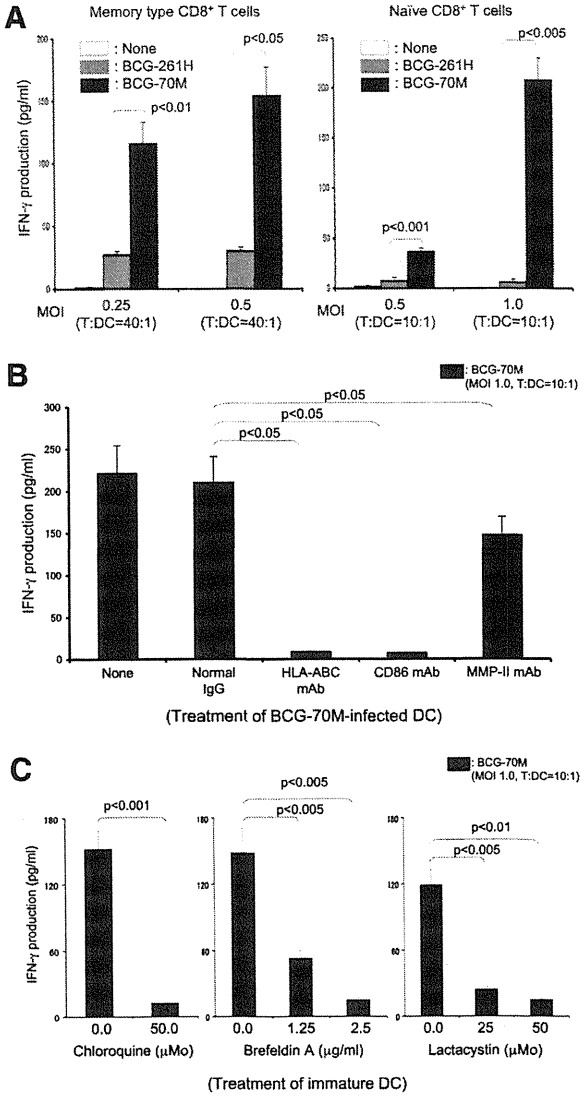

FIGURE 4. *A*, IFN-γ production from $CD8^+$ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ T cells (1×10^5) were stimulated with the indicated dose of BCG-infected DC. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t* test. *B*, Inhibition of naive $CD8^+$ T cell activation by the treatment of BCG-70M-infected DC with mAb. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG-70M at a MOI of 1.0, and subsequently treated with 10 μg/ml mAb to HLA-ABC, CD86, or MMP-II (M270-13). The DC were used as the stimulator of naive $CD8^+$ T cells (1×10^5/well) at T:DC = 10:1. IFN-γ produced from the T cells was measured by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t* test. *C*, Effect of treatment of immature DC with reagents on the activation of naive $CD8^+$ T cells. PBMCs were obtained from one donor. Monocyte-derived immature DC were treated with the indicated dose of either chloroquine, brefeldin A, or lactacystin, and subsequently infected with BCG-70M at a MOI of 1.0. These DC were used as the stimulator of naive $CD8^+$ T cells (1×10^5/well) at T:DC = 10:1. IFN-γ produced from the T cells was measured. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t* test.

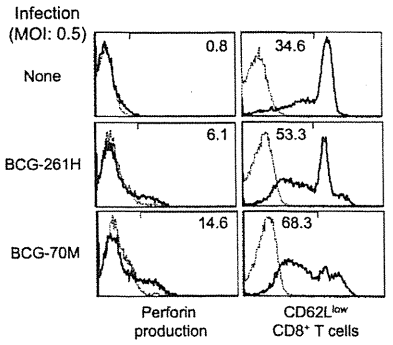

FIGURE 5. Influence of naive $CD4^+$ T cells on the activation of naive $CD8^+$ T cells. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG (MOI 0.5) and cocultured with unseparated naive T cells (T:DC = 10:1) for 7 days. The stimulated $CD8^+$ T cells were gated and analyzed for perforin production and for expression of CD62L Ag. Numbers indicate either percentage of perforin-positive $CD8^+$ T cells or $CD62L^{low}$ $CD8^+$ T cells among $CD8^+$ T cell population. A representative of three separate experiments is shown.

was inhibited (data not shown). Furthermore, pretreatment of immature DC with chloroquine before infection with BCG-70M significantly inhibited the IFN-γ production from naive $CD4^+$ T cells (Fig. 3*B*). These results indicated that the secreted HSP70-MMP-II protein or BCG-70M itself may be processed in the DC, and some of the antigenic peptides were used for the stimulation of autologous Ag-specific naive $CD4^+$ T cells. Similarly, BCG-70M-infected DC stimulated memory $CD8^+$ T cells more efficiently than BCG-261H-infected DC, although a higher dose of BCG-70M was necessary to induce a similar level of IFN-γ production from $CD8^+$ T cells than the dose of BCG-70M required to produce the cytokine from memory $CD4^+$ T cells. As reported, BCG-261H did not activate naive $CD8^+$ T cells efficiently (15); however, BCG-70M-infected DC induced a significant level of IFN-γ production from naive $CD8^+$ T cells (Fig. 4*A*). Using a higher dose of BCG-70M (MOI 1.0) and a larger number of BCG-70M-infected DC (T:DC = 10:1), a high concentration (~200 pg/ml) of IFN-γ could be produced from naive $CD8^+$ T cells. In addition, various MOIs and T:DC ratios were assessed, and a similar difference between BCG-261H and BCG-70M was observed (data not shown). To clarify the mechanism leading to the activation of naive $CD8^+$ T cells by BCG-70M, BCG-70M-infected DC were treated with mAbs. Again, the activation of naive $CD8^+$ T cells by BCG-70M-infected DC was significantly inhibited by the treatment of the DC with the mAb to HLA-ABC or CD86. However, surface treatment of the DC with the mAb to MMP-II significantly, but only partially, inhibited the T cell activation (Fig. 4*B*). These results may indicate that BCG-70M-infected DC cross-primed naive $CD8^+$ T cells in an Ag-specific manner. To elucidate the mechanisms leading to the cross-presentation by BCG-70M-infected DC, we treated immature DC with various reagents in advance of the BCG-70M infection (Fig. 4*C*). On the pretreatment of DC with chloroquine, IFN-γ production from naive $CD8^+$ T cells was significantly inhibited, indicating that protein derived from BCG-70M was degraded in presumably the phagolysosome. Furthermore, on the pretreatment of DC with brefeldin A, an inhibitor of antegrade Golgi transportation, and lactacystin, an inhibitor of proteosomal protein degradation, IFN-γ production from naive $CD8^+$ T cells was inhibited significantly in a manner dependent on the concentration of the reagents. Because BCG-70M activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, we stimulated naive $CD8^+$ T cells with BCG-70M in the presence of the $CD4^+$ T cells (Fig. 5). The expression level of CD62L on some $CD8^+$ T cells was

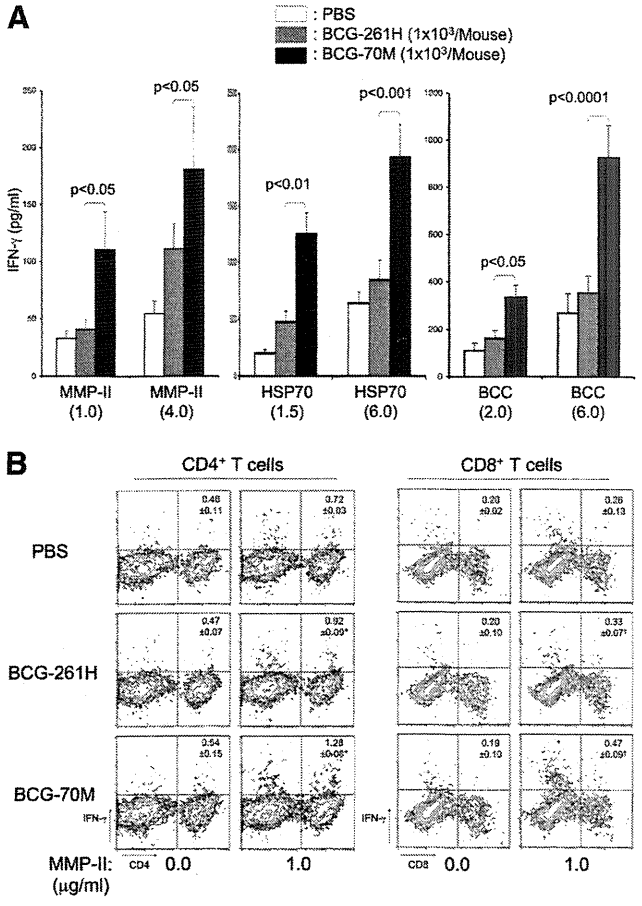

FIGURE 6. *A*, Production of memory-type T cells in C57BL/6 mice by infection with BCG. Five-week-old C57BL/6 mice were infected with 1×10^3 CFU/mouse of either BCG-261H or BCG-70M s.c. Four weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either MMP-II-, HSP70-, or BCG-derived cytosolic protein for 4 days, and IFN-γ produced in the cell supernatant was measured. Assays were performed in triplicate for each mouse, and the results of three mice per group are shown as the mean ± SD. Representative results of four separate experiments are shown. Titers were statistically compared using Student's *t* test. *B*, Intracellular IFN-γ production from $CD4^+$ T cells and $CD8^+$ T cells in C57BL/6 mice s.c. inoculated with BCG by secondary stimulation. Groups of 5-wk-old C57BL/6 mice were infected with 1×10^3/mouse BCG-261H or BCG-70M intradermally. Four weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with 1.0 μg/ml rMMP-II for 3 days. The $CD4^+$ T cells and $CD8^+$ T cells were gated separately and analyzed for intracellular production of IFN-γ. The number in the *top right-hand corner* of each panel represents the mean ± SD for three mice in the percentage of IFN-γ-producing cells among the gated T cell population. Representative results of four separate experiments are shown. Titers were statistically compared using Student's *t* test. *, $p < 0.05$; †, $p < 0.01$.

significantly reduced by stimulation with BCG-70M-infected DC, and a significant amount of intracellular perforin was produced in naive $CD8^+$ T cells by the stimulation. These changes were more efficiently induced by BCG-70M-infected DC than by BCG-261H-infected DC (Fig. 5). The $CD62L^{low}CD8^+$ T cells and perforin-producing $CD8^+$ T cells were not produced when naive $CD8^+$ T cells were stimulated in the absence of naive $CD4^+$ T cells (data not shown).

Memory T cell production by BCG-70M in vivo

Another important aspect to be studied is the production of Ag-specific memory T cells in vivo. C57BL/6 mice were infected with

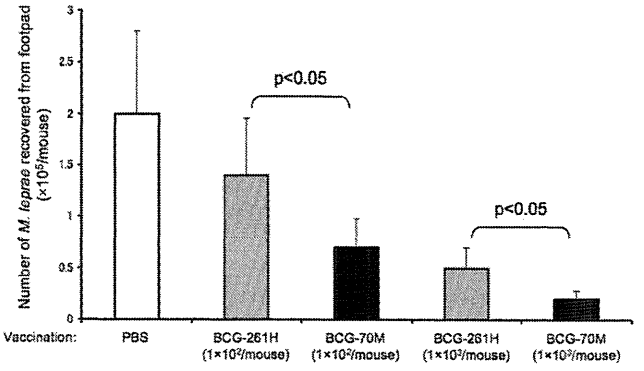

FIGURE 7. Inhibition of *M. leprae* multiplication by s.c. vaccination with BCG-70M. Five-week-old C57BL/6 mice (8 mice per group) were vaccinated with 1×10^2 or 1×10^3 CFU/mouse either BCG-261H or BCG-70M s.c., and were challenged with 5×10^3 bacilli/mouse *M. leprae* in footpad 4 wk after the vaccination. The number of *M. leprae* recovered from the footpad at 32 wk after the challenge was enumerated by Shepard's methods (29). Representative results of two separate experiments are shown. Titers were statistically compared using Student's *t* test.

1000 CFU/mouse rBCG for 4 wk, and their splenocytes were secondarily stimulated in vitro with rMMP-II protein, or recall Ags, like BCC (Fig. 6*A*). When a lower dose of MMP-II was used as a stimulator, only T cells obtained from BCG-70M-infected mice responded to the stimulator. Because BCG-Tokyo, the parental strain of the rBCGs, has the gene encoding MMP-II, a higher dose of *M. leprae*-derived MMP-II induced IFN-γ production from both T cells obtained from BCG-261H- and BCG-70M-inoculated mice; however, T cells from BCG-70M-infected mice more efficiently produced the cytokine than those from BCG-261H-infected mice. Also, T cells from BCG-70M-inoculated mice produced significantly higher level of IFN-γ than T cells from mice inoculated with BCG-261H on stimulation with HSP70 in vitro. The efficient generation of memory T cells responding to mycobacteria-derived Ags in mice infected with BCG-70M was confirmed, because only T cells from mice infected with BCG-70M significantly responded to BCC (Fig. 6*A*). To clarify the T cell subsets responsible for the IFN-γ production on secondary MMP-II stimulation, T cells producing the cytokine intracellulary were analyzed (Fig. 6*B*). Both $CD4^+$ T cells and $CD8^+$ T cells derived from not only non-BCG-inoculated mice, but also BCG-infected mice, produced intracellular IFN-γ by MMP-II stimulation. Both noninoculated and PBS-inoculated mice showed the similar responses (data not shown). However, significantly larger populations of both $CD4^+$ T cells (~1.3%) and $CD8^+$ T cells (~0.5%) obtained from BCG-70M-infected mice produced the cytokine. There were no $CD4^+$ T cells or $CD8^+$ T cells that were positively stained with the isotype control IgG (data not shown).

Effect of BCG-70M vaccination on the multiplication of M. leprae *in vivo*

C57BL/6 mice vaccinated with either BCG-261H or BCG-70M (1×10^2 or 1×10^3 CFU/mouse) for 4 wk were challenged with 5×10^3 of *M. leprae* in footpad. Thirty-two weeks later, footpad was removed and *M. leprae* recovered from the footpad was enumerated (Fig. 7). A total of 2×10^5 *M. leprae* was recovered from mice inoculated with PBS and challenged with *M. leprae*. Both mice inoculated with BCG-261H or BCG-70M inhibited the multiplication of *M. leprae* in the manner dependent on the dose of rBCG vaccinated; however, BCG-70M vaccination was significantly more efficient than BCG-261H vaccination in inhibiting the

multiplication, and only 2×10^4 *M. leprae* was recovered from mice vaccinated with 1×10^3 CFU/mouse BCG-70M.

Discussion

M. leprae is a representative mycobacterium among slow-growing prokaryotes, which needs 12–14 days for one division and 10–70 years for the manifestation of the disease, depending on the dose of bacilli entering the hosts. Host defense against *M. leprae* is chiefly conducted by adaptive immunity involving both $CD4^+$ and $CD8^+$ T cell subsets (3–5). Although $CD4^+$ T cells usually act at the initial phase of infection, $CD8^+$ T cells inhibit the multiplication of *M. leprae* in the chronic phase or when it is reactivated from a dormant state (31). Therefore, the vaccine should have an ability to competently activate not only $CD4^+$ T cells, but also $CD8^+$ T cells to produce memory T cells. To date, BCG is used as sole, but safe, vaccine against leprosy; however, nowadays, its efficacy is considered not so convincing (13). We have made several attempts to improve the potency of BCG, especially its immunostimulatory activities. We chiefly focused on achieving the fusion of BCG-infected phagosomes with lysosomes, without which the full and polyclonal activation of Ag-specific T cells cannot be expected. One approach we tried was the production of an *ure C*-deficient rBCG that successfully produces acidic phagosomes and facilitates their fusion with lysosomes (15, 28, 32, 33). Actually, the rBCG efficiently colocalized with lysosomes and effectively stimulated $CD4^+$ T cells when DC were targeted (28). However, it did not activate naive $CD8^+$ T cells. Then, we produced a second rBCG that secretes MMP-II (BCG-SM) in the phagosome (14). BCG-SM was useful in the activation of not only naive $CD4^+$ T cells, but also naive $CD8^+$ T cells to some extent (14). The T cell activation presumably occurs as a consequence of the translocation of the antigenic determinants of the secreted MMP-II to the cell surface, although the precise mechanism has not been clarified. Therefore, the intracellular secretion of immunodominant Ag by BCG is thought necessary to enhance the T cell-stimulating activity of BCG. However, BCG-SM vaccinations only partially inhibited the multiplication of *M. leprae* in the footpads of mice (our unpublished observation). These observations indicate the need for another rBCG capable of activating both naive $CD4^+$ and $CD8^+$ T cells more strongly.

Because the strong activation of naive $CD8^+$ T cells by mycobacteria required the cross-presentation of mycobacteria-derived Ags to $CD8^+$ T cells, in this study, we used BCG-derived HSP70 as a mediator facilitating the cross-presentation by DC, because HSPs of both mammalian host cells and bacterial origin are reported to have chaperone activity (34) and can effectively prime a cytolytic response (35). The usefulness of HSP65 as a convincing stimulator of $CD8^+$ T cells was revealed in animal studies to develop vaccine against *M. tuberculosis* (36). A DNA vaccine containing the *hsp65* gene activated naive $CD8^+$ T cells, and consequently inhibited the development of tuberculosis, although precisely how was not clarified. Our newly constructed rBCG (BCG-70M) secreted a fusion protein comprising HSP70 and MMP-II in vitro in the absence of any exogenous secretion signal. The secretion of HSP70-MMP-II fusion was confirmed by Western blotting analyses (Fig. 1*A*) and by the surface expression of MMP-II on DC (data not shown). The exact mechanism leading to the secretion of the fusion protein from BCG-70M is not known, but the secretion could be due to the inherent characteristics of HSP70 to be secreted (16, 37). Although we tried to enhance the secreting activity of BCG-70M by additionally inserting *M. tuberculosis*-derived secretion signal to BCG-70M, the secretion efficacy was rather inhibited and the construct stimulated naive T cells less efficiently than BCG-70M (data not shown).

BCG-70M secreted a 90-kDa fusion protein composed of HSP70 and MMP-II (14). The activation of naive $CD8^+$ T cells by BCG-70M was only partially inhibited by the treatment of DC with the mAb to MMP-II. Therefore, it can be speculated that BCG-70M activated $CD8^+$ T cells polyclonally by using various epitopes, originating from MMP-II, HSP70, or other Ags of BCG, because T cells from BCG-70M-infected mice vigorously responded to MMP-II, HSP70, and BCG-derived cytosolic protein (Fig. 6*A*). Therefore, HSP70 may alter the clonality of responding $CD8^+$ T cells, and the production of such polyclonal Ag-specific $CD8^+$ T cells might be beneficial for the broad coverage of a heterogenous MHC population.

BCG-70M induced higher level of cytokine production, including IL-12p70, TNF-α, and IL-1β, than the vector control BCG. The enhanced cytokine production by BCG-70M could be due to the intracellular secretion of HSP70 as a part of the fusion protein by the BCG. MMP-II can ligate TLR2 (6), but HSP70 is also known to bind TLRs (38); thus, the secreted HSP70-MMP-II protein seems to activate DC strongly. The contribution of TLR2 to cytokine production was confirmed by the inhibition of IL-12p70 production by antagonistic Ab to TLR2. The cytokines released from DC by BCG-70M stimulation could facilitate skewing of the direction of T cell activation to type 1 and induce the efficient and strong production of IFN-γ from naive $CD8^+$ T cells.

HSPs play a varied role in enhancing the ability of APCs to stimulate T cells (39). For the activation of Ag-specific $CD4^+$ and $CD8^+$ T cells, peptides should be loaded onto the corresponding MHC pathways. For the loading of BCG-derived Ags on these pathways, the proteins secreted from phagocytozed BCG should be transported to functional lysosomes. In the phagolysosome, some portions of HSP70-MMP-II fusion protein could be degraded, but the rest would be sequestrated into the cytosol, where they are degraded and used for cross-priming $CD8^+$ T cells. BCG-70M-infected DC expressed derivatives of MMP-II and the other proteins on their surface, and they activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells. However, both MMP-II expression on DC and the activation of the T cells by DC were inhibited by the pretreatment of DC with chloroquine (24, 40). These results indicate that secreted HSP70-MMP-II fusion protein was efficiently processed in lysosomes and its derivatives are used for the activation of both subsets of naive T cells. When naive $CD8^+$ T cells were stimulated by BCG-70M in the presence of naive $CD4^+$ T cells, $CD62L^{low}CD8^+$ T cells and perforin-producing $CD8^+$ T cells were efficiently produced. The activation of naive T cells was confirmed by the production of memory-type T cells by BCG-70M infection to unprimed mice, because both $CD4^+$ and $CD8^+$ T cell subsets from BCG-70M-infected mice responded to the restimulation with MMP-II in vitro. Furthermore, BCG-70M significantly and more efficiently inhibited the multiplication of *M. leprae*, which were challenged in footpad of mice, than BCG-261H.

There are two pathways of cross-presentation, as follows: cytosolic (ER-Golgi-dependent) and vacuolar pathways (20). It is known that HSP can enhance both pathways (20). In the present study, IFN-γ production from naive $CD8^+$ T cells was largely blocked by the treatment of DC with brefeldin A, an inhibitior of antegrade Golgi transportation and of TAP-dependent transportation, and also with lactacystin, a proteosomal protein degradation blocker (20, 40). Therefore, it can be presumed that the fusion protein was sequestered into the cytosol from the lysosome, degraded in the proteosome, and used for loading on MHC class I molecules through the TAP-dependent pathway. Furthermore, it has been reported that proteins that are intracellularly secreted are usually processed by a cytosolic (ER-Golgi-dependent) pathway, and DC prefer this pathway for cross-priming $CD8^+$ T cells with

protein Ag (20). Our present observations seem to fit well with these previous findings. Therefore, we concluded that BCG-70M activates naive CD8$^+$ T cells through the ER-Golgi-dependent cytosolic cross-presentation pathway. However, *M. tuberculosis*-derived HSP70 activated T cells through a post-Golgi, proteosomal-independent mechanism, and both brefeldin A and lactacystin may inhibit vacuolar pathway in some cases (20). Also, the vacuolar pathway is used more frequently by macrophages (20). Therefore, the possibility that BCG-70M may also use the post-Golgi pathway in vivo cannot be ruled out.

Taken together, in this study, we constructed a rBCG that secretes HSP70-MMP-II fusion protein, which effectively activates not only DC, but also naive T cells. Therefore, the combination of HSP70 and MMP-II may be useful for stimulating both subsets of naive T cells.

Acknowledgments

We acknowledge the contribution of N. Makino in the preparation of the manuscript. We also thank Y. Harada for her technical support, and the Japanese Red Cross Society for kindly providing PBMCs from healthy donors.

Disclosures

The authors have no financial conflict of interest.

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Jpn.J.Leprosy 78, 7-16(2009)

Temperature dependency for survival of *Mycobacterium leprae* in macrophages

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[Received: 4 Aug. 2008 / Accepted: 15 Oct. 2008]

Key words : human, macrophage, mouse, *Mycobacterium leprae*, survival

Hansen's disease is caused by an infection with an intracellular pathogen, *Mycobacterium leprae*, which mainly inhabits macrophages and Schwann cells. However, little is known about the survival or growth mechanisms of the bacilli in mouse and human macrophages. In the present study, by using radiorespirometry analysis for the evaluation of the viability of *M.leprae*, we observed that *in vitro* incubation of *M.leprae*-infected macrophages at 35°C was more growth permissive than at 37°C, and supplementation with the immunosuppressive cytokine IL-10 supported the survival of the bacilli in the macrophages for 3 weeks, whereas viability of the bacilli was gradually lost if cultured without IL-10. In human macrophages, *M.leprae* retained its viability when cultured at 35°C for at least 4 weeks without IL-10. However, the viability of *M.leprae* was almost lost within 2 weeks if cultured at 37°C. These data suggest that temperature is a crucial factor for the survival of *M.leprae* in host cells.

Introduction

Hansen's disease is caused by an infection with *Mycobacterium leprae*. *M.leprae* is an intracellular pathogen, mainly residing in macrophages and Schwann cells. In patients, *M.leprae* is predominantly observed in the skin, nasal mucosa and peripheral nerves, particularly the more superficial ones. This clinical observation suggests that the optimal temperature of *M.leprae* for survival in human cells is less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse footpad where the temperature is lower than the core temperature, and the optimal temperature for the growth of *M.leprae* is reported to be in the range of several degrees above and below 30°C [2].

From another aspect, the growth of *M.leprae* seems to be largely affected by the host immune

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response. Hansen's disease is characterized by a broad spectrum of the host immune response, such as lepromatous type (towards the increased load of bacteria) and tuberculoid type (towards the decreased bacterial load). In lepromatous type leprosy, Th-2 cytokines (IL-4, IL-5 and IL-10) are predominantly expressed in local lesions. In contrast, in tuberculoid type, Th-1 cytokines (IFN-γ, IL-2) are predominantly expressed [3]. Among cytokines, IFN-γ has been demonstrated to play a central role in activating macrophages to kill intracellular pathogens including *M.leprae*, whereas IL-10 is reported to inhibit the microbicidal activity of macrophages, resulting in the survival of the intracellular pathogen [4]. However, little is known about the survival and growth mechanisms of *M.leprae* in human macrophages since the viability of these uncultivable bacilli cannot be easily measured by in vitro study.

Previously we reported that metabolically active *M.leprae* were maintained in monolayer cultures of mouse peritoneal macrophages and supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system temperature is extremely important and 31-33°C incubation temperature is more growth permissive than 37°C [5]. In the present study, we observed that incubation of mouse macrophages at 35°C was also more permissive than at 37°C and supplemental IL-10, but not TGF-β, supported the metabolic activity of *M.leprae* in the macrophages for several weeks. Moreover, *M.leprae* from infected human macrophages cultured in vitro sustained metabolic activity for at least 4 weeks if cultured at 35°C but not at 37°C. Collectively, these data demonstrate that temperature is one of the crucial factors for *M.leprae* survival in human host cells.

Materials and Methods

M.leprae inoculum: The Thai-53 strain of *M.leprae* [6] was maintained in continuous passage in athymic *nu/nu* mice (Clea Co, Tokyo, Japan) by inoculation of bacilli into both hind foot pads. Experiments with mice were performed in compliance with the guidelines of the Experimental Animal Committee of the National Institute of Infectious Diseases. At approximately one year post inoculation, the foot pads were processed to recover *M.leprae* by Nakamura's method with a slight modification [7]. Briefly, tissue was minced and homogenized with Hanks' balanced salt solution (HBSS) containing 0.05% Tween 80. The homogenate was centrifuged at 150×g for 10 min and supernatant of the sample homogenate was treated with 0.05% trypsin at 37°C for 60min. The suspension was centrifuged at 4,000×g for 20min and sediment was re-suspended in HBSS followed by treatment with 1% sodium hydroxide at 37°C for 15min. The treated material was washed and re-suspended in HBSS at the desired bacillary concentration. Bacillary number in each foot pad was enumerated individually according to standard techniques [8].

Cytokines: Murine recombinant IL-10 was obtained from Genzyme Corp. TGF-β was obtained from Kurashiki Bouseki (Kurashiki, Japan). Both cytokines were stored at -80°C until use.

Mouse macrophage culture: Mouse peritoneal resident cells (approximately 50% macrophages) were harvested from retired ICR mice and suspended as previously described [9] at a concentration of 2×10^6/ml in RPMI 1640 (Gibco BRL, Invitrogen Corp., Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS, HyClone Laboratories, Logan UT), 25 mM N-2-

hydroxyethylpiperazine -N'- 2-ethanesulfonic acid (HEPES), 2 mM glutamine and 100μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). One half ml was seeded into 24 well tissue culture plates (Corning) containing 13 mm LUX coverslips (Nunc Thermanox coverslips, NalgeNunc, Thermo Scientific, Rochester, NY). After 20 hr adherence of the cells, macrophage monolayers were obtained after washing non-adherant cells from the coverslip with Hanks Balanced Salt Solution (HBSS, Sigma) leaving approximately 1×10^6 macrophages adhered per coverslip.

Human macrophage culture: Human peripheral blood was obtained under informed consent from healthy individuals. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, HP7 9NA, UK) gradient centrifugation[10]. The cells were suspended in AIM-V medium (Gibco BRL, Invitrogen Corp., Carlsbad, CA) and 1×10^6 PBMC were cultured in a well of a 24-well tissue culture plate (Falcon, Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes) containing 13 mm LUX coverslips at 37°C in a 5%-CO_2 incubator for adherence of monocytes. After 1 hr incubation, the coverslips were washed with HBSS to remove non-adherent cells. The monocytes on the coverslips were cultured in a new 24-well plate containing RPMI1640 medium (Sigma) supplemented with 20% FBS (Whittaker Co., Walkersville, MD), 25mM HEPES, 2mM L-glutamine and 100μg/ml ampicillin in the presence of 10 ng/ml of human M-CSF (R&D Systems, Minneapolis, MN) or 40 ng/ml of GM-CSF (R&D Systems). After 7 days, the M-CSF-conditioned macrophages (M-macrophages) and the GM-CSF-conditioned macrophages (GM-macrophages) were used for infection with *M.leprae*.

Infection of macrophages with *M.leprae*: Purified mouse macrophage monolayers were infected with fresh *M.leprae* suspended in 0.5 ml medium per well. After 4 hr incubation for mouse macrophages and 20 hr incubation for human macrophages, non-phagocytosed bacilli were removed by washing and the cultures were incubated in 1.0 ml media supplemented with the appropriate cytokine in 5% CO_2 at the appropriate experimental temperatures[9]. Media were changed and cytokines were replenished at 5 days interval.

Radiorespirometry: The macrophages were lysed with 0.1 N NaOH to release the phagocytosed *M.leprae*, and the viability of the bacilli was determined by evaluating the oxidation of ^{14}C-palmitic acid to $^{14}CO_2$ by radiorespirometry as described previously [11]. Total isotope release was usually analyzed after one week of incubation at 31°C [9].

Staining of *M.leprae*-infected macrophages: Coverslips of *M.leprae*-infected adherent macrophages were prefixed with absolute methanol and acid-fast stained. The specimens were observed under Nikon Optiphot light microscopy.

Results

Viability of *M.leprae* in mouse macrophages cultured *in vitro*: Mouse peritoneal resident macrophages (1×10^6 cells per well) were incubated with freshly harvested *M.leprae* (multiplicity of infection (MOI), 5:1 or 10:1) for 4 hr to allow phagocytosis. Non-phagocytosed bacilli were washed off and the culture of the macrophages continued for up to 14 days. Viability (metabolic activity) of *M.leprae* in macrophages was assessed by radiorespirometry. As shown in Fig. 1, the viability of the bacilli was gradually decreased in macrophages cultured at 35°C. In contrast, the viability was significantly lost, if the macrophages were cultured at 37°C. Next, the mouse peritoneal

resident macrophages were incubated with 3 doses of *M.leprae* (MOI, 1:1, 4:1 and 10:1) for 4 hr to allow phagocytosis, and the culture continued for longer periods up to 21 days. Viability of *M.leprae* in macrophages was assessed at 7 day intervals. As shown in Fig. 2, in each dose of *M.leprae* infection, decrease in viability was significant after 21 days.

Effects of cytokines on viability of *M.leprae* in mouse macrophages: Supplementation of IL-10 to the infected macrophage culture was

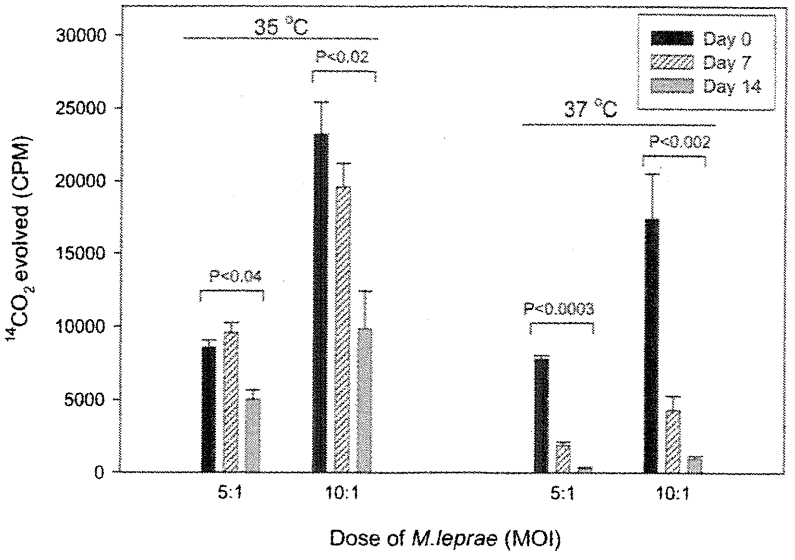

Fig.1. Viability of *M.leprae* in mouse macrophages cultured *in vitro*. Mouse peritoneal resident macrophages were incubated with 5×10^6 or 1×10^7 per well of *M.leprae* (MOI, 5:1 or 10:1), for 4 hr at 37°C to allow phagocytosis. Non-phagocytosed bacilli were washed off and the culture of the macrophages continued up to 14 days at 35°C or 37°C. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

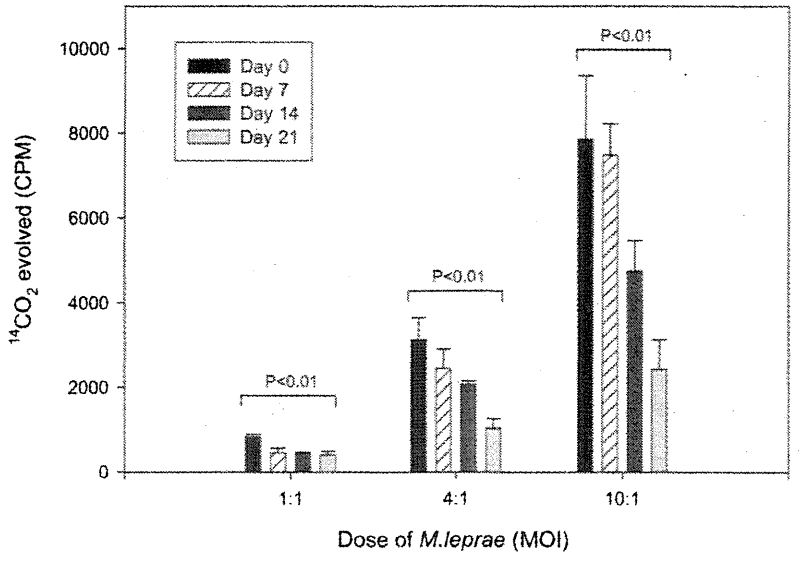

Fig.2. Viability of *M.leprae* in mouse macrophages cultured *in vitro*. Mouse peritoneal resident macrophages were incubated with 3 doses, 1×10^6, 4×10^6 and 1×10^7 per well of *M.leprae* (MOI, 1:1, 4:1 and 10:1) at 35°C for 4 hr to allow phagocytosis, and the culture continued at 35°C for longer periods up to 21 days. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

clearly associated with sustained viability of intracellular *M.leprae* cultured at 35°C (Fig.3). In the presence of 3 U/ml of IL-10, *M.leprae* maintained their viability, whereas viability was steadily lost without IL-10. We also examined the effect of TGF-β, another suppressive cytokine for macrophage activation, on the viablility of the bacilli. To the contrary, supplementation of TGF-β

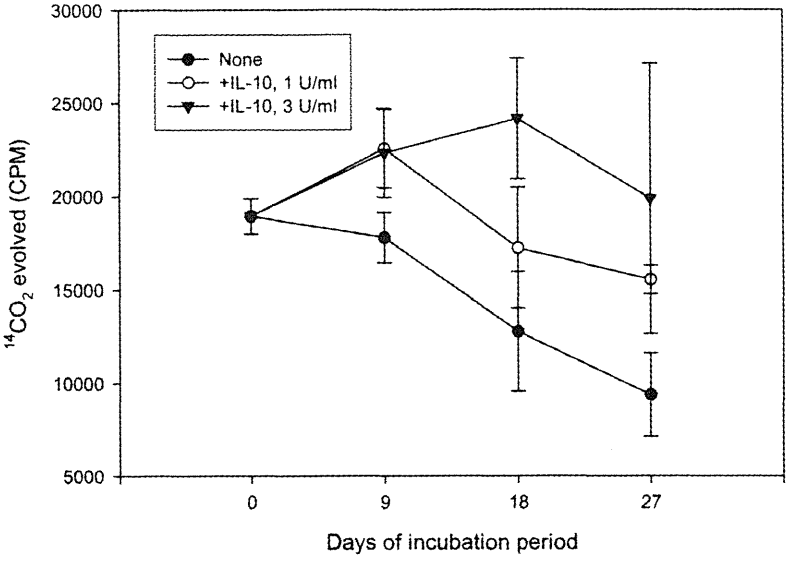

Fig.3. Effect of IL-10 on *M.leprae* survival in mouse macrophages. Mouse peritoneal resident macrophages were incubated with 1×10^7 per well of *M.leprae* (MOI, 10:1) at 35°C for 4 hr to allow phagocytosis, and the culture continued at 35°C for 9, 18 and 27 days. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.

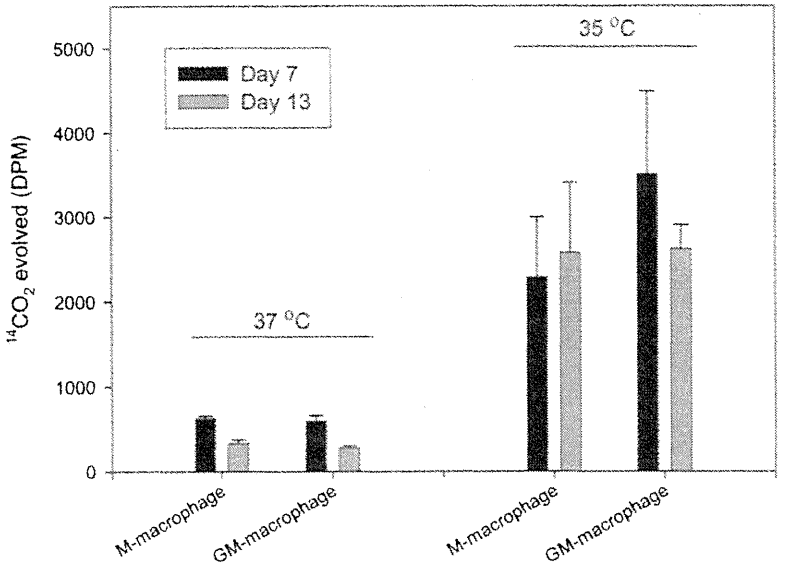

Fig.4.Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated at the same temperatures for indicated periods. By observation of the acid fast-stained cells under light microscopy, no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured between at 35°C and at 37°C. So the viability at day 0 is considered equal. After 7 days and 13 days incubation period, the cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry (dpm: disintegrations per minute).

significantly decreased the viability of *M.leprae*, when incubated for longer than 28 days post infection (Table 1).

Viability of *M.leprae* in human macrophages cultured *in vitro*: Human macrophages were obtained by culturing monocytes in the presence of either M-CSF or GM-CSF for 7 days. These macrophages (1×10^5 cells per well) were incubated with *M.leprae* (MOI, 50:1) for 20 hr either at 35°C or 37°C for infection and incubated again at the same temperatures. By observation of the acid fast-stained cells under light microscopy,

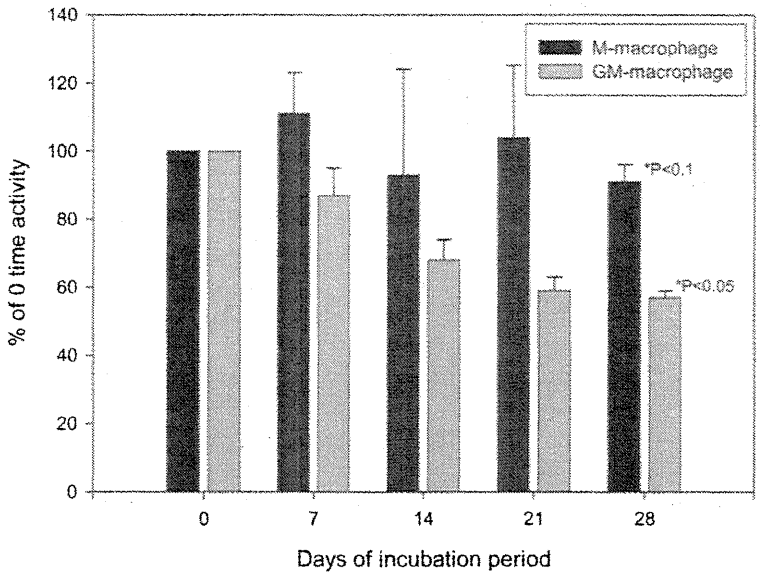

Fig.5. Viability of *M.leprae* in human macrophages cultured *in vitro*. Human M- or GM-macrophages were infected with *M.leprae* (MOI, 50:1) for 20 hr at 35°C and incubated again at 35°C for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry. The results at day 7, 14, 21 and 28 are expressed as percentages of *M.leprae* metabolic activity at time 0. Radiorespirometry data obtained from *M.leprae* in M-macrophages at time 0 was 5,932±399 and those in GM-macrophages was 3,084±78. *P values calculated in comparison to day 0 viability.

Table 1. Effect of TGF-β on survival of *M.leprae* in mouse macrophages cultured at 35°C[a]

Experiment 1				
Days of incubation period	At time 0	7	14	28
Medium only	5,222±936[b]	2,774±295	3,086±425	2,828±1,815
+TGF-β		2,919±535	3,119±1,339	1,973±126
Experiment 2				
Days of incubation period	At time 0	14	28	49
Medium only	26,791±1,428	19,103±621	7,420±2,986	5,713±1,144
+TGF-β		14,306±2,240	3,728±410	1,594±317

[a]Mouse peritoneal resident macrophages were incubated with *M.leprae* (MOI, 1:10) for 4 hr to allow phagocytosis, and the culture continued for indicated periods. The cells were lysed to obtain *M.leprae* and metabolism of the bacilli was assessed by radiorespirometry.
[b]Radiorespirometry data, cpm.
Dose of TGF-β, 500pg/ml.
N.D., not determined.

no difference was recognized in the number of *M.leprae* phagocytosed by macrophage cultured at 35°C and at 37°C (data not shown). Viability of *M.leprae* was assessed after 7 and 13 days. The results clearly showed that the viability of *M.leprae* incubated at 35°C was maintained, whereas the viability was lost if cultured at 37°C (Fig. 4). Next, *M.leprae*-infected human M- and GM-macrophages were cultured for prolonged periods at 35°C. Viability was sustained well for 4 weeks in human macrophages, especially in M-macrophages (Fig. 5).

Discussion

In vivo M.leprae is able to enter and survive in a wide variety of tissues and cell types[12]. The preferred host cell for the leprosy bacillus appears to be the macrophages and a number of unsuccessful attempts have been made to grow *M.leprae* in macrophages *in vitro*. For example, Sharp and Banerjee[13] employed macrophages from conventional mice and rats, *nu/nu* mice or *nu/nu* rats and armadillos. The *M.leprae* inocula were derived from 3 sources (human leproma, *nu/nu* mouse footpad and frozen infected armadillo tissue). Incubation temperature was varied from 31°C to 35°C and *M.leprae*-infected cells were maintained for up to 200 days. Fieldsteel and McIntosh [14] employed a range of rat, mouse and human tissue. The conclusion of these reports is that no significant multiplication of *M.leprae* occurred in any of the cells or tissues.

Previously, we reported that metabolically active *M.leprae* could be maintained in monolayer cultures of mouse peritoneal macrophages and that supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system, temperature is an extremely important factor for growth and 31-33°C incubation temperature is more permissive than 37°C [5]. In the present study, we further observed that incubation of mouse macrophages infected with *M.leprae* at 35°C was also more growth permissive than at 37°C. We chose 35°C as the incubation temperature, and not 31°C, because the maintenance of the integrity of the macrophage monolayer was better at 35°C than at 31-33°C. Moreover, the monolayer of *M.leprae*-infected human macrophages at 31-33°C could not be maintained for longer than one week. We observed that maintenance of the monolayer was good at 35°C, and *M.leprae* at 35°C was also more growth permissive than those at 37°C in human macrophages (Fig. 4 and 5). Our starting inoculum of *M.leprae* was freshly obtained for each experiment from infected *nu/nu* mice. We also were able to rapidly quantify the metabolic activity of *M.leprae* using the radiorespirometry technique adapted by Franzblau [11]. This assay is accurate and highly sensitive with the results available in a short duration of 1 wk (compared to 6-12 months when titrated in mouse footpads). Radiorespirometry data correlates well with other *in vitro* systems [11] but, more importantly, the data correlated well with "viability" as observed in the mouse footpad system [12].

Various clinical evidence suggests that *M.leprae* prefer a growth temperature of less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse foot pad where the temperature is lower than the body temperature [2]. In addition, *Dasypus novemcinctus*, the nine-banded armadillo has a core temperature of ~33°C, which renders it permissive as a host for the leprosy bacillus [13]. Mononuclear phagocytes in virtually every organ of the natural or experimentally infected armadillo become heavily parasitized with propagating *M. leprae* [14]. Whether intracellular or extracellular, *M.leprae* clearly prefers temperatures cooler than

normal human body temperature [12], and 37°C appeared to be highly detrimental to *M.leprae* viability. The *in vitro* results obtained in the present study confirmed the preference of lower temperature (35°C) by *M.leprae* residing in human macrophages.

In this study, supplemental IL-10, but not TGF-β supported the metabolic activity of *M.leprae* in mouse macrophages for several weeks, similar to the results obtained previously [5]. In choosing TGF-β and IL-10 as the cytokines that might bolster the intracellular survival of *M.leprae,* we were attempting to down-regulate any innate ability of the normal macrophages to cope with the organism. TGF-β is produced by activated macrophages and other inflammatory cells and has a broad array of modulatory functions on the immune response. TGF-β has been shown to interfere with macrophage antimicrobial mechanisms including the generation of reactive oxygen intermediates [15] and reactive nitrogen intermediates [16], and has been shown to enhance the intracellular growth of *M.tuberculosis* in human monocytes [17]. However, in the present studies with mouse macrophages, exogenous TGF-β had no detectable effect on sustaining intracellular *M.leprae* viability, and in fact decreased the viability (Table 1). In contrast, supplementing media with IL-10 clearly affected the long term viability of *M.leprae* in mouse macrophages (Fig. 3). IL-10 is produced by T cells, B cells and macrophages [18, 19]. IL-10 has been shown to be a potent down-regulator of cell-mediated immunity to intracellular pathogens [20]. *In vivo*, endogenous IL-10 dampened the cell-mediated immune response to avirulent mycobacterial infection [4] and appeared to lead to loss of control of *M.tuberculosis* infection with widespread dissemination [21]. IL-10 functions in part at the level of the macrophage by attenuating iNOS mRNA expression, iNOS activity and, by inference, NO production [22]. In human macrophages, however, the viability of *M.leprae* was maintained for 4 weeks in the absence of IL-10 (Fig. 5), suggesting that human cells seem to be better hosts than mouse cells for *M.leprae* survival. Viability of *M.leprae* in M-macrophages seems to be maintained for a longer period (up to one month) than that in GM-macrophages (Fig. 5). One of the reasons for this may be due to the production of IL-10 by M-macrophages [23], although the mechanism by which IL-10 contributes to the maintenance and growth of *M.leprae* is unclear.

In conclusion, the present study showed that the metabolism, and presumably the viability, of *M.leprae* could be sustained under culture conditions at 35°C, which is also a moderate temperature necessary to maintain the integrity of macrophages.

Acknowledgments

The study was supported partly by a Health Science Research Grant of Emerging and Re-emerging Infectious Diseases, from the Ministry of Health, Labour and Welfare of Japan. We are also grateful to the Japanese Red Cross Society for kindly providing PBMCs from healthy donors.

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マクロファージ内におけるらい菌生存の温度依存性

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〔受付：2008年8月4日、掲載決定：2008年10月15日〕

キーワード：生存、ヒト、マウス、マクロファージ、らい菌

ハンセン病は細胞内寄生菌であるらい菌によって引き起こされる感染症である。らい菌は主にマクロファージとシュワン細胞に感染する。しかしながら、マウスやヒトマクロファージ内における生存・発育機構について詳細は明らかになっていない。本研究では放射性同位元素を用いた方法によりらい菌の生存率を評価した。そして、らい菌感染マクロファージを35度で培養する方が37度で培養するよりもらい菌の生存率を高い状態に保つことができることが判明した。また、免疫抑制性サイトカインであるIL-10を添加することにより3週間程度生存が維持されることが分かった。一方、IL-10未添加の場合、生存率は徐々に低下した。ヒトマクロファージの場合は、IL-10未添加の場合でも少なくとも4週間生存は維持された。しかしながら、37度で培養すると2週間以内に生存率は著明に低下した。これらの結果から、らい菌の細胞内における生存には温度が決定的な要因のひとつであることが判明した。

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A Signaling Polypeptide Derived from an Innate Immune Adaptor Molecule Can Be Harnessed as a New Class of Vaccine Adjuvant[1]

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Modulation of intracellular signaling using cell-permeable polypeptides is a promising technology for future clinical applications. To develop a novel approach to activate innate immune signaling by synthetic polypeptides, we characterized several different polypeptides derived from the caspase recruitment domain (CARD) of IFN-β promoter stimulator 1, each of which localizes to a different subcellular compartment. Of particular interest was, N′-CARD, which consisted of the nuclear localization signal of histone H2B and the IFN-β promoter stimulator 1CARD and which localized to the nucleus. This polypeptide led to a strong production of type I IFNs and molecular and genetic analyses showed that nuclear DNA helicase II is critically involved in this response. N′-CARD polypeptide fused to a protein transduction domain (N′-CARD-PTD) readily transmigrated from the outside to the inside of the cell and triggered innate immune signaling. Administration of N′-CARD-PTD polypeptide elicited production of type I IFNs, maturation of bone marrow-derived dendritic cells, and promotion of vaccine immunogenicity by enhancing Ag-specific Th1-type immune responses, thereby protecting mice from lethal influenza infection and from outgrowth of transplanted tumors in vivo. Thus, our results indicate that the N′-CARD-PTD polypeptide belongs to a new class of vaccine adjuvant that directly triggers intracellular signal transduction by a distinct mechanism from those engaged by conventional vaccine adjuvants, such as TLR ligands. *The Journal of Immunology,* **2009, 182: 1593–1601.**

Accumulating evidence from basic research and from clinical studies clearly indicates that type I IFNs are key to the elimination of viral infection (1, 2), suppression of tumor progression (3, 4), and to vaccine immunogenicity (5). Type I IFNs, such as IFN-α and -β, are produced from a wide variety of cell types upon viral infection or in response to foreign nucleic acids, such as DNA and RNA (6–8). Recent research has dissected and elucidated the molecular basis of the ability of the immune system to sense a variety of nucleic acids as pathogen-associated molecular patterns (9) or to sense the presence of aberrant self-DNA under dangerous situations (10, 11). RIG-I-like helicases (RLHs)[4] mediate innate immune signaling in human cells induced by immunostimulatory RNAs, such as 5′-triphosphate RNA or dsRNA, or right-handed B-form DNA (B-DNA) (12–14). RLHs trigger cellular signaling through adaptor molecules, such as IFN-β promoter stimulator 1 (IPS-1, also known as MAVS/VISA/Cardif), TNFR-associating factor (TRAF) 3, and TRAF family member-associated NF-κB activator (TANK), thereby coordinating the activation of IκB kinase (IKK) family members, such as NF-κB essential modulator, IKK-α, IKK-β, TANK-binding kinase 1 (TBK1), and inducible IKK (IKKi). Once activated by such cytoplasmic kinases, NF-κB, IFN regulatory factor 3 (IRF3), and IRF7 transmigrate into the nucleus and act as master regulators of type I IFN-related gene promoters (15).

These signaling molecules contain distinct domains, and thereby associate with specific target molecules and modulate downstream signal transmission. IPS-1 plays a central role in this signaling pathway and its caspase recruitment domain (CARD) forms the death domain fold, which is structurally similar to domains of Fas-associated via death domain and caspase family members (16). The CARD of IPS-1 is essential for signal transmission through homotypic interactions with the CARDs of upstream RLHs (9). Mitochondrial sorting of IPS-1 is also crucial for its canonical

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Received for publication July 24, 2008. Accepted for publication November 19, 2008.

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[1] This work was supported, in part, by the Strategic Research Project of Yokohama City University (K18022 to F.T.), the Advancement of Medical Sciences from Yokohama Medical Foundation (to F.T. and K.K.), the National Institute of Biomedical Innovation (to K.O.), the Yasuda Medical Foundation (to F.T.), the Uehara Memorial Foundation (to F.T.), and a Grant-in-aid for Scientific Research (20590477 to F.T.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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[4] Abbreviations used in this paper: RLH, RIG-I-like helicase; B-DNA, B-form DNA; IPS-1, IFN-β promoter stimulator 1; TRAF, TNFR-associating factor; TANK, TRAF family member-associated NF-κB activator; TBK1, TANK binding kinase 1; IKK, IκB kinase; IKKi, inducible IKK; IRF3, IFN regulatory factor 3; CARD, caspase recruitment domain; N′-CARD, fusion of the NH_2-terminal nuclear localization signal of histone H2B to the IPS-1 CARD; PTD, protein transduction domain; TMD, transmembrane domain; NLS, nuclear localization signal; NDH, nuclear DNA helicase II; ODN, oligodeoxynucleotide; flu vax, influenza split-product vaccine; DC, dendritic cell; FL, full length; BM-DC, bone marrow-derived dendritic cell.

signaling because human hepatitis C virus NS3/4A protease inactivates IPS-1 by cleaving a region adjacent to the transmembrane domain (TMD), which is required for IPS-1 localization to the mitochondrial outer membrane (17).

To develop a novel approach to modulate innate immune signaling by synthetic polypeptides, we generated several different IPS-1 CARD-fusion polypeptides, each of which localizes to a different subcellular compartment. Of interest, the nuclear localization of a fusion polypeptide between the nuclear localization signal (NLS) of histone H2B and the IPS-1 CARD (hereafter referred to as N′-CARD) activated a distinct signaling pathway initiated from the nucleus and led to a strong production of type I IFN. Molecular and genetic analyses showed that nuclear DNA helicase II (NDH) is critically involved in this signaling pathway. Fusion of N′-CARD to the protein transduction domain (PTD), originally derived from the HIV Tat protein (18), facilitated transduction of N′-CARD from outside to inside the cell without loss of its original intracellular function. Finally, we demonstrate that the N′-CARD-PTD polypeptide acts as a novel vaccine adjuvant by directly triggering innate intracellular immune signaling to augment vaccine immunogenicity. Such a mechanism is distinct from TLR-mediated signaling, which is engaged in innate immune activation by conventional vaccine adjuvants, such as monophosphoryl-lipid A (an LPS derivative) and CpG oligodeoxynucleotide (ODN).

Materials and Methods

Cells and reagents

HEK293, HeLa, RAW264.7, and TC-1 cells were purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% FCS and 50 μg/ml penicillin/streptomycin. Sf9 cells were maintained in Sf900 II SFM (Invitrogen). LPS was purchased from Sigma-Aldrich. CpG ODN, 5′-ATC GAC TCT CGA GCG TTC TC-3′, was synthesized by Gene Design. Mouse GM-CSF and Flt3L were purchased from PeproTech. Influenza split-product vaccine (flu vax) was prepared at The Research Foundation for Microbial Diseases of Osaka University (Kanon-ji city, Kagawa, Japan) from the purified influenza virus A/New Caledonia/20/99 strain (H1N1) by sequential treatment with ether and formalin, according to the method of Davenport et al. (19, 20).

Expression plasmids

The IPS-1 expression plasmid was described previously (21). The IPS-1 CARD, aa 1–100 of the IPS-1 ORF, was PCR-amplified. Fusion cDNAs were generated by ligating aa 1–100 and 514–540 of IPS-1 ORF (CARD-TMD), aa 1–37 of histone H2B ORF and aa 1–100 of IPS-1 ORF (N′-CARD), N′-CARD and aa 514–540 of hIPS-1 ORF (N′-CARD-TMD), and were amplified by PCR. These fragments were introduced in-frame into pFLAG CMV5b (Sigma-Aldrich) or pGEX6P-2 (GE Healthcare). GST-N′-CARD was further fused to the PTD (Tyr-Ala-Arg-Ala-Ala-Ala-Arg-Gln-Ala-Arg-Ala) and introduced into pFastBac HT-B (Invitrogen). TBK1, IKKi, NDH, and chloride channel 1A (CC1A) cDNAs were amplified by PCR using a human spleen cDNA library (Takara). These fragments were introduced in-frame into pFLAG-CMV4 (Sigma-Aldrich), pCIneo-HA, pCAGGS-Flag-m1SECFP, pCAG-His Venus, or pcDNA3-RFP. The N′-CARD T54A expression plasmid was generated by site-directed mutagenesis, as described previously (22). The sequences of the PCR products were confirmed using an ABI PRISM Genetic Analyzer (PE Applied Biosystems).

Luciferase assay

The luciferase assay was conducted as described previously (23).

Confocal microscopy

HeLa cells were transfected with CARD-YFP, CARD-TMD-YFP, N′-CARD-YFP, N′-CARD-TMD-YFP, IPS-1-YFP, YFP-IKKi, YFP-TBK1, and/or mRFP-NDH and incubated for 48 h. In some cases, the cells were treated with Hoechst 33258 (Invitrogen) and/or MitoTracker reagent (Invitrogen) at 37°C for 15 min. Alternatively, HeLa cells were treated with CARD or N′-CARD-PTD for 30 min. Cells were treated with Hoechst 33258 for 15 min before fixation and incubation with mouse anti-FLAG M2-Cy3. After washing with PBS containing 1% BSA, the cells were examined under an FV 500 confocal microscope (Olympus).

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting was performed as described previously (24) using anti-FLAG M2 (Sigma-Aldrich), anti-FLAG M2-HRP (Sigma-Aldrich), anti-HA (Covance), anti-HA-HRP (Roche Diagnostics), anti-ubiquitin-HRP (Santa Cruz Biotechnology), anti-NDH (provided by J. D. Parvin, Brigham and Women's Hospital, Boston, MA), anti-p-JNK, anti-p-p38, anti-p-ERK, and anti-β-actin (Cell Signaling Technology).

RNA interference

An siRNA targeting NDH mRNA (stealth RNAi) was chemically synthesized by Invitrogen (Carlsbad, CA): sense, 5′-AUU GCU UGC AAA UCA UGA UCC UGU U-3′; antisense, 5′-AAC AGG AUC AUG AUU UGC AAG CAA U-3′. HEK293 cells (6×10^5) were transfected with 120 pmol of control or NDH siRNA using Lipofectamine RNAi MAX reagent (Invitrogen) according to the manufacturer's protocol.

Purification of recombinant polypeptides

DH10Bac competent cells (Invitrogen) were transformed with pFastBac HT-B-GST or with GST-N′-CARD-PTD to generate recombinant Bacmids. Sf9 cells were transfected with Bacmid-encoding GST or GST-N′-CARD-PTD to generate recombinant seed baculoviruses. Seventy-two hours after infection, the Sf9 cells were washed once with PBS and suspended in sonication buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 10% Triton X-100. After sonication, cell lysates were centrifuged at 15,000 rpm, at 4°C for 30 min. The supernatants were collected and dialyzed with sonication buffer. Recombinant polypeptides were purified using GSTrap (GE Healthcare) according to the manufacturer's protocol. In brief, after the column was equilibrated with 2 ml sonication buffer, the cell lysate was applied and the column then washed three times with 10 ml PBST (PBS containing 0.5% Triton X-100) and with PBS once. Recombinant polypeptide (GST or GST-N′-CARD-PTD) was eluted with sonication buffer containing 10 mM reduced glutathione and then dialyzed with PBS. Recombinant proteins (1 μg) used in all the experiments contained <20 fg endotoxins (*Limulus* J Single Test, Wako).

ELISA and RT-PCR

Bone marrow-derived dendritic cells (DCs) were generated by 5 days of culture with GM-CSF (20 ng/ml) (GM-DCs) or Flt3L (100 ng/ml) (FL-DCs). GM-DCs or FL-DCs were treated with or without 1, 3, or 10 μg/ml N′-CARD-PTD or 1 μM of CpG ODN for 24 h and the supernatants were subjected to ELISA for mouse IFN-α, IFN-β (PBL Biomedical Laboratories), or IL-12 p40 (Invitrogen). RAW264.7 cells were treated with 1 μg/ml LPS or 10 μg/ml N′-CARD-PTD for 3, 6, 12, 18, 24, and 48 h. The levels of mRNA for TNF-α, IL-6, IFN-α, IFN-β, IP-10, and β-actin were examined by RT-PCR as described previously (5, 22).

Immunization

Eight-week-old female BALB/c mice were administered s.c. with N′-CARD-PTD (5 μg), CpG ODN (5 μg), or flu vax (0.7 μg) alone, flu vax (0.7 μg) plus N′-CARD-PTD (5 μg), or flu vax (0.7 μg) plus CpG ODN (5 μg) at 0 and 10 days. Blood was drawn at 20 days and serum Ab titer was measured by ELISA as described previously (25). Alternatively, 8-wk-old female C57BL/6 mice were immunized with E7 peptide (E7, Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe, 3 μg), E7 plus N′-CARD-PTD (5 μg), or E7 plus CpG ODN (5 μg) at 0 and 2 wk. Splenocytes were harvested 2 wk after final immunization. The cells were incubated with 1 μg/ml E7 or NP peptide (Ala-Ser-Asn-Glu-Asn-Met-Glu-Thr-Met) for 18 h at 37°C. Total RNA was isolated and real-time PCR was performed as described previously (22).

Influenza challenge

Ten days after final immunization, mice were challenged intranasally with 2×10^4 pfu (8 LD_{50}) of influenza virus A/PR/8/34 (25). The body weights and mortality of the challenged mice were monitored for the next 14 days.

Tumor transplantation

Eight week-old C57BL/6 mice were administered subcutaneously with TC-1 (1×10^5 cells/mouse), a mouse lung carcinoma expressing E7 Ag (25). Mice were immunized with control NP peptide (3 μg), E7 (3 μg), N′-CARD-PTD (5 μg), or E7 (3 μg) plus N′-CARD-PTD (5 μg) at 3, 4, 5, 6, and 7 day after TC-1 inoculation. The sizes of local tumor mass were monitored for the next 20 days.

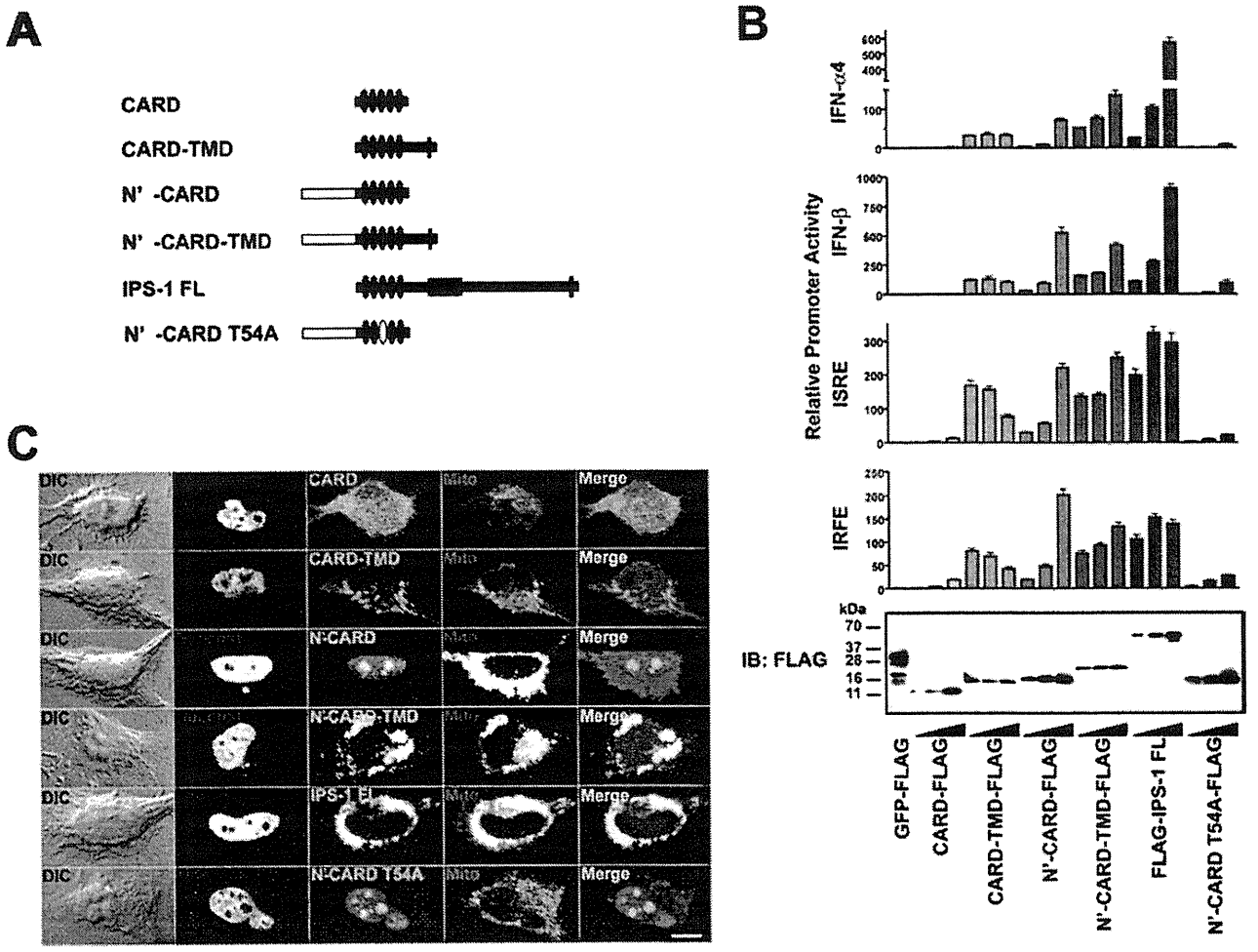

FIGURE 1. Synthetic IPS-1 CARD fusion molecules induce activation of type I IFN-related promoters. *A*, Schematic diagram of synthetic fusion molecules consisting of domains derived from IPS-1 and histone H2B. *B*, HEK293 cells were transfected with the expression plasmids, GFP-FLAG, CARD-FLAG, CARD-TMD-FLAG, N′-CARD-FLAG, N′-CARD-TMD-FLAG, FLAG-IPS-1 FL, and N′-CARD T54A-FLAG in the presence of TK-RL plus a reporter plasmid expressing firefly luciferase under the control of either the IFN-α4 promoter (*top panel*), the IFN-β promoter (*second panel from the top*), the ISRE-dependent promoter (*third panel from the top*), or the IRFE-dependent promoter (*fourth panel from the top*). Data represent means ± SD of the relative luciferase activity of six samples. Cell lysates were also subjected to immunoblot analysis to examine levels of target polypeptide expression (*bottom panel*). *C*, HeLa cells were transfected with the expression plasmids, YFP-CARD, YFP-CARD-TMD, YFP-N′-CARD, YFP-N′-CARD-TMD, YFP-IPS-1 FL, and YFP-N′-CARD T54A. Genomic DNA or mitochondria were stained with Hoechst 33258 or Mitotracker reagent, respectively, and then analyzed under a confocal microscope. The data represent one of three independent experiments with similar results. Scale bar, 10 μm.

Statistical analysis

The Student's *t* test or the Mantel-Cox log rank test was used for statistical analysis.

Results

The nuclear redistribution of IPS-1 CARD elicits type I IFN promoter activation

To elucidate the mechanisms underlying IPS-1 CARD-mediated signaling, plasmids encoding either the IPS-1 CARD alone or the IPS CARD fused to the IPS-1 TMD or to the NLS of histone H2B were generated and their abilities to induce type I IFN-related promoter activation were characterized (Fig. 1*A*). Although the CARD alone had minimal activity in eliciting such promoter activation, fusion of the TMD to the CARD (CARD-TMD) resulted in a significant activation, suggesting that the TMD facilitates CARD-mediated signaling, consistent with previous data (Fig. 1*B*; Ref. 26). Of interest, fusion of the NH_2-terminal NLS of histone H2B to the IPS-1 CARD (N′-CARD) conferred strong promoter activation, suggesting that nuclear localization of N′-CARD triggers signal activation. Indeed, N′-CARD induced phosphorylation of IRF3 at a comparable level to full length IPS-1 (FL) (Supplemental Fig. 1).[5] The mutant polypeptide N′-CARD T54A, in which the third α-helical structure of the CARD was disrupted (22), induced significantly lower levels of promoter activation, suggesting that the conformation of the IPS-1 CARD is also critical for its activity. Although N′-CARD fused to the IPS-1 TMD (N′-CARD-TMD) induced significant levels of promoter activation, the levels were comparable to those induced by N′-CARD or CARD-TMD, suggesting that the effects of CARD distribution mediated by the NLS and the IPS-1 TMD are redundant.

N′-CARD localizes to the nucleus and signals through NDH

To elucidate the signaling mechanisms triggered by N′-CARD and CARD-TMD, we examined the subcellular localizations of these fusion molecules. Confocal microscopy analysis showed that CARD-TMD fused to YFP (YFP-CARD-TMD) was present in

[5] The online version of this article contains supplemental information.

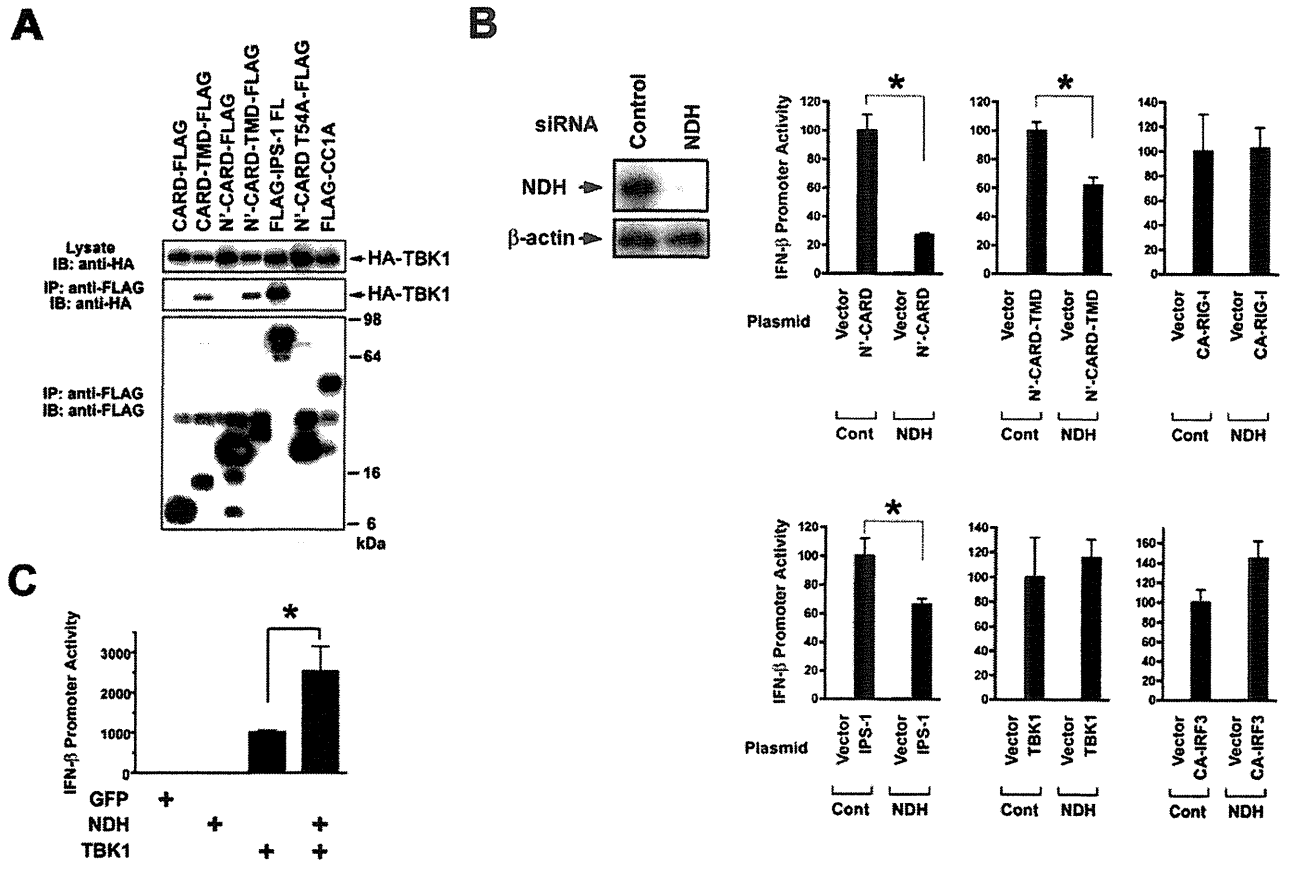

FIGURE 2. Role of NDH in N′-CARD-mediated signaling. *A*, Cell lysates from HEK293 cells transfected with the expression plasmids for HA-TBK1 plus CARD-FLAG, CARD-TMD-FLAG, N′-CARD-FLAG, N′-CARD-TMD-FLAG, FLAG-IPS-1 FL, N′-CARD T54A-FLAG, or FLAG-CC1A were prepared and immunoprecipitated with anti-FLAG Ab. The immune complexes were analyzed by immunoblotting using anti-HA or anti-FLAG Ab. *B*, After HEK293 cells were transfected with control or NDH siRNA, the levels of NDH protein were examined by immunoblotting. The cells were further transfected with the expression plasmid for N′-CARD, N′-CARD-TMD, CA-RIG-I, IPS-1, TBK1, and CA-IRF3 in the presence of TK-RL plus a reporter plasmid expressing firefly luciferase under the control of the IFN-β promoter. *C*, HEK293 cells were transfected with the expression plasmid(s) for GFP, NDH, and/or TBK1 in the presence of TK-RL plus a reporter plasmid expressing firefly luciferase under the control of the IFN-β promoter. *B* and *C*, Forty eight hours after transfection, luciferase assay was performed. Data represent means ± SD of the relative luciferase activity of eight samples. *, $p < 0.05$.

mitochondria, with a localization pattern similar to that of IPS-1 FL (YFP-IPS-1 FL), while N′-CARD fused to YFP (YFP-N′-CARD) was mostly present in the nuclear interchromosomal space (Fig. 1*C*). Because CARD alone (YFP-CARD) was present diffusely within the cell and both YFP-N′-CARD and YFP-N′-CARD T54A localized to the nucleus, it was suggested that the NLS directed the nuclear distribution of the IPS-1 CARD (Fig. 1*C*). These results implied that N′-CARD triggers cellular signaling pathways that originate in the nucleus and that are distinct from those triggered by CARD-TMD or IPS-1 FL, which originate from mitochondria.

TBK1, and its closely related IKK family member IKKi, are kinases acting downstream of IPS-1 and are required for a type I IFN production (21, 26, 27). We next examined the molecular interactions between each CARD-fusion molecule and TBK1 by immunoprecipitation analysis. As a control, TBK1 was coprecipitated with IPS-1 FL (Fig. 2*A*). A significant amount of TBK1 was also detected after precipitation with CARD-TMD or N′-CARD-TMD, but not after precipitation with CARD, N′-CARD, or N′-CARD T54A, suggesting that the TMD supports the association of the CARD with TBK1 (Fig. 2*A*).

To examine the signaling mechanisms triggered by N′-CARD, we tried to identify cellular molecules that associate with N′-CARD using a tandem-affinity purification system and TOF-MS analysis (data not shown). Among the N′-CARD interacting molecules identified, we were particularly interested in nuclear DNA helicase II (NDH, also known as RNA helicase A), a 1270 amino acid protein containing two copies of a dsRNA binding domain, a DEIH (Asp-Glu-Ile-His) helicase core, and an RGG (Arg-Gly-Gly) box nucleic acid-binding domain.

To examine the functional role of NDH in the signaling pathway leading to type I IFN production, NDH mRNA was ablated by RNA interference. Endogenous NDH protein was specifically decreased by NDH siRNA but not by control siRNA treatment (Fig. 2*B*). Knockdown of NDH resulted in a suppression of N′-CARD-induced IFN-β promoter activation by 73%. The level of promoter activation induced by IPS-1 or N′-CARD-TMD was also partially suppressed in NDH-knockdown cells by 33 and 38%, respectively. The levels were comparable when a constitutively active form of RIG-I (RIG-I 2CARDs), TBK1, or a constitutively active form of IRF3 (IRF3CA) was examined (Fig. 2*B*). Although over-expression of NDH had no effect, and over-expression of TBK-1 had a minimal effect on IFN-β promoter activation, over-expression of NDH plus TBK1 synergistically activated the IFN-β promoter, suggesting that NDH had the ability to up-regulate TBK1 activity (Fig. 2*C*). These results, taken together, suggest that NDH is involved in the events downstream of N′-CARD, and partially in

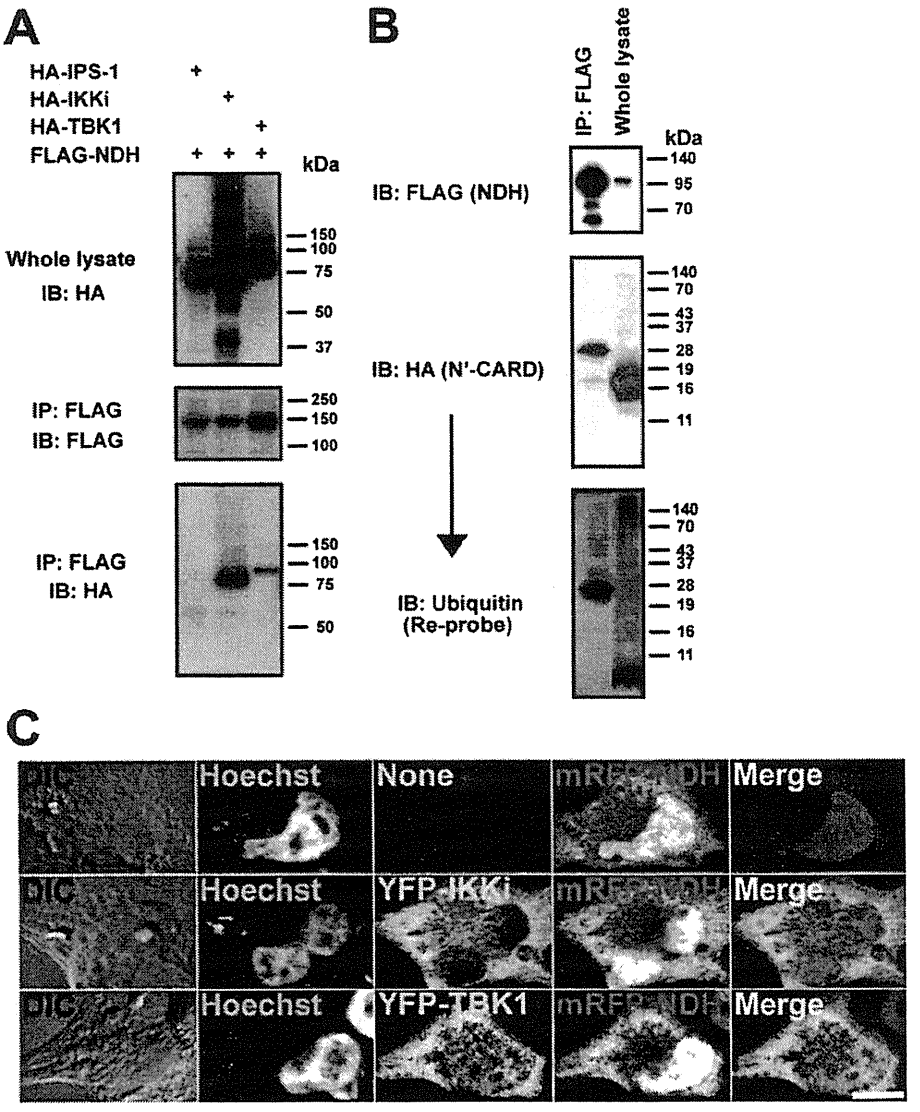

FIGURE 3. NDH associates with N′-CARD, TBK1, and IKKi. *A* and *B*, The lysates of HEK293 cells transfected with the expression plasmids for FLAG-NDH plus HA-IPS-1, HA-IKKi, HA-TBK1 (*A*) or N′-CARD-HA (*B*) were prepared and immunoprecipitated with anti-FLAG Ab. The immunoblots were probed with anti-HA or anti-FLAG Ab (*A* and *B*) or sequentially probed with anti-HA and anti-ubiquitin Ab (*B*). *C*, HeLa cells were transfected with an expression plasmid for mRFP-NDH alone or with those for mRFP-NDH and YFP-IKKi or YFP-TBK1. After staining with Hoechst 33258, the cells were examined under a confocal microscope. Data represent one of three independent experiments with similar results. Scale bar, 10 μm.

those downstream of IPS-1, and that it plays a role in signaling upstream of TBK1.

To confirm the physical interactions among NDH, IKKi, TBK1, and N′-CARD, immunoprecipitation analysis was performed. A strong interaction was detected between NDH and IKKi or TBK1, while there was no apparent association of NDH with IPS-1 in this assay (Fig. 3*A*). By contrast, NDH was confirmed to interact with N′-CARD. Of interest, the mobility of N′-CARD coprecipitated with NDH was retarded in SDS-PAGE (~25 kDa) when compared with that in whole cell lysate (~18 kDa) (Fig. 3*B*). The retarded N′-CARD was detected by anti-ubiquitin Ab, suggesting that mono-ubiquitinated N′-CARD, directly or indirectly, has the ability to associate with NDH (Fig. 3*B*). We also examined the subcellular localization of NDH, IKKi, and TBK1 by confocal microscopy analysis (Fig. 3*C*). Both YFP-IKKi and YFP-TBK1 were mostly present in the cytoplasm, while mRFP-NDH was diffusely present within the cell. Most NDH present within the cytoplasm colocalized with IKKi or TBK1 (Fig. 3*C*).

Recombinant N′-CARD polypeptide fused to the protein transduction domain (N′-CARD-PTD) induces type I IFN production and exerts innate immune responses in vitro

To examine the potent ability of N′-CARD in modulating innate immune responses, we generated a recombinant N′-CARD polypeptide fused to the PTD, which enables transduction of extracellular protein into intracellular compartments. When the N′-CARD-PTD polypeptide was added to the culture medium of HeLa cells, it entered the nucleus within 30 min (Fig. 4*A*). By contrast, when the same amount of CARD polypeptide was added, only a minimal level of the polypeptide was observed inside the cell (Fig. 4*A*). The addition of the N′-CARD-PTD polypeptide alone induced significant levels of IFN-β promoter activation in HEK293 cells, suggesting that N′-CARD-PTD has the ability to transmigrate into the cell and trigger NDH-mediated cellular signaling to elicit type I IFN production (Supplemental Fig. 2).

We next examined whether administration of the N′-CARD-PTD polypeptide activates immune cells in vitro. As shown in Fig. 4*B*, N′-CARD-PTD induced production of a proinflammatory cytokine (TNF-α), type I IFNs (IFN-α and -β), and an IFN-stimulated gene product (IP-10) in a mouse macrophage cell line, RAW264.7. The expression of IFN-α and -β mRNAs was detected within 18 h; the expression of IFN-α mRNA continued for more than 48 h after N′-CARD-PTD treatment. By contrast, LPS, an activator of TLR4-mediated innate immune responses, induced IFN-β mRNA within 3 h, but this induction lasted for less than 18 h. The overall level of IFN-α mRNA production was higher in cells stimulated with N′-CARD-PTD compared with those stimulated with LPS, while that of IFN-β was lower in those stimulated

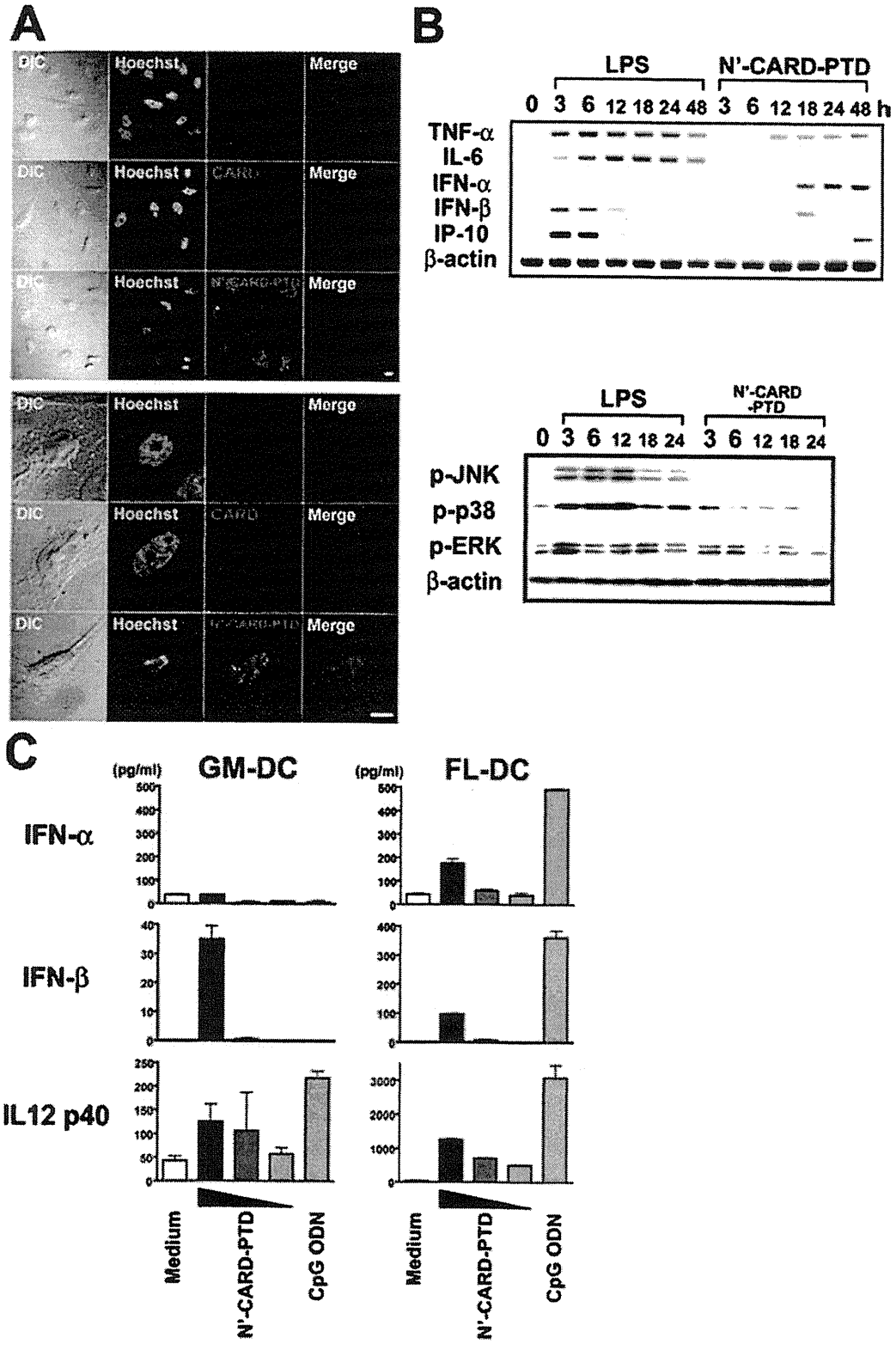

FIGURE 4. The N′-CARD-PTD polypeptide induces type I IFN production and DC maturation. *A*, Recombinant CARD or N′-CARD-PTD polypeptide was administered into the culture medium of HeLa cells. Thirty minutes after addition, the cells were permeabilized, stained with anti-FLAG M2-Cy3 and Hoechst 33258, and subjected to confocal microscopy analysis. *Upper panel*, Lower magnification. *Lower panel*, Higher magnification. Scale bar, 10 μm. *B*, RAW264.7 cells were treated with 1 μg/ml LPS or 10 μg/ml N′-CARD-PTD for 3, 6, 12, 18, 24, and 48 h. The levels of mRNA expression for TNF-α, IL-6, IFN-α, IFN-β, IP-10 and β-actin were examined by RT-PCR (*upper panel*). The levels of phosphorylated JNK, p38, or ERK were examined by immunoblotting (*lower panel*). *C*, GM-DCs or FL-DCs were treated with or without 1, 3, or 10 μg/ml N′-CARD-PTD or 1 μM of CpG ODN for 24 h and the supernatants were subjected to ELISA for mouse IFN-α, IFN-β, or IL-12 p40. Data represent one of two or three independent experiments with similar results.