-γ mAb R4-6A2 and IFN-γ bound was detected by means of biotinylated anti-IFN-γ mAb XMG1.2. To detect IL-10, anti-IL-10 mAb JES5-2A5 and biotinylated anti-IL-10 mAb SXC-1 were used as the coating antibody and detecting antibody, respectively. The detection limits for IFN-γ and IL-10 were 1 U/ml and 1 U/ml, respectively.

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Abbreviations: APC, antigen-presenting cell; Bt_2cAMP, N^6,O^2-dibutyryl cyclic adenosine 3′,5′-monophosphate; IFN-γ, interferon-γ; IL, interleukin; MHC, major histocompatibility complex; TCR, T cell receptor

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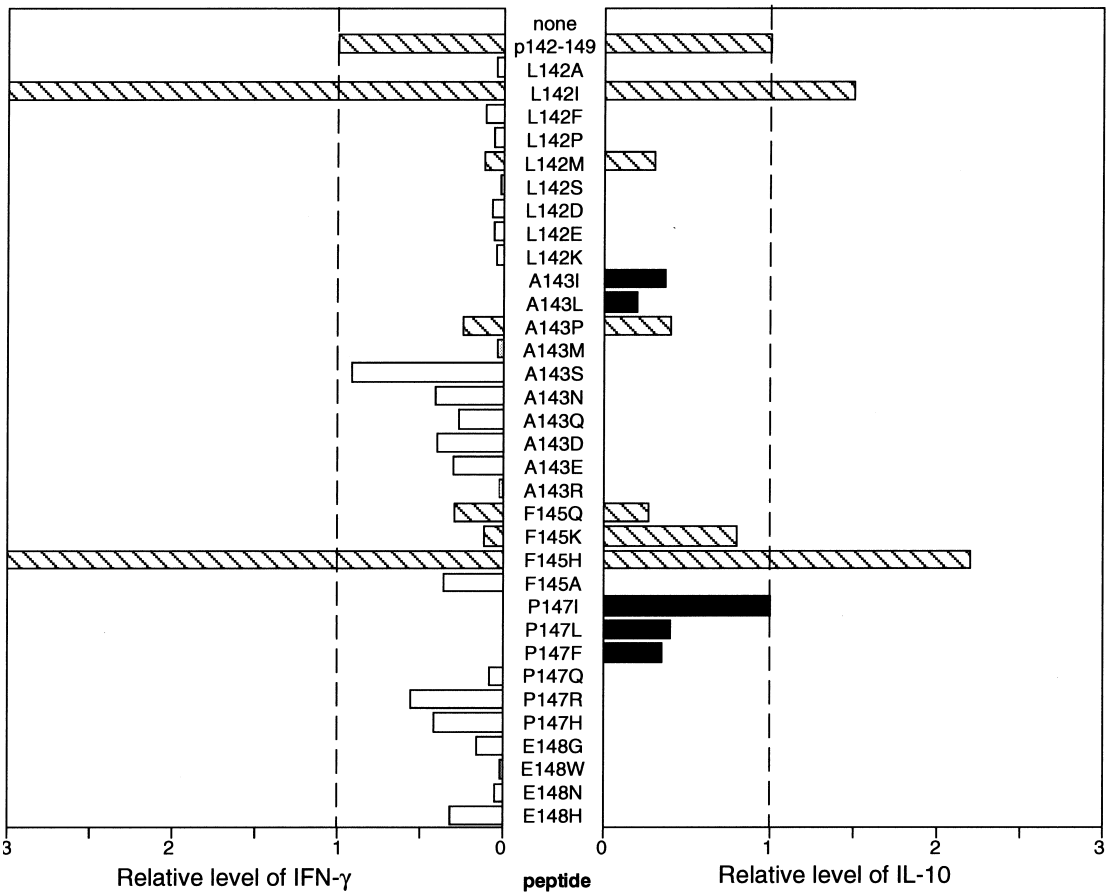

Fig. 1. Analog peptides change the cytokine production pattern of 5F1 cells. 5F1 cells (4×10^4/well) were stimulated with APC (2×10^5/well) and 10 μM p142–149 or analog peptides. After a 48-h culture period, the levels of IFN-γ and IL-10 in the culture supernatants were assessed by ELISA. Shown here are representative results for analog peptides which induced IL-10 and/or IFN-γ production: peptides inducing production of both IL-10 and IFN-γ (hatched bars), IL-10 but not IFN-γ (closed bars), or IFN-γ but not IL-10 (open bars). The data are expressed as relative values compared with the level of each cytokine produced upon stimulation with p142–149.

2.5. Cytotoxicity assay

Cytotoxicity was assayed by measuring the release of lactate dehydrogenase (LDH) into supernatants using an enzymatic assay kit (Cyto Tox 96 Assay kit: Promega, Madison, WI, USA) according to the manufacturer's instructions. Data are expressed as the percentage of maximal release observed, after subtraction of the release induced by targets pulsed with no peptide. Ten thousand EL-4 cells (ATCC) were used as the target cells at an effector:target ratio of 10.

2.6. T cell proliferation assay

The proliferation of T cells was determined by measuring the uptake of [^{3}H]thymidine. T cells (4×10^4/well) were stimulated by irradiated spleen cells (2×10^5/well) and each peptide in 200 μl of medium. The cells were cultured for 48 h and pulsed with 18.5 kBq of [^{3}H]thymidine (248 GBq/nmol; New England Nuclear, Boston, MA, USA) during the last 20 h.

3. Results

3.1. Analog peptides selectively induced IL-10 production or IFN-γ production in an α_{s1}-casein-specific CD8$^+$ T cell clone

The α_{s1}-casein specific CD8$^+$ T cell clone 5F1 produces IL-10 and IFN-γ in response to region 142–149 of α_{s1}-casein complexed with a K^b molecule [20]. The sequence of this region (Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu) is in complete accordance with the proposed K^b motif sequence (X-X-**Tyr**-X-**Phe/Tyr**-X-X-**Leu/Met**, where bold letters indicate anchor residues) [21]. From this motif, the potential TCR contact residues of p142–149 are assumed to be Leu at position 142, Ala at position 143, Phe at position 145, Pro at position 147, and Glu at position 148. On the basis of this information, we designed 90 analog peptides each with a substitution in these TCR contact residues (for a list of these peptides, see Table 1).

To evaluate the effects of these single amino acid substitutions, the amounts of IL-10 and IFN-γ produced by 5F1 in response to stimulation with each of the 90 analog peptides were determined by ELISA. Fig. 1 shows representative data for each analog peptide capable of triggering IL-10 and/or IFN-γ production. We can categorize the analog peptides into four groups: (1) peptides triggering production of IL-10 but not IFN-γ such as A143L or P147I, (2) peptides triggering production of IFN-γ but not IL-10 such as A143S or E148H, (3) peptides triggering production of both IL-10 and IFN-γ such as L142I or F145H, and (4) peptides failing to trigger production of either of the cytokines examined. This is the first demonstration of discrimination of the signal requirement for IL-10 production from that for IFN-γ production by analog peptides.

Next, the relationship between peptide concentration and the induction of IL-10 and IFN-γ production was examined using A143L, a peptide classified into group 1, and p142–149.

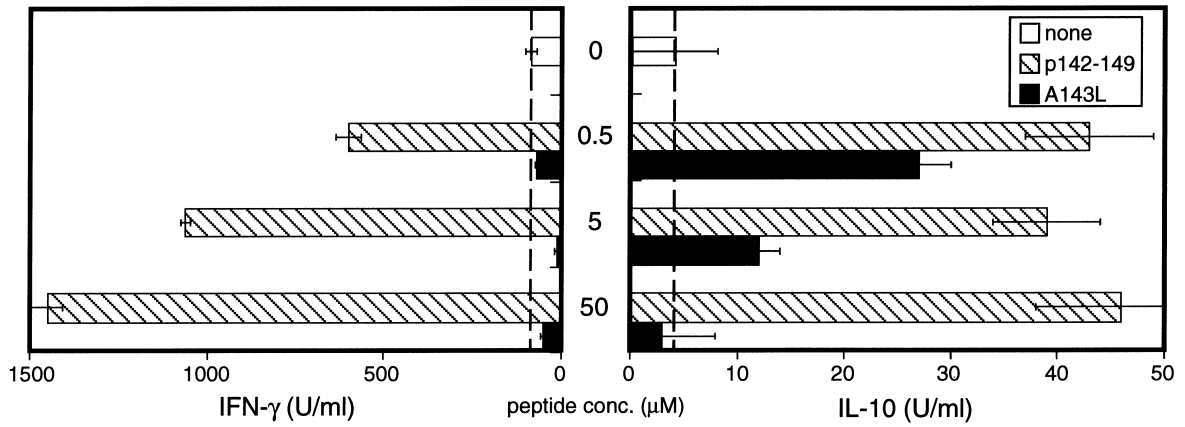

Fig. 2. IL-10 and IFN-γ production by 5F1 cells upon stimulation with various concentrations of A143L or p142–149. 5F1 cells (4×10^4/well) were stimulated with the indicated concentrations of A143L or p142–149 in the presence of APC (2×10^5/well). After a 48-h culture period, the levels of IL-10 and IFN-γ in the culture supernatants were assessed by ELISA.

As shown in Fig. 2, A143L optimally induced IL-10 production at 0.5 μM and induced no IFN-γ production even at high peptide concentrations. In contrast, p142–149 induced both IL-10 and IFN-γ production at each concentration examined, as shown in Fig. 2. At lower concentrations, p142–149 induced weak production of both IL-10 and IFN-γ, and when the concentration of p142–149 was further reduced neither of them was produced (data not shown). Thus, selective induction of IL-10 was not observed when 5F1 cells were stimulated with p142–149.

3.2. *Analog peptides which triggered only IL-10 production induced neither cytotoxicity nor a proliferative response*

We next assessed the relationship between the putative IL-10 specific cellular signals and the signals for cytotoxicity. 5F1 cells showed significant cytolytic activity for p142–149-pulsed EL4 cells (Fig. 3a). Each of five peptides, A143I, A143L, P147I, P147L, and P147F, which induced only IL-10 production, was unable to induce cytolytic activity, whereas analog peptides which induced IFN-γ production could induce cytotoxicity (Fig. 3a).

5F1 cells showed a proliferative response to trypsin-digested α_{s1}-casein [20] and to p142–149 (Fig. 3b). The analog peptides which induced only IL-10 production did not induce proliferation, whereas the analog peptides which triggered IFN-γ production induced a proliferative response (Fig. 3b). These results indicate that peptides A143I, A143L, P147I, P147L and P147F selectively activated the signaling pathway for IL-10 production, which is independent of that for cytotoxicity and proliferation of the responding $CD8^+$ T cells.

4. Discussion

In this study, we have shown that single amino acid sub-

Table 1
List of peptides used in this study

Peptide designation	Sequence[1)]	Peptide designation	Sequence[1)]	Peptide designation	Sequence[1)]	Peptide designation	Sequence[1)]	Peptide designation	Sequence[1)]
p142-149[2)]	LAYFYPEL								
L142G	G-------	A143G	-G------	F145G	---G----	P147G	-----G--	E148G	------G-
L142A	A-------	A143V	-V------	F145A	---A----	P147A	-----A--	E148A	------A-
L142V	V-------	A143I	-I------	F145V	---V----	P147V	-----V--	E148V	------V-
L142I	I-------	A143L	-L------	F145I	---I----	P147I	-----I--	E148I	------I-
L142F	F-------	A143F	-F------	F145L	---L----	P147L	-----L--	E148L	------L-
L142P	P-------	A143P	-P------	F145P	---P----	P147F	-----F--	E148F	------F-
L142M	M-------	A143M	-M------	F145M	---M----	P147M	-----M--	E148P	------P-
L142S	S-------	A143S	-S------	F145S	---S----	P147S	-----S--	E148M	------M-
L142T	T-------	A143T	-T------	F145T	---T----	P147T	-----T--	E148S	------S-
L142Y	Y-------	A143Y	-Y------	F145Y	---Y----	P147Y	-----Y--	E148T	------T-
L142W	W-------	A143W	-W------	F145W	---W----	P147W	-----W--	E148Y	------Y-
L142N	N-------	A143N	-N------	F145N	---N----	P147N	-----N--	E148W	------W-
L142Q	Q-------	A143Q	-Q------	F145Q	---Q----	P147Q	-----Q--	E148N	------N-
L142D	D-------	A143D	-D------	F145D	---D----	P147D	-----D--	E148Q	------Q-
L142E	E-------	A143E	-E------	F145E	---E----	P147E	-----E--	E148D	------D-
L142K	K-------	A143K	-K------	F145K	---K----	P147K	-----K--	E148K	------K-
L142R	R-------	A143R	-R------	F145R	---R----	P147R	-----R--	E148R	------R-
L142H	H-------	A143H	-H------	F145H	---H----	P147H	-----H--	E148H	------H-

[1]Dashes indicate identity with the p142–149 sequence.
[2]A peptide corresponding to region 142- -149 of bovine α_{s1}-casein.

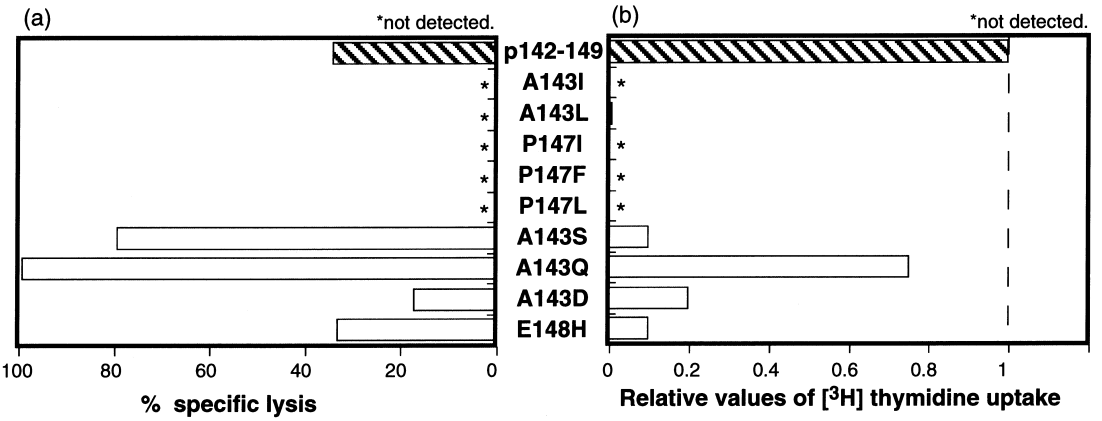

Fig. 3. Cytolytic activity (a) and proliferation (b) of 5F1 cells in response to the analog peptides. a: EL-4 cells (1×10^4/well) were pulsed for 1 h with each peptide at a concentration of 10 μM, and plated with 5F1 cells (1×10^5/well) in 96-well plates. After a 4-h incubation period, supernatants were recovered and assayed for the presence of LDH released by the target cells. The standard deviation was less than 10% of maximum in each instance. b: 5F1 cells (4×10^4/well) were cultured with APC (2×10^5/well) and 10 μM p142–149 or analog peptides for 48 h, and [^{3}H]thymidine uptake during the last 20 h was measured. The results are expressed as relative values compared with the level of [^{3}H]thymidine uptake observed upon stimulation with p142–149.

stituted analogs of an antigenic peptide could induce separate production of either IL-10 or IFN-γ from a CD8$^+$ T cell clone. This indicates that a single amino acid substitution in an antigenic peptide can modulate cytokine production by a CD8$^+$ T cell clone in an antigen-specific manner. Furthermore, analog peptides triggering production of IFN-γ induced cytotoxicity and proliferation as well, while those inducing IL-10 production without triggering IFN-γ production did not. Our results demonstrate that the signal transduction pathway for triggering of IL-10 production and the signaling requirements for induction of IFN-γ production, cytotoxicity and proliferation are distinct in CD8$^+$ T cells.

We demonstrated in our previous study that production of IL-10 and IFN-γ requires distinct signals in a CD8$^+$ T cell clone [12]. Stimulation with Bt_2cAMP enhanced the production of IL-10 but not IFN-γ. This indicates that IL-10 production but not IFN-γ production requires activation of a cAMP-dependent pathway. The analog peptides triggering IL-10 production alone may selectively activate this cAMP pathway. Recently, Madrenas et al. [22] and Sloan-Lancaster et al. [23] analyzed intracellular signaling events in T cells responding to altered peptide ligands and reported a distinct pattern of ζ chain phosphorylation accompanied by a failure to activate ZAP-70 kinase. Such a difference in signals through TCR probably influences the different activation patterns of the CD8$^+$ T cells observed in this study. Refined experiments using selective inhibitors for each signaling pathway may clarify whether these pathways are involved in the selective induction of IL-10 production.

For CD8$^+$ T cell clones, it has been reported that altered peptide ligands selectively induced cytotoxicity but not proliferation and cytokine production [16,24]. Valitutti et al. have demonstrated that different biological responses of human CD8$^+$ T cells, e.g. cytotoxicity, cytokine production, and proliferative response, are elicited depending upon the level of TCR occupancy [25]. They observed that cytotoxicity could be induced by the lowest occupancy, implying that induction of cytotoxicity has the least signal requirements. Therefore, the above examples of partial activation may be attributed to insufficient signals transduced through TCR engaged with altered ligands. In the present study, we could dissect IL-10 production from the rest of CD8$^+$ T cell responses; some peptides induced only IL-10 production, while others induced the responses other than IL-10 production. Furthermore, a peptide which induced only IL-10 production did not induce IFN-γ production even at high peptide concentrations, while p142–149 did not show selective induction of IL-10 at low concentrations. Therefore, the selective induction of CD8$^+$ T cell responses cannot be explained by hierarchical induction of TCR-mediated signal transduction pathways, but indicate that distinct signaling pathways were selectively induced by the variant peptides. Our results clearly demonstrate a novel type of activation of CD8$^+$ T cells induced by altered peptide ligands.

The selective induction of CD8$^+$ T cell responses by altered antigenic peptides as shown here not only facilitates understanding of the relationship between signaling pathways specific for production of a cytokine and antigenic stimulation, but also could be applied as a novel means of controlling the cytokine balance via CD8$^+$ T cells. 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, and allergies are Th2-dominant diseases [26]. It has been reported that IFN-γ selectively inhibits Th2-type responses [6], while IL-10 inhibits responses of Th1 cells [27]. Thus, CD8$^+$ T cells secreting IL-10 and/or IFN-γ have the potential to correct the imbalance in Th1-Th2 responses that may be responsible for the deleterious immune responses. Moreover, peptides which are recognized by CD8$^+$ T cells have a low possibility of being recognized by CD4$^+$ T cells, because of the distinct MHC-molecule requirements. Therefore, peptides inducing CD8$^+$ T cell responses could be used without the risk of accidental activation of CD4$^+$ T cells. If we can choose suitable analog peptides capable of modifying the cytokine production pattern of CD8$^+$ T cells in particular immune diseases, this strategy will lead to clinical application of altered peptide ligands as a CD8$^+$ T cell-mediated immunotherapy.

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