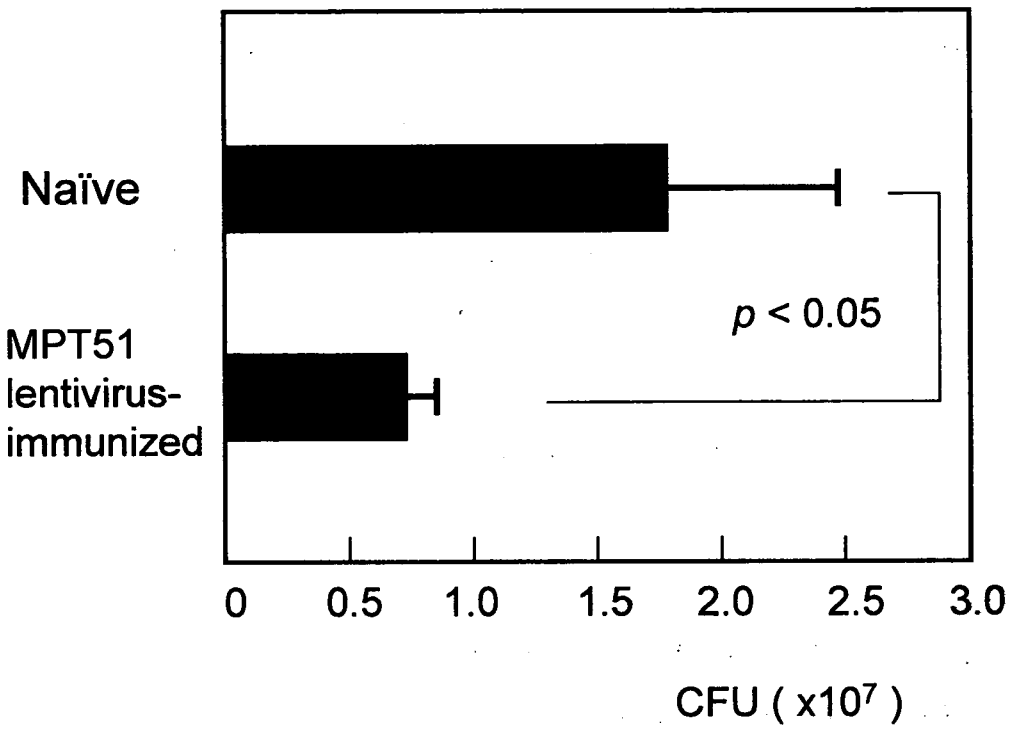

Fig. 4, Hashimoto et al.

In vivo hierarchy of individual T-cell epitope-specific helper T-cell subset against an intracellular bacterium

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Abstract

Cellular immunity is indispensable for efficient protection against intracellular bacterial infection. $CD4^+$ and $CD8^+$ T cells specific for a variety of antigenic peptides derived from particular bacteria are induced after the infection. T cells recognizing different antigenic peptides have been speculated to have different functions in terms of the protective immunity. We here induced individual $CD4^+$ T cells specific for each antigenic peptide derived from *Listeria monocytogenes* independently with DNA vaccines using gene gun bombardment and compared the $CD4^+$ T-cell populations for their ability on the specific protective immunity against lethal listerial challenge and analyzed their characteristics.

Key words: DNA immunization; Th epitope; *Listeria monocytogenes*

1. Introduction

Listeria monocytogenes is a facultative Gram-positive intracellular bacterium. Murine infection with *L. monocytogenes* is an excellent model system for studying cellular immunity against intracellular microorganisms [1]. For the protection against the microorganism, $CD4^+$ helper T-lymphocytes (Th), in addition to $CD8^+$ cytotoxic T-lymphocytes (CTL), which are specifically amplified at listerial infection, have been shown to play a critical role in the protective immunity against challenge by lethal dose of *L. monocytogenes* [2-5].

$CD4^+$ T-cell epitopes as well as $CD8^+$ T-cell epitopes, derived from *L. monocytogenes* have been identified so far. Especially, T-cell epitopes derived from two critical virulent factors of *L. monocytogenes*, listeriolysin O (LLO) and p60 protein have been intensively studied. LLO is a sulfhydryl-activated pore-forming exotoxin. It allows the bacterium to escape from the phagosome and to replicate in the cytoplasm [1]. LLO 215-226 was first identified as a dominant $CD4^+$ Th epitope restricted to H2-E^k molecule [6, 7]. p60 protein is a murein hydrolase and has been shown to be involved in the invasion of mammalian cells [1]. Following LLO 215-226 peptide, p60 301-312 was then reported as an H2-A^d-restricted $CD4^+$ Th epitope [8]. Further, Geginat and colleagues [9] reported several T-cell epitopes of LLO and p60 protein in BALB/c and C57BL/6 mice using an approach for the direct ex vivo identification and characterization of T-cell epitopes based on the screening of peptide spot libraries with ex vivo isolated spleen cells in a highly sensitive enzyme-linked immunospot (ELISPOT) assay. They found in their system, four $CD8^+$ T-cell epitopes and six $CD4^+$ T-cell epitopes in BALB/c mice and two $CD8^+$ T-cell epitopes and five $CD4^+$ T-cell epitopes in C57BL/6 mice including previously identified ones [9].

In our previous works, we have investigated individual T-cell epitope-specific T-cell responses against *L. monocytogenes* using minigene DNA vaccine system [10-12]. For $CD8^+$ T-cell

epitope-specific responses, we showed that interaction between T cells against dominant and subdominant epitopes does not operate in the generation of the hierarchy among individual $CD8^+$ T-cell epitopes with minigene DNA vaccination [11]. For $CD4^+$ T-cell epitope-specific responses, we have reported that LLO 215-226-specific T-cell response evoked by DNA vaccination was capable of inducing protective immunity against lethal listerial challenge in C3H mice [13].

Here, we compared four different H2-A^d-, or H2-E^d-restricted $CD4^+$ T-cell epitope-specific responses in terms of the protective immunity in BALB/c mice by immunization of invariant chain (Ii) cDNA whose major histocompatibility complex (MHC) class II-associated Ii peptide (CLIP) region was replaced by the antigenic peptides. The antigenic peptide/CLIP-replaced Ii gene immunization would be an efficient method for presenting antigenic peptides of interest to Th *in vivo* [13-16]. Using this system, each individual epitope peptide has been expected to be expressed in similar amounts *in vivo* [11] and would be feasible for evaluation of each $CD4^+$ T-cell epitope peptide for induction of the specific T-cell responses including the protective ability against following listerial challenge.

2. Materials and methods

2.1. Animals

BALB/c mice (Japan SLC; Hamamatsu, Japan) were maintained in the Animal Facility of Hamamatsu University School of Medicine. Mice between 6 and 18 weeks of age were used for immunization. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

pCI-mIi p41-LLO215m, a DNA vaccine plasmid for LLO 215-226 (SQLIAKFGTAFK), has been reported previously [13]. DNA vaccine plasmids for LLO 189-200 (WNEKYAQAYPNV; pCI-mIi p41-LLO 189m), p60 367-378 (SSASAIIAEAAQK; pCI-mIi p41-p60 367m), and p60

301-312 (EAAKPAPAPSTN; pCI-mIi p41-p60 301m) were constructed similarly (Fig. 1). In brief, double-stranded oligonucleotides coding for each T-cell epitope peptide replaced HindIII–NspI DNA fragment coding for CLIP region of murine Ii p41 molecule in pCI-mIi p41 plasmid [13, 15]. The oligonucleotides for T-cell epitopes derived from *L. monocytogenes* were codon-optimized to mouse. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing, using ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA). During the cloning procedure, the DNA fragments were purified from agarose gels using GeneClean II kit (Bio 101, La Jolla, CA, USA). Large-scale purification of plasmids was conducted with Qiagen plasmid mega kit system (Qiagen, Valencia, CA, USA) and endotoxin was removed by Triton X-114 phase separation.

2.3. DNA immunization

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA), preparation of the cartridge of DNA-coated gold particle cartridge was followed to the manufacturer's instruction manual. Finally, 0.5 mg of gold particles was coated with 1 μg of plasmid DNA and the injection was carried out with 0.5 mg gold per shot twice. Mice were injected with 2 μg of plasmid DNA four times at 1-week intervals.

2.4. Lymphocyte proliferation assay

Spleen cells (5×10^5 per well) from the immunized mice were maintained with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere. They were incubated for 48 h at 37°C in 96-well round-bottom tissue culture plates in the presence or absence of 1 μM of individual Th-epitope peptide. After 48 h in culture, *de novo* DNA synthesis was assessed by adding 0.5 μCi per well [methyl-^{3}H] thymidine (10 Ci mmol^{-1}; ICN Biochemicals, Irvine, CA, USA) for the last 12 h of culture. Triplicate cultures were harvested onto glass fiber filters, and the [methyl-^{3}H] thymidine incorporation was determined by counting the radioactivity (cpm) using liquid scintillation counter.

2.5. Bacterial infection

L. monocytogenes EGD strain was kept virulent by in vivo passage. For the inoculation, a seed of *L. monocytogenes* was cultured overnight in trypticase soy broth (BBL, Sparks, MD, USA) at 37°C in a bacterial shaker and suitably diluted with phosphate-buffered saline. The exact infection dose was assessed retrospectively by plating. Mice were immunized four times with DNA vaccine plasmids as described above. One month later, the mice were challenged with 2×10^4 CFU of *L. monocytogenes* by intravenous injection. Bacterial numbers of the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates (BBL).

2.6. Cytokine assay for spleen cells from immunized mice

One month after the last immunization, spleen cells were harvested from the immunized mice. Recovered cells were plated in 24-well plates at 2×10^6 cells per well in the presence or absence of 1 μM of individual T-cell epitope peptide for 5 days. Concentration of cytokines (interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-5, IL-4, IL-2) in culture supernatants was determined by cytometric bead assay (CBA). Fifty micro liter of culture supernatants were assayed for these cytokines using mouse Th1/Th2 cytokine CBA kit (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's instruction.

2.7. Statistics

Data from multiple experiments were expressed as the means ± SE. Statistical analyses were performed by using StatView-J 5.0 statistics program (Abacus Concepts, Berkeley, CA, USA). Data were analyzed with one factor-analysis of variance followed by the Fisher's protected least significant difference test.

3. Results

3.1. Proliferative responses of spleen cells of mice immunized with expression plasmids for Th

epitopes

In order to examine $CD4^+$ T-cell responses against four different Th epitopes of *L. monocytogenes* in BALB/c mice, we constructed four DNA vaccine plasmids for in vivo antigen presentation of LLO 215-226, LLO 189-200, p60 367-378, and p60 301-312 (Fig. 1). The DNA vaccine constructs are expression plasmids for recombinant murine Ii cDNA whose CLIP region were replaced by an oligonucleotide encoding $CD4^+$ T-cell epitope peptide [13, 15]. The plasmid DNAs were injected by gene gun bombardment into BALB/c mice as shown in Materials and methods section. We chose the immunization method as it was a very reliable and reproducible method from our previous experience [17].

First, we performed lymphocyte proliferation assay one month after the last immunization. As shown in Fig. 2, immunization with each Th-epitope peptide expression plasmid induced individual peptide-specific proliferative responses of the spleen cells. The stimulation indexes were similar for four T-cell epitope peptides. These results indicated that the DNA vaccination successfully induced individual epitope-specific T-cell responses.

3.2. Induction of protective immunity against listerial infection after immunization with Th epitope expression plasmids

In order to examine whether the immunity evoked by immunization with each Th-epitope peptide expression plasmid is associated with an increased resistance to infection of virulent *L. monocytogenes*, the *in vivo* protection experiment was carried out. Seventy-two hours after listerial challenge, mice were sacrificed and CFU from the spleens were counted. As shown in Fig. 3, bacterial number in spleens of mice immunized with plasmids for p60 367-378 and LLO 215-226 tended to be reduced compared with that of naïve mice. On the contrary, bacterial number in spleens of mice immunized with plasmids for p60 301-312 and LLO 189-200 tended to be similar with that of naïve mice. Immunization with plasmid for p60 301-312 even increased the bacterial number in spleens compared with that of naive mice.

3.3. Cytokine production from spleen cells of mice immunized with expression plasmids for individual Th epitopes

We are interested in what caused different protective effects by immunization with these different Th-epitope peptide expression plasmids. One of the reasons may be cytokine profiles produced by specific Th. We therefore analyzed cytokine profiles in the supernatants of immune spleen cell culture after 5-day *in vitro* stimulation with individual Th-epitope peptides. As shown in Fig. 4, spleen cells from mice immunized with plasmids for LLO 215-226 and p60 367-378 tended to express TNF-α and IFN-γ after the Th-epitope peptide stimulation. Whereas, immune spleen cells with plasmids for LLO 189-200 and p60 301-312 tended to withdraw the expression of IFN-γ, although these tendencies were rather moderate.

4. Discussion

Following infection from a variety of pathogenic microorganisms, specific $CD4^+$ and $CD8^+$ T-cell responses are induced against various antigens of the microorganisms. $CD8^+$ T-cell responses are evoked mainly by microorganisms located in the cytoplasm of host cells, e.g., in the case of infection of viruses, as well as *L. monocytogenes*. $CD4^+$ T-cell responses are induced mainly by infection of extracellularly-located microorganisms or microorganisms located in the phagosome, e.g., in the case of infection of *Salmonella*, *Legionella*, and *Mycobacterium* spp. [18]. $CD4^+$ T-cell responses have been also shown to be induced by *L. monocytogenes* and play an important role in the protective immunity against listerial challenge. In murine *Listeria* infection model, $CD4^+$ T-cell responses have been studied. MHC class II gene-deficient mice were reported to be more sensitive to lethal listerial challenge compared with their control heterozygous littermates [19]. Geginat and colleagues [8] reported that adoptive transfer of p60 301-312-specific IFN-γ-producing $CD4^+$ T-cell line into BALB/c mice induced protective immunity against lethal listerial challenge, suggesting that $CD4^+$ T cells are positively involved in

protective immunity. On the contrary, Kursar and colleagues [20] reported that depletion of $CD4^+$ T cells during immunization with nonviable *L. monocytogenes* enhanced $CD8^+$ T cell-mediated protection against listeriosis, suggesting involvement of regulatory T cell population in failure of induction of protective immunity by nonviable *Listeria* vaccination.

There has been reported functional diversity of helper T cells [21]. Especially, so-called T helper 1 (Th1) / T helper 2 (Th2) dichotomy has been reported to be important for determining the following immunological outcome [22]. Th1-type $CD4^+$ T cells produce abundant IFN-γ, TNF-α, or IL-2. These cytokines were known to be involved mainly in cell-mediated immunity. Preexisting memory $CD4^+$ Th1, but not Th2 T-cell subset at the time of $CD8^+$ T-cell priming resulted in increased $CD8^+$ T-cell responses to bacterial and viral pathogens [23]. On the contrary, Th2-type $CD4^+$ T cells produce IL-4, IL-5, or IL-10. These cytokines were known to be involved in humoral immunity. Induction of Th1 or Th2 $CD4^+$ T cells would be affected by many variable factors. They include, immunization method used, type of antigen-presenting cells and/or density of costimulatory molecules on the cells, factors evoking innate immunity such as adjuvants and infectious agents. Further, antigenic peptide dose may be critical for determining Th1/Th2 balance. Hoskens and colleagues [24] investigated the relationship of antigenic peptide dose and Th1/Th2 selection using an in vitro system with naive T cells from ovalbumin-specific T-cell receptor transgenic mice. In the system, they showed that low peptide dose (0.01-0.04 μM) induced Th2 responses (dominant IL-4 production and less IFN-γ production) and high peptide dose (3.7-100 μM) induced Th1 responses (dominant IFN-γ production).

In addition to them, several reports suggested antigenic peptide affinity to MHC may also be involved in determining Th1/Th2 selection [25-27]. The peptides of higher affinity for a given MHC class II molecule elicited a shift towards the Th1 subset. In our work, exact affinity of peptides to MHC was not clear, but RANKPEP MHC-binding peptide prediction program

(http://bio.dfci.harvard.edu/Tools/rankpep.html) predicted the affinity by calculating MHC-binding scores for each peptide. The scores for antigenic peptides studied here were as follows. LLO 215-226, 11.829 for H2-E^d; LLO 189-200, 7.104 for H2-A^d; p60 367-378, 11.261 for H2-A^d; p60 301-312, 11.44 for H2-A^d. The scores seemed not so different from each other except for LLO 189-200, whose score showed somewhat lower than those of other peptides. T-cell receptor affinity for the peptide-MHC and T-cell receptor repertoire (which may affect T-cell receptor Vβ chain usage) may also affect the Th1/Th2 selection.

In this study, we compared four $CD4^+$ T-cell epitope-specific T-cell responses against *L. monocytogenes* using gene gun DNA vaccine system. We used DNA vaccines of $CD4^+$ T-cell epitope peptide-Ii chain cDNA chimeric DNA constructs for that purpose as described in our previous work [13, 15]. The results in this study showed that individual epitope-specific $CD4^+$ T-cell responses are different in terms of protective immunity and cytokine production profiles although the same DNA vaccination system was performed for each epitope-specific $CD4^+$ T cells. In results of the in vivo protection experiment, p60 367-378-specific and LLO 215-226-specific T cells tended to have protective ability against listeriosis, but p60 301-312-specific and LLO 189-200-specific T cells did not (Fig. 3). Related with the result, p60 367-378 and LLO 215-226 peptides had tendency to induce IFN-γ production, whereas, p60 301-312 and LLO 189-200 peptides did not in the cytokine assay (Fig. 4). These apparent results suggest that the level of IFN-γ production may affect the protective ability of each peptide-specific T cells.

We tried to evaluate cytokine profiles of each T-cell epitope-specific T cells using CBA system, but the cytokine expression levels were rather moderate. This may be caused by several reasons. BALB/c mice may induce relatively low $CD4^+$ T-cell responses when compared with $CD8^+$ T-cell responses to LLO and p60 molecules of *L. monocytogenes* [9]. Further evaluation would be definitely necessary.

In conclusion, selection of $CD4^+$ T-cell epitopes would be critical for construction of

multi-epitope vaccination. Minigene DNA vaccination would serve a feasible system for evaluation of each T-cell epitope for induction of protective immunity against various pathogens.

Acknowledgements

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Figure legends

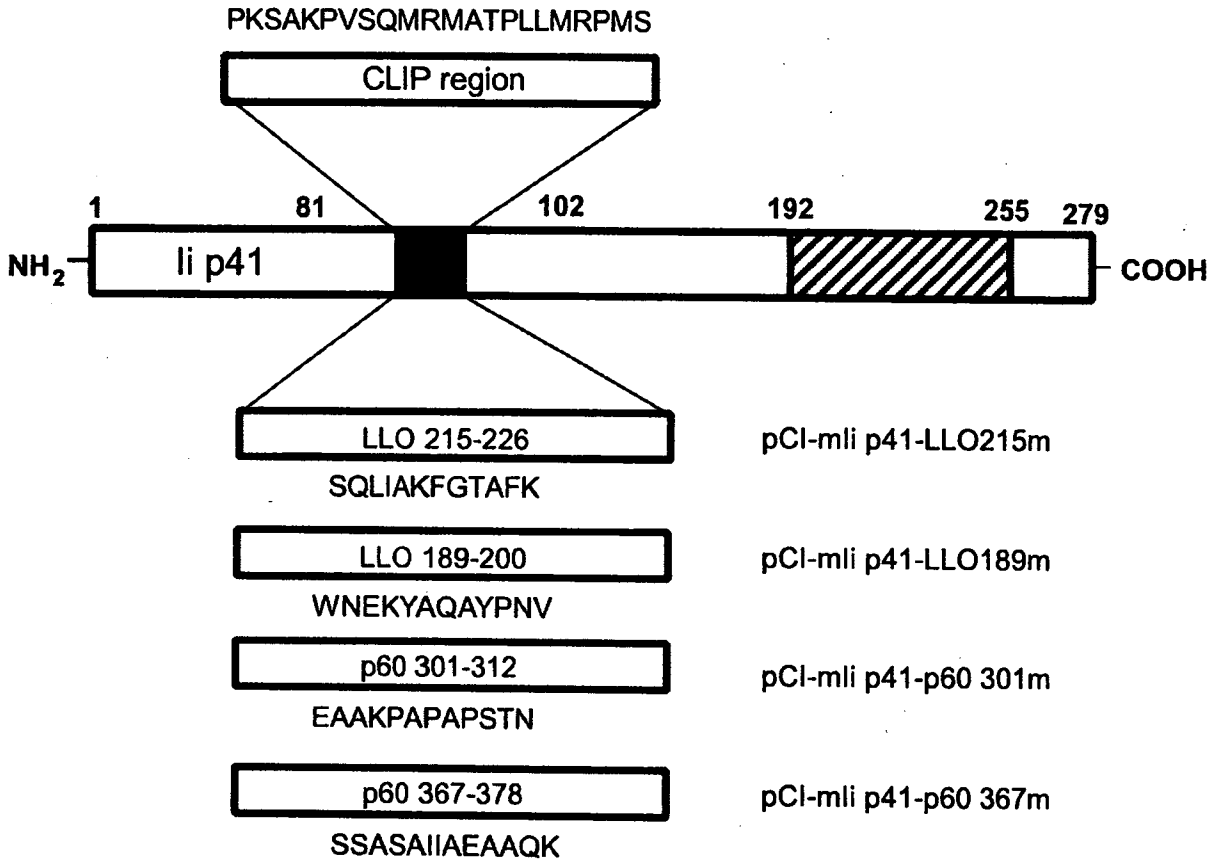
Fig. 1. The schema of murine Ii p41 molecule whose CLIP is replaced by LLO 215-226, LLO 189-200, p60 367-378, or p60 301-312 deduced from the cDNA construct (pmIi p41-LLO215m, pmIi p41-LLO189m, pmIi p41-p60 367m, or pmIi p41-p60 301m, respectively). The deduced amino acid sequences of the replaced CLIP region and the Th-epitope peptides are shown. Amino acid numbers of each domain of murine Ii p41 molecule are also shown.

Fig. 2. Individual Th-epitope-specific proliferative responses of spleen cells from mice immunized with Th-epitope expression plasmids. BALB/c mice were immunized with each plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested one month after the last immunization and cultured in vitro (5×10^5 per well) in the presence or absence of 1 μM of each Th-epitope peptide for 2 days and pulsed with 0.5 μCi of [methyl-^{3}H] thymidine for last 12 h. Results of control wild-type Ii p41 expression plasmid-immunized mice are also shown as a control. The means ± SE of stimulation index (cpm in the presence of the peptide divided by cpm in the absence of the peptide) of three mice per group are shown except for two mice for LLO 215-227 group. Asterisks indicate statistical significance ($p < 0.05$) compared with the value of control mice.

Fig. 3. Evaluation of protective immunity induced by immunization with Th-epitope expression plasmids. Mice were immunized with each Th-epitope expression plasmid four times at 1-week intervals. One month after the last immunization, the immunized mice were challenged i.v. with 2×10^3 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating ten-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naive mice are also shown as a control (Naive). Results are expressed as the

mean ± SE for three to four mice for each group except for two mice for LLO 189-200 group. Asterisk indicates statistical significance ($p < 0.05$) compared with the value of mice immunized with p60 301-312 DNA vaccine.

Fig. 4. Cytokine productions by spleen cells from Th-epitope expression plasmid-immunized mice. The spleen cells of mice immunized with each Th-epitope expression plasmid were harvested one month after the last immunization and cultured in vitro in the presence or absence of 1 μM of each Th-epitope peptide for 5 days, and the culture supernatants were analyzed by CBA. The values represent the means of Δpg/ml (the value in the presence of the peptide minus the value in the absence of the peptide) of two mice per each group.



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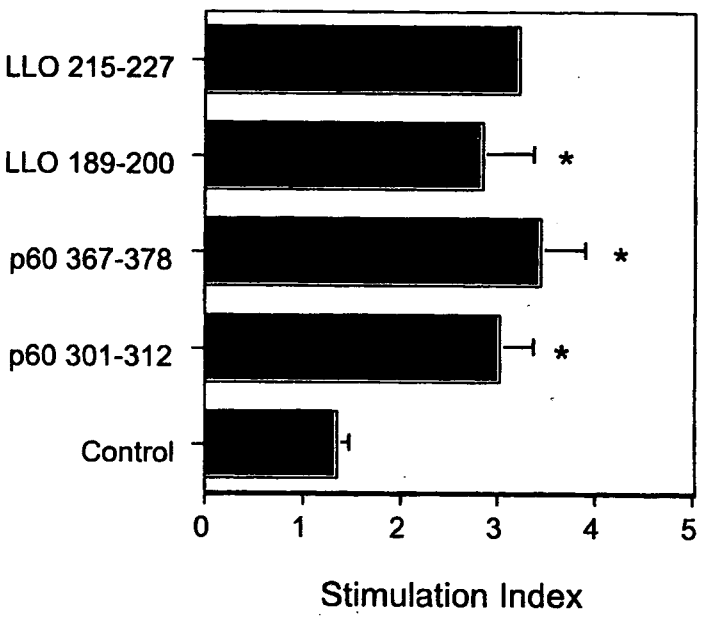

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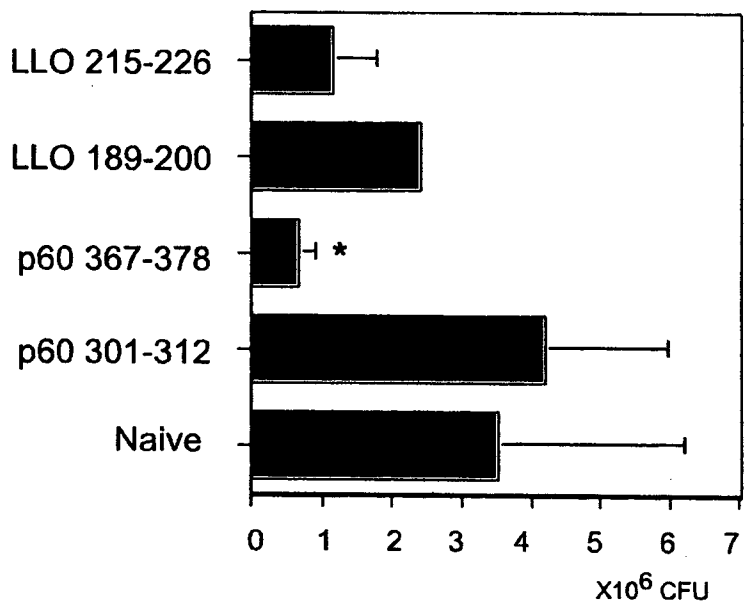

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