ing the retrovirus was used to infect P815 cells in the presence of 8 μg/ml polybrene (Sigma-Aldrich Japan, Tokyo, Japan). After 16 h, the P815 cells were washed in phosphate-buffered saline (PBS) and cultured in the presence of 400 μg/ml G418. After 2–3 weeks, surviving stable transformants were obtained and used for the study.

2.6. Semi-quantitative reverse transcription (RT) PCR

RT-PCR was performed as described previously (Yoshida et al., 1995). Total RNA was prepared from the G418-resistant transformants using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Single-stranded cDNA was synthesized with SuperScript II reverse transcriptase (Gibco BRL) and then used for PCRs. The PCR products were separated by 8% polyacrylamide gel electrophoresis and visualized by staining with ethidium bromide (Sigma-Aldrich Japan). The images were recorded using a densitograph AE-6900M (ATTO, Tokyo, Japan). The sequences of the primers used in this study were as follows:

Primer pairs for MPT51-F1, MPT51-F2, and MPT51-F3 were described above.

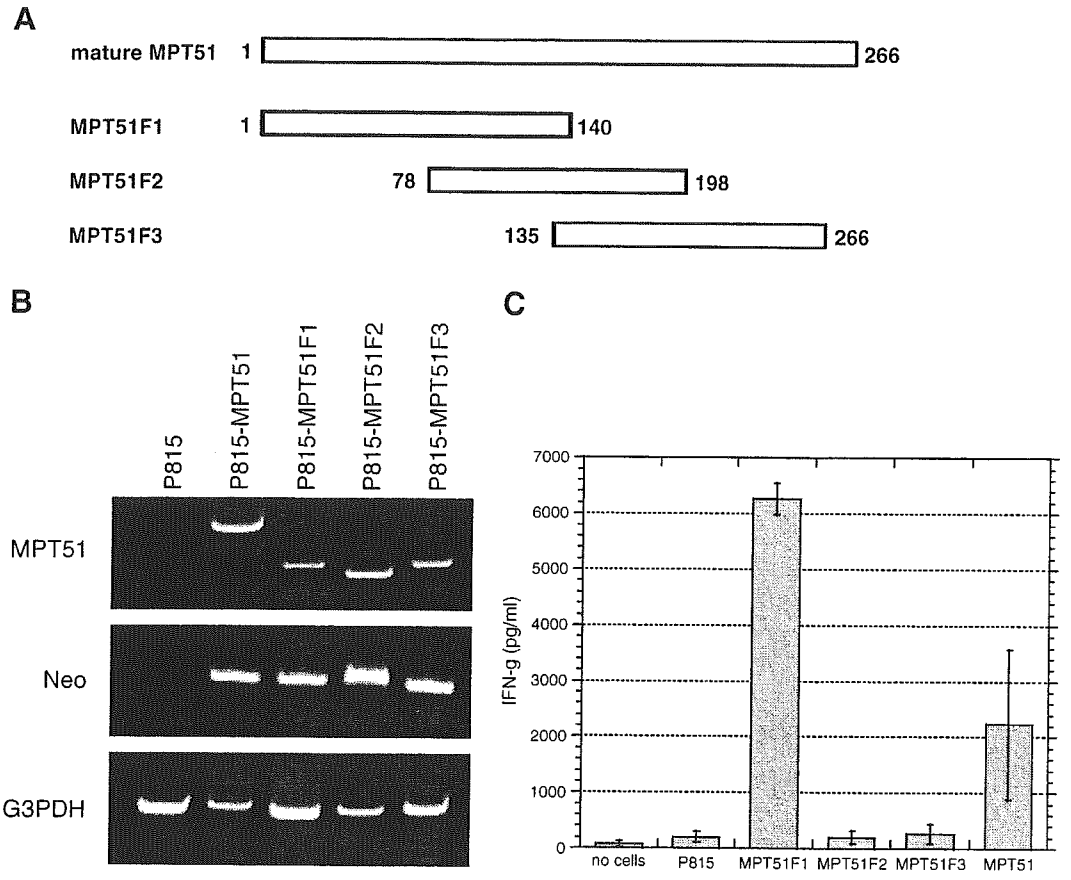

Fig. 2. Identification of fragments of a portion that contains a T cell epitope(s). (A) Regions of MPT51 protein, F1, F2, and F3 that were cloned into the retroviral expression vector. The numbers next to the indicated fragments correspond to the amino acid numbers of mature MPT51 protein. (B) P815 cells were infected with retroviruses encoding the indicated MPT51 fragments and G418-resistant stable transformants were obtained. Then, mRNA expression of the MPT51 gene, and MPT51F1, MPT51F2, and MPT51F3 gene fragments in the transformants were assessed by RT-PCR analysis. *Neo* and G3PDH mRNA expression were also assessed by RT-PCR. (C) The immune splenocyes were stimulated with either wild-type P815 cells or MPT51 fragment-expressing P815 cells for 24 h, and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

Neo forward 5′-CGACCTGTCCGGTGCCCT-GAATGAA-3′;
Neo reverse 5′-GTCCCTTCCCGCTTCAGTGA-CAACG-3′;
G3PDH forward 5′-ACCACAGTCCATGCCAT-CAC-3′;
G3PDH reverse 5′- TCCACCACCCTGTT-GCTGTA-3′.

2.7. Immunization of mice

Mice were immunized by employing a gene gun system with a plasmid DNA vaccine encoding the mature MPT51 molecule. For DNA immunization with the Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), DNA-coated gold particles were prepared according to the manufacturer's instruction manual. The gold particles were coated with plasmid DNA in a ratio of 0.5 mg gold particles/1 μg DNA. To immunize the mice, the shaved abdominal skin area was wiped with 70% ethanol. The spacer of the gene gun was held directly against the abdominal skin, and the device was discharged at a helium discharge pressure of 400 psi. Each mouse received two immunizations containing 1 mg of plasmid DNA-coated gold particles in total.

2.8. Preparation of splenocyte culture supernatants for evaluation of IFN-γ production

Spleen cell suspensions (1×10^6 cells/well) from mice immunized with a DNA vaccine encoding the MPT51 protein were stimulated by P815 stable

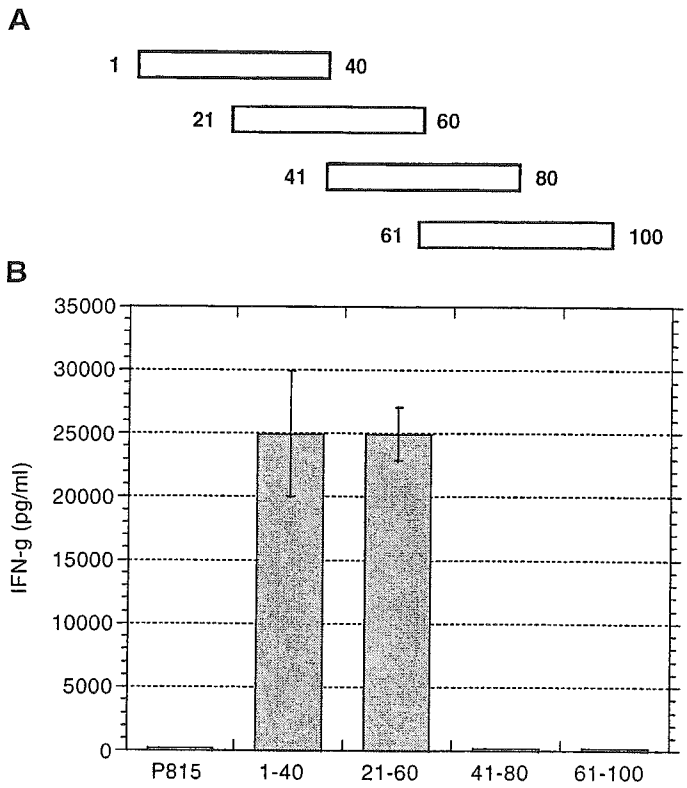

Fig. 3. MPT51 1–60 contains a T cell epitope(s). (A) Four 40-mer fragments of MPT51 overlapping 20 aa were cloned into the retroviral expression vector. (B) The immune splenocytes were stimulated with the P815 cells expressing the indicated 40-mer and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

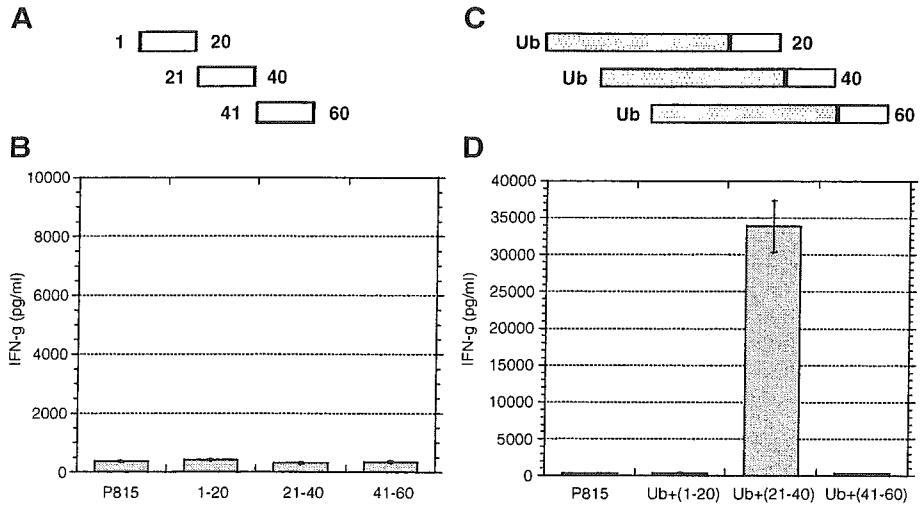

Fig. 4. Epitope expression on the 20-mer of MPT51 requires ubiquitination. (A) Three 20-mer fragments of MPT51 were inserted into the retroviral vector. (B) The immune splenocytes were stimulated with the P815 cells expressing the indicated 20-mer, and IFN-γ concentrations were measured by ELISA. (C) Construction of cotranslational ubiquitination of the 20-mer fragments. (D) The immune splenocytes were stimulated with the P815 cells expressing the ubiquitinated 20-mer and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

transformants (2.5×10^4 per well) in RPMI/10FCS in 96-well plates at 37 °C in 5% CO_2. The IFN-γ concentration was measured by a sandwich enzyme-linked immunosorbent assay (ELISA).

2.9. Quantification of IFN-γ with cytokine ELISA

IFN-γ production was measured by ELISA. The 96-well ELISA plates (EIA/RIA Plate A/2; Costar, Cambridge, MA) were coated with 2 μg/ml capture antibody (anti-murine IFN-γ monoclonal antibody (mAb) R4-6A2; BD PharMingen, San Jose, CA) at 4 °C overnight, washed with PBS containing 0.05% Tween-20, and blocked with Block Ace (Dainippon Seiyaku, Tokyo, Japan) at 37 °C for 2 h. After washing, the culture supernatant to be tested and IFN-γ standards were added to the plates and incubated at 4 °C overnight. After further washing, 0.5 μg/ml biotin-labeled anti-murine IFN-γ detection mAb (XMG1.2; BD PharMingen) was added to the plates, which were then incubated at room temperature for 1 h. After washing the plates, 0.1 μg/ml horseradish peroxidase (HRP)-conjugated streptavidin (Vector Laboratories, Inc., Burlingame, CA) was added, followed by a 30-min incubation at room temperature. After washing, bound HRP-conjugated streptavidin was detected using the substrate 3-, 3′-, 5-, 5′-tetramethylbenzene dihydrochloride (Sigma-Aldrich Japan). After 5 min, the enzyme reaction was stopped by adding 2 M H_2SO_4, followed by measuring the absorbance at 450 nm using an EZS-ABS Microplate Reader (Iwaki, Tokyo, Japan).

2.10. Analysis of $CD8^+$ T cells using H2-D^d–peptide tetramer complexes

An H2-D^d–peptide tetramer complex was kindly supplied by the NIH Tetramer Facility. Spleen cells

Table 1
Candidates of T cell epitope in MPT51 21–40

	Prediction score (9-mer)							
	SYPEITHI		BIMAS			RANKPEP		
	K^d	L^d	K^d	D^d	L^d	K^d	D^d	L^d
21–29				0.4		25.0		
23–31	16.0	15.0	57.6	20.0	5.0	10.0	103.0	53.0
24–32	13.0	12.0	48.0	400.0	7.5		76.0	40.0
25–33		13.0					35.0	48.0
27–35				0.4	10.0			
29–37	20.0		120.0			34.0		

from immunized mice were treated with ACK lysis buffer for 5 min at room temperature to remove RBC and washed twice with RPMI-1640 medium and resuspended in the RPMI/10FCS. 1×10^6 cells were stained with phycoerythrin-conjugated H2-D^d–peptide tetramer complexes, fluorescein isothiocyanate-conjugated anti-CD8 (53-6.7; BD PharMingen), and CyChrome-conjugated anti-CD4 (RM4-5; BD PharMingen) mAbs for 0.5 h at 4°C. After washing, the cells were resuspended in PBS containing 0.1% sodium azide and 1% bovine serum albumin, and then analyzed on an EPICS digital flow cytometer (EPICS XL; Beckman Coulter, Miami, FL).

3. Results

3.1. P815 cells retrovirally transduced with MPT51 can stimulate MPT51 immune splenocytes from BALB/c mice

We examined whether MPT51-transduced P815 cells were capable of stimulating splenocytes from BALB/c mice immunized with pCI-MPT51. The immune splenocytes were stimulated with MPT51 gene-transduced or nontransduced P815 for 24 h, and the IFN-γ concentrations in the culture supernatants were determined by ELISA. As shown in Fig. 1, robust IFN-γ production was observed after stimula-

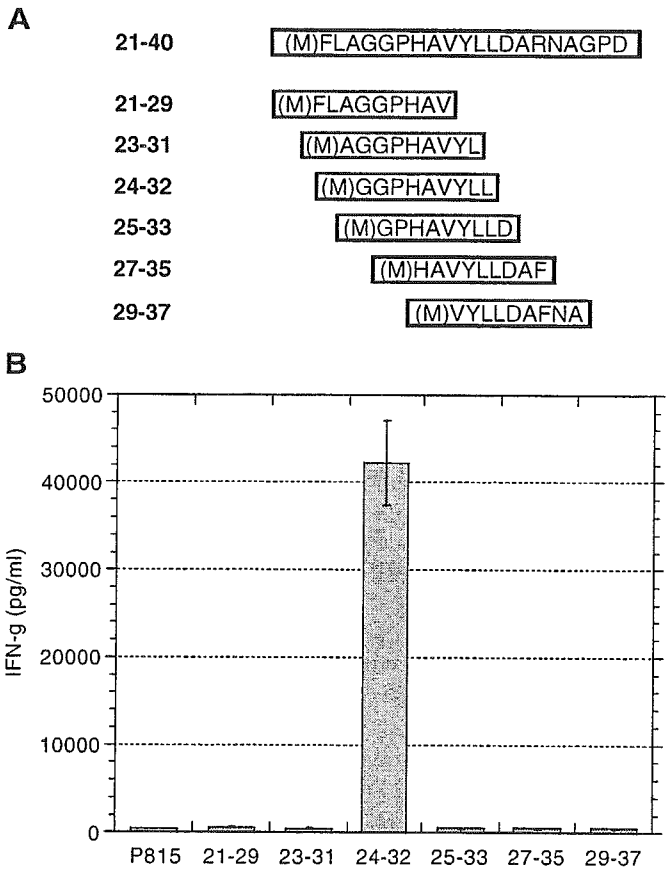

Fig. 5. Identification of a T cell epitope on MPT51. (A) Six 9-mer aa sequences predicted by algorithms. Double-stranded synthetic oligonucleotides encoding these sequences were inserted into the retroviral vector. (B) The immune splenocytes were stimulated with the P815 cells expressing the indicated putative epitopes and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

tion with P815 cells that had been transduced with MPT51. However, no significant IFN-γ production was observed in response to wild-type P815 cells. This shows that the stable transformant is able to express and process MPT51 peptides on MHC, which are then recognized by MPT51 immune splenocytes.

3.2. IFN-γ production in response to P815 cells expressing three overlapping MPT51 fragments

To map the approximate location of MPT51 peptides presented by MHC class I, three retrovirally transduced P815 transformants were produced (P815F1, P815F2, and P815F3) that expressed overlapping stretches of MPT51. These include F1 (aa 1–140), F2 (aa 78–198), and F3 (aa 136–266) (Fig. 2A). We confirmed the expression of each transgene by RT-PCR analysis. As shown in Fig. 2B, P815F1, P815F2, and P815F3 cells expressed almost the same amount of mRNA derived from each MPT51 gene fragment. Since these stable transformants were G418-resistant, they also expressed the *neo* gene. Employing these transformants as stimulators, we observed vigorous IFN-γ production by MPT51-immune spleen cells after stimulation with P815F1, suggesting that the 1–140 aa region probably contained T cell epitope(s) (Fig. 2C), while P815F2 and P815F3 did not induce IFN-γ production, suggesting that no class I-restricted MPT51 peptides were present in either of these regions.

3.3. IFN-γ production in response to P815 cells expressing shorter MPT51 peptides

In order to further map the T cell epitope within the first 140 amino acid section, we constructed plasmids encoding 40-mer stretches of MPT51 that were overlapping by 20 aa (Fig. 3A). As shown in Fig. 3B, P815 cells transduced with retrovirally encoded MPT51 aa 1–40 and MPT51 aa 21–60 were able to stimulate MPT51-immunized splenocytes to produce substantial amounts of IFN-γ. In contrast, P815 transformants that expressed the peptides MPT51 41–80 and 61–100 failed to stimulate the immune splenocytes. These results defined the T cell epitope(s) residing within aa 1–60, probably within aa 21–40.

3.4. Responses of immune splenocytes to P815 cells expressing 20-mer peptides

To further define the MPT51 epitope, we constructed three additional P815 cell lines that expressed the MPT51 peptides 1–20, 21–40, and 41–60 (Fig. 4A). However, MPT51-immune splenocytes failed to produce IFN-γ in response to P815 cells transduced with these products (Fig. 4B). Therefore, we fused the gene encoding murine ubiquitin to these products (Fig. 4C) to improve entry of the 20-mer peptides into the proteasome degradation pathway and thus enhance presentation via MHC class I. As shown in Fig. 4D, the P815 cell line expressing the fused construct containing ubiquitin and MPT51 aa 21–40 stimulated IFN-γ production by immune splenocytes, while the other fused products containing aa 1–20 and 41–60 failed to stimulate the immune splenocytes. These data confirmed that MPT51 21–40 probably contained the $CD8^+$ T cell recognized epitope.

3.5. Identification of $CD8^+$ T cell epitope on MPT51 21–40

Using three computer-based programs that are used to predict peptide binding to MHC molecules,

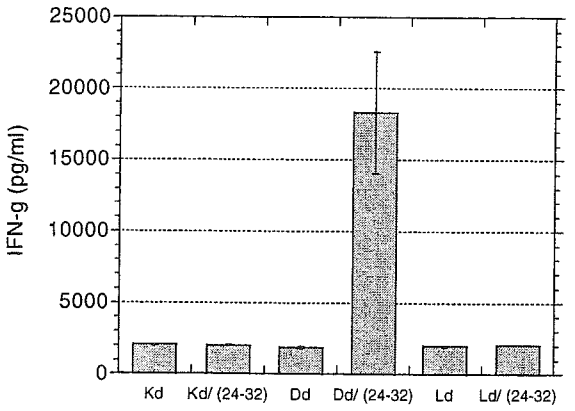

Fig. 6. MHC class I restriction molecule for the epitope, MPT51 24–32. BW5147 cells were cotransfected with MPT51 24–32 and the indicated cDNA encoding MHC class I molecules. The immune splenocytes were stimulated with the P815 cells expressing the indicated MHC class I molecules, and the epitope and IFN-γ concentrations were measured by ELISA. Data are presented as the mean±S.D. of triplicate wells. All data are representative of at least three independent experiments.

SYFPEITHI Epitope Prediction (http://www.syfpeithi.de/), BIMAS HLA Peptide Binding Prediction (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform), and RANKPEP (http://www.mifoundation.org/Tools), we predicted several candidate T cell epitopes within the 20-mer peptide. Table 1 illustrates the results of screening the interaction of different peptides with $H2^d$ class I ligands using these programs. Since rank orders of score were not always the same depending on the algorithm used, we selected six candidate T cell epitopes that had the top three scores according to each algorithm, and then constructed oligonucleotides for each into a retroviral package for expression in the P815 cell line. As shown in Fig. 5B, IFN-γ production was observed solely in response to P815 cells expressing MPT51 24–32, indicating that the 24–32 aa contains the T cell epitope.

3.6. Identification of the MHC class I restriction molecule for MPT51 24–32

To determine the MHC class I restriction molecule for MPT51 24–32, we prepared three BW5147 ($H2^k$) lymphoma cell lines retrovirally transduced with one of the genes encoding H2-K^d, H2-D^d, or H2-L^d, followed by transfection of each line with the construct encoding MPT51 24–32. IFN-γ production by immune splenocytes was only observed after stimulating with the H2-D^d cell line (Fig. 6), indicating that MPT51 24–32 is presented by H2-D^d molecules.

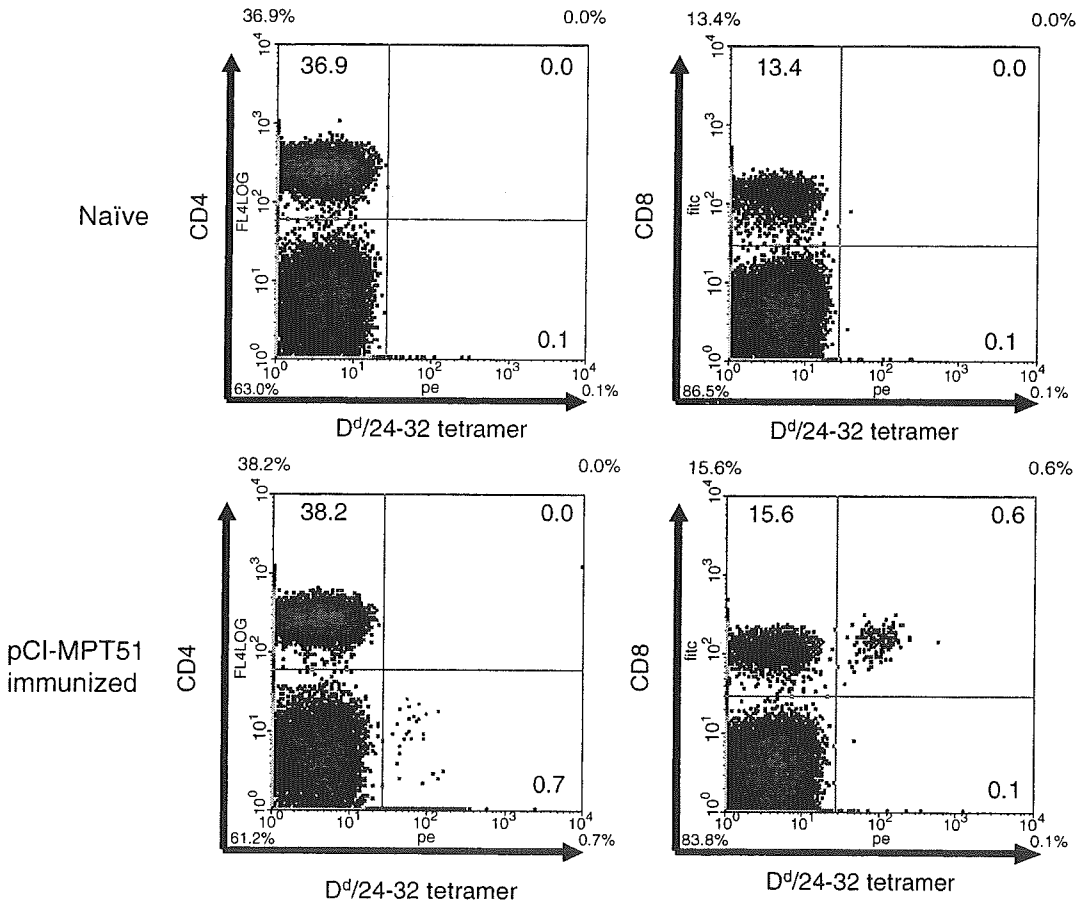

Fig. 7. Flow cytometric analysis for H2-D^d/MPT51 24–32 tetramer staining. Naïve (upper row) and immune (lower row) splenocytes were stained with PE-conjugated D^d–peptide tetramer complex and FITC-conjugated anti-CD4 (left column) or anti-CD8 (right column).

3.7. Detection of MPT51-specific CD8$^+$ T cells using MHC class I/peptide tetramer

Since we had identified both the CD8$^+$ T cell epitope of MPT51 and its class I restriction molecule, it became possible to detect MPT51-specific CD8$^+$ T cells using MHC class I/peptide tetramer. Three-color flow cytometric analysis of MPT51-immune splenocytes employing the tetramer (PE-conjugated H2-D^d/MPT51 24–32 [GGPHAVYLL]), and CyChrome-conjugated anti-CD4 and FITC-conjugated anti-CD8 mAbs revealed that 3.7% of CD8$^+$ T cells was specific for MPT51 (Fig. 7). As expected, no cells were stained by the tetramer in the CD4$^+$ T cell population in MPT51-immune mice or in naïve splenocytes.

4. Discussion

In this study, we have used a molecular approach for the identification of CD8$^+$ T cell epitopes in the MPT51 protein of *M. tuberculosis*, a novel secreted antigen that was previously shown to be protective in vaccinated mice (Miki et al., 2004). Employing the retroviral expression system, we successfully identified one CD8$^+$ T cell epitope, MPT51 24–32 (GGPHAVYLL), in BALB/c mice.

Progressing from longer expressed fragments (120–140 aa in length) of MPT51 to shorter peptides, we were able to narrow down the MPT51 epitope within the first N-terminal 60 amino acids. Attempts to use constructs that expressed this region in 20-mer stretches did not reveal the epitope, a problem that was resolved by creating fusion constructs of these peptides with ubiquitin. One possible reason is that the 20-mer products are poorly ubiquitinated by ubiquitin ligase. Our observation that cotranslational ubiquitination of the 20-mer fragment overcame the problem supports this possibility. Cotranslational ubuquitination assists the polyubiquitination of the 20-mer, which has been shown to be required for recognition by the proteasome (Thrower et al., 2000).

Ubiquitination is a pivotal step in cytosolic protein degradation and MHC class I antigen presentation. Proteins destined for degradation by the 26S proteasome are commonly modified by a multi-ubiquitin chain anchored to an internal ϵ-NH_2 group by one or more lysine residues. There are several proteins that are ubiquitinated at the amino-terminal α-NH_2 group rather than at an internal lysine residue for its degradation (Hou et al., 1994; Treier et al., 1994; Ikeda et al., 2002). In the case of the MPT51 20-mer products, aa 21–40 is probably ubiquitinated at the amino-terminal end since this peptide contains no lysine residue. It is unclear whether 20-mer products resist ubiquitination. However, the length seems to be a critical factor for processing because a 40-mer product, MPT51 1–40, which also contains no lysine residues, was successfully processed and presented to T cells (Fig. 3B). Further investigation is needed to resolve this length effect. It seems likely that the poor ubiquitination of 20-mer products has hampered the identification of T cell epitopes by the expression library system thus far.

Once the T cell epitope was defined within aa 21–40, we utilized three of the available epitope prediction programs to try to identify the most likely stretch of amino acids that contained the minimal T cell epitope. Of the six candidates that scored in the top three using each algorithm, one of these, aa 24–32, induced IFN-γ production in MPT51-immune spleen cells when stimulated with a P815 line expressing this 9-mer peptide. As expected, ubiquitination was not required for presentation of the 9-mer peptide (Figs. 5B and 6) (Uchijima et al., 1998). We have concluded that MPT51 has only one CD8$^+$ T cell epitope in BALB/c mice using this retroviral expression system. This is consistent with previous results obtained using a conventional overlapping peptide library composed of 20-mer peptides (Suzuki et al., 2004).

Various factors are involved in epitope selection, such as antigen processing and the T-cell repertoire, in addition to a peptide's affinity for MHC molecules. In general, the programs used can only predict the potential epitope candidates from the whole sequence. In fact, when the whole protein sequence was scanned by these programs, there were a number of other peptides that were predicted as having high scores, although we found no evidence of T-cell response to these regions of the protein. In some cases, peptides showing lower scores can be used to eliminate some of these possible candidates. However, there may be cases where peptides with lower scores could represent dominant or sub-dominant epitopes. Although the use of such programs may be useful in the selection of

potential peptide candidates for testing, all of these peptides should be tested individually to confirm their immunologic relevance.

It is possible that some of the transformants poorly expressed MPT51 fragments, but this seems unlikely since we employed G418-resistant stable transformants. In our experience, most of transformants that survive under G418 selection conditions expressed the transduced gene as well as the *neo* gene as have been reported by other investigators (Gubin et al., 1997; Ward et al., 2003). In fact, the majority of the transformants used in this study expressed their transduced MPT51 gene fragments well (Fig. 2B). The use of a protein expression tag, such as a His tag, may help in monitoring gene expression. However, we did not employ such a tag system because the addition of this tag to protein fragments had the potential to alter the processing of the protein and to change its recognition (Del Val et al., 1991; Mo et al., 2000).

The strategy described in this report has several advantages over other methods in identifying $CD8^+$ T cell epitopes. The retroviral expression library construction can be performed simply using PCR cloning and several oligonucleotides can be prepared at the same time to cover the entire protein. It is obvious that this approach has a certain cost saving over choosing to synthesize overlapping peptides, particularly if the protein is of a larger size. In addition, strategies that use synthetic 15- to 20-mer peptides may fail to detect a $CD8^+$ T cell response due to the necessity of trimming the peptide to the proper 8–10-mer length required for MHC class I presentation. In our method, protein fragments can be properly processed inside cells and loaded on the MHC class I naturally and is thus suitable for assessing a natural $CD8^+$ T cell response. It also appears effective for detecting epitopes produced by protein splicing. It has been shown that CTL against tumors recognize, in certain cases, antigenic peptides produced by post-translational protein splicing in the proteasome (Hanada et al., 2004; Vigneron et al., 2004). Such epitopes cannot be detected using a conventional peptide library. The present retroviral mapping system has the potential to detect such spliced epitopes.

One of the other impediments to T cell epitope characterization is the determination of the MHC class I restriction molecules. The present method circumvents this problem because the potential MHC class I molecules of interest can be co-transfected with the recognized T cell epitope. Using this co-transfection strategy, we identified H2-D^d as a restriction molecule for the MPT51 24–32 epitope. This finding allowed us to employ the H2-D^d/MPT51 24–32 tetramer to measure the size of the T cell population specific for MPT51 24–32 after immunization with a DNA vaccine encoding MPT51.

Our results allowed us to simplify the retroviral epitope detection system. It is possible to skip the 40-mer step and directly prepare DNA encoding ubiquitin-conjugated 20-mers spanning the candidate fragment, which means that this method could theoretically replace the synthesis of an overlapping conventional peptide library for $CD8^+$ T cell epitope mapping.

Taken together, our epitope mapping method using a retroviral expression system is comparable in efficiency and accuracy to a conventional peptide library for $CD8^+$ T cell epitope mapping and is much more cost-effective. Using this system, the preparation of a large peptide library or synthesis of numerous peptides predicted by program algorithms is not necessary. In conclusion, the use of this methodology could find a wide application in quickly identifying immunologically relevant $CD8^+$ T cell epitopes important in infectious diseases and cancer.

Acknowledgments

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Immunization with a gene encoding granulocyte-macrophage colony-stimulating factor inserted with a single helper T-cell epitope of an intracellular bacterium induces a specific T-cell subset and protective immunity

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Abstract

We evaluated here the effect of immunization with a gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a helper T cell (Th) epitope, listeriolysin O (LLO) 215–226 derived from *Listeria monocytogenes* on induction of a specific Th by gene gun bombardment. Immunization of C3H/He mice with pGM215m plasmid encoding murine GM-CSF inserted with LLO 215–226 Th epitope gave the epitope-specific proliferative responses of $CD4^+$ T lymphocytes. In addition, specific interferon-γ production from the splenocytes was observed. Concomitantly, pGM215m-immunized mice showed significant protective immunity against lethal listerial challenge. These results suggest that immunization of a gene for GM-CSF inserted with a Th epitope is useful for eliciting a specific Th subset in vivo.

Keywords: DNA immunization; GM-CSF; Th epitope

1. Introduction

Helper T cells (Th) play pivotal roles in many aspects of infection immunity, especially for modulating immune responses by producing special sets of cytokines. For protection against intracellular bacteria, activation of macrophages is indispensable and type 1-helper T cells (Th1) are important for the activation. The DNA vaccination method which induces only a particular Th population without production of antibodies may be advantageous as antibodies could, in some cases, give undesirable consequences [1]. Here, we evaluated the effect of immunization with a gene encoding murine granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a single H2-E^k-restricted Th epitope [residues 215–226 of listeriolysin O (LLO)] derived from *Listeria monocytogenes* [2] by gene gun bombardment.

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2. Materials and methods

2.1. Animals

C3H/He mice (between 6 and 18 weeks of age; Japan SLC, Hamamatsu, Japan) were maintained at the Institute for Experimental Animals, Hamamatsu University School of Medicine. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

The eukaryotic expression vector, pCI (Promega, Madison, WI) was used as a backbone plasmid for construction of plasmids for DNA immunization. The oligonucleotides used for p215m plasmid were, 5′-CCCGGG ATG AGC CAG CTG ATC GCC AAG TTT GGC ACC GCC TTT AAG TAG CCCGGG-3′ and the opposite-strand oligonucleotide,

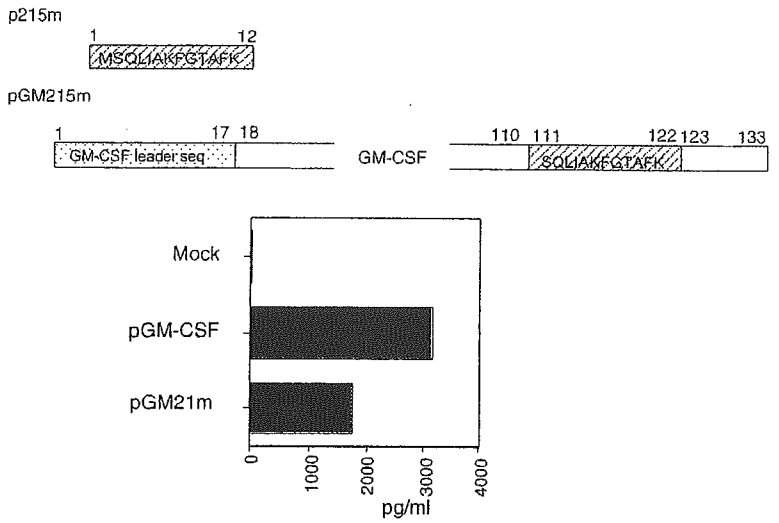

Fig. 1. (A) The schema of gene products deduced from the cDNA constructs prepared in this study (p215m and pGM215m). The hatched boxes indicate LLO 215–226 peptide and a dotted box indicate murine GM-CSF leader sequence. Amino acid numbers of each domain were shown above each schema. (B) Expression of GM-CSF inserted with LLO 215–226 peptide (GM215) in transfected cells. Supernatants of pGM-CSF- or pGM215m-transfected 293T cells were examined with ELISA specific to murine GM-CSF.

5′-CCCGGG CTA CTT AAA GGC GGT GCC AAA CTT GGC GAT CAG CTG GCT CAT CCCGGG-3′, which encode amino acid residues 215–226 of LLO, MSQLIAK-FGTAFK and a termination codon. These oligonucleotides were annealed and inserted into the SmaI site of pCI (Fig. 1A). The codon usage of the oligonucleotide for LLO 215–226 peptide was optimized to that of *Mus musculus* [3]. pGM-CSF was constructed by inserting murine GM-CSF gene into the EcoRI/NotI sites of pCI. For pGM215m plasmid, a double-stranded oligonucleotide encoding LLO 215–226 was inserted in the unique EcoRV site of murine GM-CSF gene in pGM-CSF (Fig. 1A). The region is located in the region which should not affect the function of GM-CSF [4,5]. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing by using ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA).

2.3. Mice immunization

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), 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. Enzyme-linked immunosorbent assay (ELISA) for GM-CSF

293T cells (human embryonal kidney cells) (approximately 5×10^6) were transfected with 2 μg of pGM-CSF or pGM215m using SuperFect Transfection Reagent (QIAGEN GmbH, Hilden, Germany). The supernatants were prepared 48 h after transfection and were assayed for GM-CSF using AN'ALYZA mouse GM-CSF Immunoassay Kit (G-T, Mineapolis, MN) according to the instruction manual.

2.5. Lymphocyte proliferation assay

Spleen cells (5×10^5 cells per well) from the immunized mice were incubated in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 atmosphere for 48 h at 37 °C in 96-well round-bottom tissue culture plates in the presence or absence of 1 μM of LLO 215–226 peptide. After 48 h in culture, de novo DNA synthesis was assessed by adding 0.5 μCi/well of [methyl-^{3}H] thymidine (10 Ci/mmol; ICN Biochemicals, Irvine, CA) 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 a liquid scintillation counter.

2.6. ELISA for IFN-γ

Spleen cells were harvested from the immunized mice. Recovered cells were plated in 24-well plates at 2×10^6 cells/well in the presence or absence of 1 μM of LLO 215–226 peptide for 5 days. Concentration of IFN-γ in the culture supernatants was determined by a sandwich ELISA as described in our previous report [6].

2.7. Intracellular IFN-γ staining

The number of LLO 215–226-specific $CD4^+$ T-cell subset was examined by intracellular IFN-γ staining. Spleen cells

from the immunized mice were treated with Tris-buffered ammonium chloride solution to remove red blood cells as described in our previous report [7].

2.8. Bacterial infection

A seed of *L. monocytogenes* EGD strain was cultured overnight in trypticase soy broth (Beckton Dickinson and Company, Cockeysville, MD) at 37 °C in a bacterial shaker and suitably diluted with PBS. The exact infection dose was assessed retrospectively by plating. Mice were immunized four times with DNA vaccine plasmids as described above, or immunized by a single intraperitoneal injection with a sub-lethal dose (1×10^4 CFU) of *L. monocytogenes*. One month later, the mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. 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 (Beckton Dickinson and Company).

2.9. Statistics

Data from multiple experiments were expressed as the mean ± S.D. Statistical analyses were performed by using StatView-J 5.0 statistics program (SAS Institute, Inc., Cary, NC). Data were analysed with one factor-analysis of variance followed by the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. Construction of plasmids for DNA immunization

In order to evaluate vaccination with plasmid DNA encoding murine GM-CSF inserted with a dominant Th epitope, we constructed pGM215m plasmid (Fig. 1A). As a control, we prepared another plasmid, p215m, a minigene plasmid for expression of LLO 215–226 peptide alone (Fig. 1A). In order to confirm expression of the GM-CSF-LLO 215–226 protein (GM215) by transfection of pGM215m, we transiently transfected 293T cells with pGM215m or pGM-CSF control plasmid and prepared the culture supernatants. As shown in Fig. 1B, we were able to detect expression of GM215 protein in the culture supernatant of pGM215m-transfected 293T cells by using GM-CSF-specific ELISA.

3.2. Proliferative responses of spleen cells of mice immunized with pGM215m plasmid and the epitope-specific IFN-γ production by the spleen cells

In order to examine the effect of immunization with pGM215m, we performed lymphocyte proliferation assay after immunization of C3H/He mice with the plasmid by using gene gun bombardment. We chose the immunization method as it is an appropriate vaccination route to evaluate the effect of GM-CSF expression plasmid on Langerhans cells

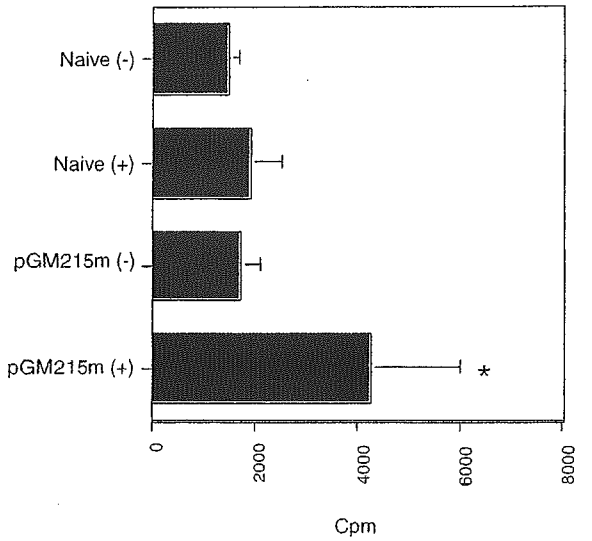

Fig. 2. Specific proliferative responses of spleen cells from pGM215m-immunized mice. C3H/He mice were immunized with pGM215m plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested three weeks after the last immunization and cultured in vitro (5×10^5 cells/well) in the presence or absence of 1 μM of LLO 215–226 peptide for 2 days and pulsed with 0.5 μCi of [methyl-^{3}H] thymidine for last 12 h. Results of naïve C3H/He mice are also shown as a control. The values indicate cpm per well. The mean ± S.D. of cpm of four mice per group are shown. Asterisks indicate statistical significance ($p \leq 0.001$) compared with the value of spleen cells of naïve mice without LLO 215–226 stimulation.

and also it is a reliable and reproducible method from our previous experience [8]. As shown in Fig. 2, immunization with pGM215m plasmid induced LLO 215–226-specific proliferative responses of spleen cells from the immunized mice. Immunization with p215m plasmid showed less proliferative responses to LLO 215–226 peptide stimulation, which were not significantly different from those observed in naïve mice (data not shown).

Furthermore, we analyzed IFN-γ amounts in the supernatants of spleen cell culture after 5-day in vitro stimulation with LLO 215–226 peptide. Again, immunization with pGM215m induced higher amounts of IFN-γ than those of mice immunized with p215m after the in vitro stimulation (Table 1). We did not detect IL-4 pro-

Table 1
IFN-γ production by splenocytes from C3H/He mice immunized with pGM215m plasmid

Immunization	Stimulation[a]	IFN-γ (pg/ml)[b]
Naïve	–	85.1
	LLO 215	74.1
pGM215m	–	95.9
	LLO 215	1318.7

[a] Spleen cells of immunized mice (2×10^6 cells/well) were cultured in the absence (–) or presence of 1 μM of LLO 215–226 peptide (LLO 215).

[b] After 4 days, cytokine concentrations in culture supernatants were quantified with sandwich ELISA. The mean of duplicate wells of representative data was shown.

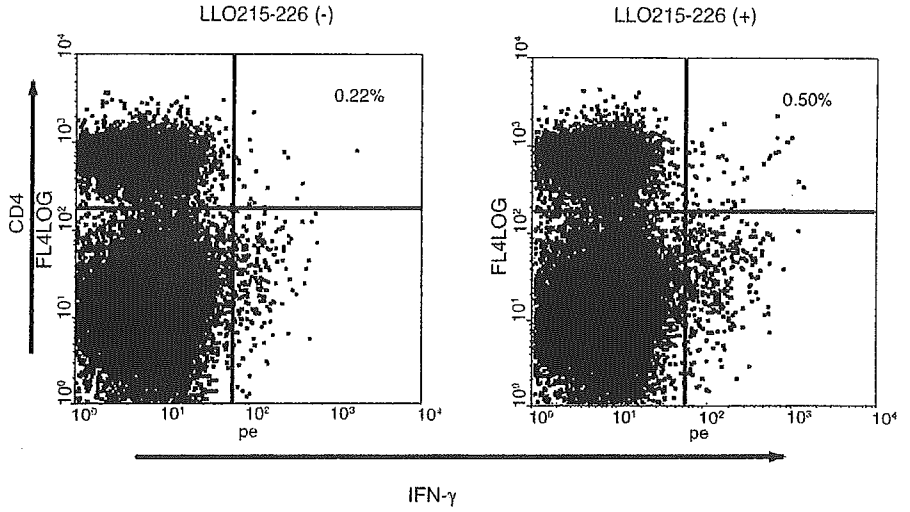

Fig. 3. Induction of LLO 215-specific $CD4^+$ T cells after pGM215m immunization. Intracellular IFN-γ staining on $CD4^+$ T-cell subset was performed using spleen cells after pGM215m immunization in the presence or absence of LLO 215–226 peptide. The percentages of IFN-γ-positive cells in $CD4^+$ T cells are shown.

duction from spleen cells of the immunized mice with ELISA (the detection limit of IL-4 in our ELISA system was approximately 100 pg/ml; data not shown). In addition, we performed intracellular IFN-γ staining with spleen cells of mice immunized with pGM215m. After in vitro stimulation with LLO 215–226 peptide, $CD4^+$ IFN-γ-producing cells was induced in the immune spleen cells (Fig. 3).

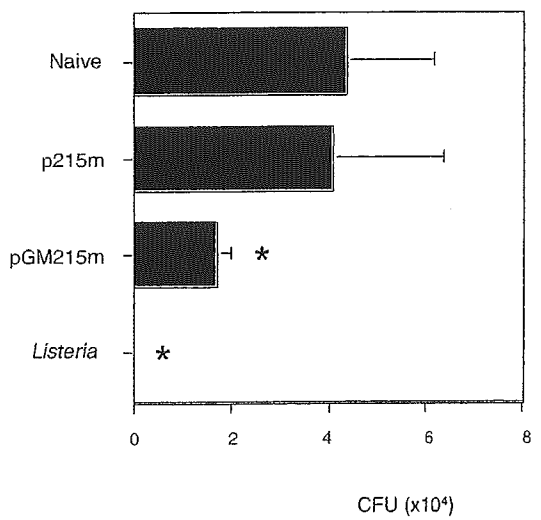

Fig. 4. Protective immunity induced by immunization with pGM215m. Mice were immunized with pGM215m four times at one-week intervals. One month after the last immunization, the immunized mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naïve mice and mice immunized with a sublethal dose of *L. monocytogenes* are also shown as controls. Results are expressed as the means ± S.D. for three mice for each group. Asterisks indicate statistical significance ($p < 0.05$) compared with the value of naïve mice.

3.3. Induction of protective immunity against listerial infection after immunization with pGM215m plasmid

In order to examine whether the immunity evoked by immunization with pGM215m 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 immunized with pGM215m plasmid were sacrificed and CFU from the spleens were counted. As shown in Fig. 4, immunization with p215m did not show significant protective effects. On the contrary, mice immunized with a sublethal dose of *L. monocytogenes* were able to eliminate challenged *L. monocytogenes* from the spleens. Immunization with pGM215m conferred moderate, but significant protective immunity against lethal listerial challenge.

4. Discussion

DNA vaccination may work through direct transfection of antigen presenting cells (APCs), or by secretion of the encoded protein by muscle or skin cells for the uptake by APCs. Therefore, two different strategies have been considered to induce a particular epitope-specific Th by DNA vaccination. One is an intracellular targeting of antigens [9–11]. Another strategy is taking an advantage of secreted proteins. In general, secreted proteins are phagocytosed by APCs and presented on MHC class II molecules. In this study, we used a plasmid expressing murine GM-CSF, a cytokine which is indispensable for development of APCs such as dendritic

cells and macrophages. GM-CSF is one of most studied cytokines for vaccine adjuvants [12,13]. GM-CSF expression plasmid injected into mouse muscle has been reported to lead to a local infiltration of potential APCs [14]. We therefore reasoned that immunization of a gene for GM-CSF-Th epitope fusion molecule may work well for induction of the epitope-specific Th subset.

Several reports showed that immunization with a DNA vaccine co-expressing both antigen and GM-CSF [15] or a bicistronic plasmid DNA for antigen and GM-CSF [16,17] is superior to co-immunization of DNA vaccines for antigen alone and for GM-CSF in terms of strength of the specific immunity induced by the vaccines. Linking antigen and GM-CSF expression closely in vivo may provide a microenvironment suitable for the uptake and presentation of antigen by dendritic cells or macrophages. Immunization of pGM215m plasmid fits this condition.

It has been reported that $CD4^+$ T cells, especially LLO 215–226-specific T cells, are involved in protective immunity against listerial challenge. Verma et al. [18] demonstrated that induction of $CD4^+$ T cell population responsive to LLO 215–226 in vivo elicits partial protective immunity by using *Salmonella* carrier system. They showed one-log order reduction in numbers of the bacterium in spleens and livers of the immunized mice. In another approach, we showed that significant induction of protective immunity to *L. monocytogenes* by immunization with a plasmid DNA expressing LLO 215–226 Th epitope that replaces the class II-associated invariant chain (Ii) peptide (CLIP) of the Ii [10] or immunization with a plasmid encoding LLO 215–216 Th epitope fused with the endosomal/lysosomal targeting signal of lysosome-associated membrane protein (LAMP)-1 [11]. pGM215m immunization shown here was more effective than LLO215–LAMP fusion DNA immunization [11] in terms of induction of the protective immunity (data not shown).

The immunization strategy shown here, i.e., immunization with GM-CSF gene inserted with a double-stranded oligonucleotide encoding a Th epitope, would be applicable to DNA vaccination for induction of CTL or antibodies as a molecular adjuvant for supplying Th. Investigators add a universal Th epitope such as 13 aminio-acid Pan HLA-DR Epitope (PADRE) [19] in multi-CTL epitope plasmid DNA construction for efficient CTL induction [20]. GM-CSF-Th epitope DNA vaccination would be the alternative strategy for induction of Th.

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Immunization with dendritic cells retrovirally transduced with mycobacterial antigen 85A gene elicits the specific cellular immunity including cytotoxic T-lymphocyte activity specific to an epitope on antigen 85A

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Abstract

In the present study, we evaluated antigen 85A (Ag85A) gene-transduced dendritic cells (DCs) vaccine against *Mycobacterium tuberculosis*. Murine bone marrow-derived DCs were retrovirally transduced with mycobacterial Ag85A gene and injected to BALB/c mice intravenously. The DC vaccine was capable of inducing purified protein derivative (PPD)- and the antigen-specific spleen cell proliferation and IFN-γ production from both $CD4^+$ and $CD8^+$ T cells in spleens of the immune mice. In addition, the DC vaccination induced cytotoxic T-lymphocytes (CTL) and IFN-γ-producing cells specific for a 9-mer CTL epitope on Ag85A molecule. This eliciting cellular immunity led to protection against wasting disease due to *M. tuberculosis* infection and induction of moderate bacterial clearance.

Keywords: Antigen 85A; Dendritic cells; *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) remains one of most serious public health problems being prevailed worldwide along with AIDS and malaria, resulting in 8 million new cases and 2 million deaths each year [1]. The appearance of multidrug-resistant *Mycobacterium tuberculosis* strains has worsened the problem. The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG), which has been reported to have a variable protective efficacy especially in adult TB [2]. Therefore, there remains an urgent need for more effective vaccines for TB [3].

Protection against intracellular bacteria such as *M. tuberculosis* critically depends on induction of cellular immune responses. Administration of soluble proteins would be insufficient to stimulate these responses. The reason why BCG vaccine has been utilized for decades is that the vaccine is able to induce specific cellular immunity although the efficacy is controversial as mentioned before. Immunization with dendritic cells (DCs) is one of promising strategies for eliciting effective cellular immunity against intracellular pathogens as DCs are the most potent antigen-presenting cells (APCs). DCs capture the pathogens or apoptotic cells. Then they migrate to regional lymphoid organs, where they present antigens to naïve T cells [4,5]. DCs possess the distinct ability to prime naïve helper T-lymphocytes (Th) and cytotoxic T-lymphocytes (CTL). Thus there has been much interest in the

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use in immune modulation of infectious diseases and cancer. Vaccination with DCs pulsed with tumor-associated antigens has been shown to generate specific anti-tumor immunity in vivo in murine tumor models [6–9]. DC vaccination has been also examined in the field of infectious diseases [10–13]. We also showed that vaccination with DCs retrovirally transduced with a gene for a dominant CTL epitope derived from *Listeria monocytogenes* elicited significant protective immunity against lethal listerial challenge infection [14].

Promising candidate antigens for TB vaccines include antigen (Ag) 85 family molecules such as Ag85A or Ag85B, heat shock proteins such as Hsp60 and ESAT-6 (reviewed in [15]). We used Ag85A as a vaccine target in this study. Ag85A molecule is a mycobacterial major secreted protein which belongs to the Ag85 family consisting of three structurally related components, Ag85A (p32A; 32 kDa), Ag85B (p30, MPT59, α antigen; 30 kDa), and Ag85C (reviewed in [16]). The Ag85 family molecules are cross-reactive antigens and are highly conserved among *Mycobacterium* spp. The genes encode proteins with fibronectin-binding capacities [17] and mycolyltransferase activities, which are involved in the final stage of mycobacterial cell wall assembly [18]. Ag85A protein was reported to stimulate B- and T-cell responses in TB patients and immunization with Ag85A protein induced the protective immunity against *M. tuberculosis* in guinea pigs [19]. In addition, reports of naked DNA vaccines against TB employing Ag85A gene have accumulated [20–24]. In addition, we reported recently that vaccination with attenuated *Listeria* carrying Ag85A expression plasmid elicited significant protective immunity against *M. tuberculosis* challenge [25]. More recently, vaccination with Ag85A-expressing vaccinia virus was shown to be effective in boosting antimycobacterial immunity in human trial [26]. According to these reports, Ag85A molecule seems to be one of the most promising candidates for future subunit TB vaccines.

In the present study, we developed a retrovirally transduced DC vaccine expressing Ag85A, and assessed its ability to generate the antigen-specific cellular immunity and to induce protective immunity against murine *M. tuberculosis* infection.

2. Materials and methods

2.1. Recombinant retroviral vector

BCG Ag85A gene was amplified from a plasmid, pMB49 [27] by PCR with following primers: 5′-ATAAGAATGCGGCCGCACCATGCAGCTTGTTGACAGG-3′ (forward primer) and 5′-ATAGTTTAGCGGCCGCTGTTCGGAGCTAGGCGC-3′ (reverse primer) (underlined letters indicate NotI sites). These PCR fragments were digested with NotI and inserted into a NotI site of pMX [28]. The nucleotide sequence designed in the plasmid was confirmed by DNA sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Large-scale purification of the plasmid was conducted using the Qiagen Plasmid Mega Kit System (Qiagen, Chatsworth, CA) and endotoxin was removed by Triton X-114 phase separation. Retroviral supernatant was generated by transfection of pMX-Ag85A proviral construct into Phoenix ecotropic packaging cell line [purchased from American Type Culture Collection (Manassas, VA) and used with the permission of Dr. GP Nolan (Stanford University School of Medicine, Stanford, CA)].

2.2. Reverse transcription (RT)–PCR analysis for Ag85A gene detection

Bone marrow-derived DCs transduced with pMX-Ag85A were harvested and total RNA was prepared from the cells by Isogen RNA extraction solution (Nippon Gene, Tokyo, Japan). The single-stranded cDNA was synthesized with Molony murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) and then used for PCR analysis. The images were recorded using AE-6900M densitograph (ATTO, Tokyo, Japan). Primers used for Ag85A gene detection are: 5′-AGGCCAACAGGCACGTCAA-3′ (forward primer) and 5′-ACATGTCGGAGGCCTTGTA-3′ (reverse primer). As a control, the same RT–PCR was performed with primers for glycelaldehyde-3-phosphate dehydrogenase (G3PDH).

2.3. Mice

BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan). These mice were maintained in a specific-pathogen-free condition at the Experimental Animal Institute, Hamamatsu University, School of Medicine. All mice used in this study were between 8 and 14 weeks of age. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University, School of Medicine.

2.4. Peptides and protein

Lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, CA). The three Ag85A CTL-epitope candidate peptides are synthesized based on Denis et al. [29]. They are, pep1 (MPVGGQSSF; corresponding to amino acid residues (aa) 70–78 of Ag85A which is predicted to bind H2-L^d according to SYFPEITHI computer algorithm [http://www.syfpeithi.de]), pep2 (WYDQSGLSV; aa 60–68 of Ag85A predicted to bind H2-K^d), and pep3 (VYAGAMSGL; aa 144–152 of Ag85A predicted to bind H2-K^d). The purity of peptides was confirmed by mass spectrometry. All peptides were dissolved in 5% dimethyl sulfoxide in distilled water to a concentration of 1 mM and were stored at −80 °C until used. Purified recombinant (r) Ag85A protein was kindly provided by Dr. John T. Belisle (Colorado State University, Fort Collins, CO) through the

NIH, NIAID Contract NO1 AI-75320 entitled "Tuberculosis Research Materials and Vaccine Testing".

2.5. Culture of bone marrow-derived DCs and transduction with retrovirus

Bone marrow-derived DCs were cultured using a method described by Inaba et al. [30] with some modifications as in our previous work [14]. To determine the phenotype of cultured DCs, we stained them with PE-, or FITC-conjugated monoclonal antibodies (mAbs) against cell surface molecules [CD40, CD80, CD86, H2-A^d (all from BD Biosciences, San Diego, CA)] and analyzed using EPICS Profile-II (Beckman Coulter, Fullerton, CA). Transduction of retroviruses was also carried out as in our previous work [14]. Briefly, 1×10^6 bone marrow-derived DCs were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (RPMI/10FCS) for 48 h and resuspended in 1 ml of the retroviral supernatant supplemented with 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO), 1000 units/ml of murine rGM-CSF, and 1000 units/ml of murine rIL-4. These cells were centrifuged at $2500 \times g$ at 32 °C for 2 h. After centrifugation, cells were cultured in RPMI/10FCS in 5% CO_2 atmosphere. The transduction process was repeated on days 3 and 4.

2.6. Immunization

After washing twice in phosphate-buffered saline (PBS), 1×10^5 transduced DCs in 0.2 ml of PBS were injected intravenously into mouse twice at a 2-week interval. As a control, mice were also immunized with 2×10^6 CFU of BCG (substrain Tokyo; Japan BCG Inc., Tokyo, Japan) subcutaneously twice at a 2-week interval. In some experiments, 2 μg of Ag85A expression plasmid (pCI-Ag85A) was immunized with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA) four times at 1-week intervals as in our previous work on MPT51 molecule [31].

2.7. Detection of PPD-, or Ag85A-specific antibodies (Abs) by ELISA

The 96-well ELISA plates (EIA/RIA plate A/2; Costar, Cambridge, MA) were coated with 25 μg/ml of purified protein derivative (PPD; Japan BCG Inc., Osaka, Japan) or 5 μg/ml of purified Ag85A protein at 4 °C overnight, washed with PBS containing 0.05% Tween 20 (PBS/Tween), and blocked with 30% Block Ace (Dainippon Seiyaku, Tokyo, Japan) solution in PBS at 37 °C for 2 h. After washing, the sera diluted with RPMI1640 medium were added to the plates and incubated at 4 °C overnight. After washing, horseradish peroxydase (HRP)-conjugated goat anti-mouse IgG Abs were added to the plates at room temperature for 2 h. After washing, the bound HRP-conjugated Abs were detected by HRP substrate reagent (Techne, Minneapolis, MN). The absorbance at 450 nm was determined with EZS-ABS microplate reader (Iwaki, Tokyo, Japan).

2.8. Lymphocyte proliferation assay

Spleen cells were prepared from the immune mice and treated with Tris-buffered 0.83 M NH_4Cl buffer for 1 min at room temperature to remove red blood cells. Then the spleen cells (5×10^5 per well) were incubated for 48 h at 37 °C in 96-well round-bottom tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in the presence or absence of 5 μg/ml of PPD (Japan BCG Inc.). The de novo DNA synthesis was assessed by adding 0.5 μCi/well of [methyl-^{3}H] thymidine (6.7 Ci/mmol; ICN Biochemicals, Irvine, CA) for the last 12 h of culture. The cultured cells were harvested onto glass fiber filters, and the radioactivity was counted by a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan). The [methyl-^{3}H] thymidine incorporation was calculated in counts per minute (cpm).

2.9. Quantification of IFN-γ by sandwich ELISA

Pools of spleen cell suspensions (2×10^6 ml^{-1}) from groups of mice immunized with DCs were cultured in RPMI/10FCS in 24-well plates in the presence of PPD (Japan BCG Inc.) (10 μg/ml), Ag85A protein (5 μg/ml), or peptides (5 μM) at 37 °C in 5% CO_2 atmosphere. The culture supernatants were harvested after 5 days, aliquoted, and stored at −20 °C until assayed for IFN-γ. Concentration of IFN-γ in the culture supernatants was determined by sandwich ELISA as described in our previous work [31]. Briefly, the 96-well ELISA plates (EIA/RIA plate A/2; Costar) were coated with 2 μg/ml of capture Ab (anti-murine IFN-γ mAb, R4-6A2; BD Biosciences) at 4 °C overnight and washed with PBS/Tween and blocked with PBS/Tween containing Block Ace (Dainippon Seiyaku) at 37 °C for 2 h. After washing, the culture supernatants to be tested and serially diluted IFN-γ standard solutions were added to the plates and incubated at 4 °C overnight. After washing, 0.5 μg/ml of detection Ab (biotinylated anti-murine IFN-γ mAb, XMG1.2; BD Biosciences) was added to the plates. The plates were incubated at room temperature for 2 h and washed. The plates were then added with 0.1 μg/ml of HRP-conjugated streptavidin (Vector laboratories Inc., Burlingame, CA) and incubated at room temperature for 30 min. After washing, bound HRP-conjugated streptavidin was detected by HRP substrate reagent (Techne). The absorbance at 450 nm was determined with EZS-ABS microplate reader (Iwaki).

2.10. Preparation of $CD4^+$ and $CD8^+$ T cell subsets from immune splenocytes with Ag85A gene-transduced DCs

Spleen cells were prepared from the immune mice and treated with Tris-buffered 0.83 M NH_4Cl buffer for 1 min at room temperature to remove red blood cells. Then they were

washed twice with RPMI 1640 medium. $CD4^+$ and $CD8^+$ T cell subsets were prepared from spleen cells of immune mice using murine $CD4^+$ or $CD8^+$ T cell isolation kit according the manufacturer's instruction (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The $CD4^+$ or $CD8^+$ T cells (1×10^6 cells) and Ag85A gene-transduced DCs (1×10^5 cells) were cultured in 96-well round-bottom tissue plates (Greiner Bio-One GmbH) for 4 days. The culture supernatants were harvested and stored at −20 °C until assayed.

2.11. CTL assay

Eight weeks after the last immunization, immune spleen cells were cocultured in 12-well plates at density of 2×10^7 cells/well for 5 days with 2×10^7 cells/well syngeneic splenocytes that had been pretreated with 100 μg/ml of mitomycin C and pulsed with 1 μM of Ag85A pep3 peptide (VYAGAMSGL) for 1 h at 37 °C. Each well received also 10 units/ml of human rIL-2 (Hoffmann-La Roche, Nutley, NJ). Cell-mediated cytotoxicity was measured using a conventional ^{51}Cr release assay. The target cells used in this study were RAW264.7 (mouse macrophage cell line; $H2^d$) pulsed with 1 μM of the peptide for 1.5 h at 37 °C. Target cells at a concentration of 1×10^4 cells/well were incubated for 5 h in triplicate at 37 °C with serial dilutions of effector cells, and the specific lysis was determined as calculated by the formula: percent specific lysis = [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100.

2.12. Bacterial infection and evaluation of protective ability of Ag85A gene-transduced DC vaccine

Immunized BALB/c mice were infected with 5×10^5 CFU of *M. tuberculosis* H37Rv i.v. 2 months after the last immunization. Mice were sacrificed 4 weeks later and the bacterial numbers in the spleens, livers, and lungs were counted in CFU on Middlebrook 7H11 plates (BD Biosciences).

2.13. Statistical analysis

Data from multiple experiments were expressed as mean ± standard deviations (S.D.). Statistical analyses were performed with the StatView-J5.0 statistics program (SAS Institute Inc., Cary, NC). Data were analyzed by Fisher's protected least significant difference (PLSD).

3. Results

3.1. Retroviral transduction of bone marrow-derived DCs and expression of Ag85A gene in the cells

DCs were generated from murine bone marrow by culturing with rGM-CSF and rIL-4, as previously described [30]. DCs transduced with Ag85A-encoding retrovirus (Ag85A gene-transduced DCs) and control untransduced DCs expressed similar amounts of CD40, CD80, CD86, and MHC class II molecules (data not shown), indicating that retroviral transduction to DCs did not affect the phenotype of the DCs.

In order to confirm the expression of Ag85A gene in transduced DCs, RT–PCR was performed. As shown in Fig. 1A, an Ag85A gene-specific band was detected in the retrovirus-transduced DCs, but not in control untransduced DCs, indicating Ag85A gene expression in the transduced DCs.

We next examined the antigen presentation capacity of Ag85A gene-transduced DCs. When Ag85A gene-transduced DCs or untransduced DCs were incubated with spleen cells derived from Ag85A DNA vaccine-immune mice, Ag85A-transduced DCs, but not untransduced DCs rendered the spleen cells to produce IFN-γ (Fig. 1B), indicating that Ag85A gene-transduced DCs were capable of presenting the antigen (Ag85A) to T cells.

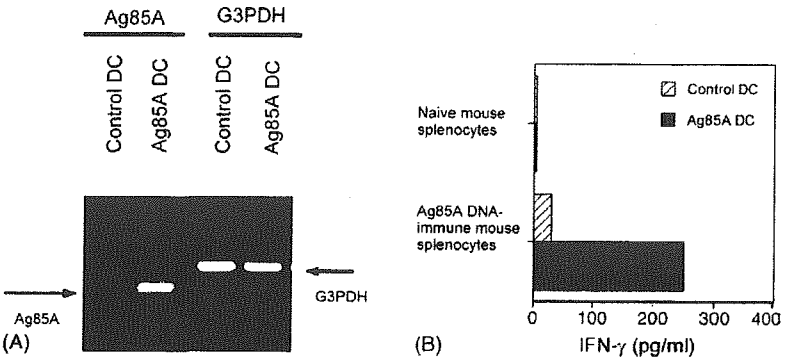

Fig. 1. Ag85A gene expression in Ag85A-transduced DCs and antigen presentation capacity of the cells. (A) Ag85A gene expression by DCs transduced with pMX-Ag85A. DCs were transduced with Ag85A-expressing retrovirus and harvested to prepare total RNA. Ag85A gene expression was evaluated by RT–PCR with Ag85A-specific primers. (B) Antigen presentation capacity of DCs transduced with Ag85A-expressing retrovirus. DCs transduced with or without Ag85A-expressing retrovirus were incubated with spleen cells of Ag85A DNA-immune mice of naïve mice for 2 days and the culture supernatant was examined for IFN-γ amounts with ELISA. Average values from two independent experiments are shown.