Establishment of an *In Vitro* Model using NR8383 Cells and *Mycobacterium Bovis* Calmette-Guérin that Mimics a Chronic Infection of *Mycobacterium Tuberculosis*

MAMI HINO[1], MUTSUMI ODA[1], AYA YOSHIDA[2], KAZUE NAKATA[1],
CHIE KOHCHI[1,3], TAKASHI NISHIZAWA[1], HIROYUKI INAGAWA[1,3,4],
HITOSHI HORI[2], KIMIKO MAKINO[3], HIROSHI TERADA[3] and GEN-ICHIRO SOMA[1,3]

[1]*Institute for Health Sciences, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima-shi, 770-8514;*
[2]*Faculty of Engineering, The University of Tokushima, Minamijyousanjima-cho, Tokushima-shi, 770-8506;*
[3]*Institute for Drug Delivery Systems, Tokyo University of Science, Yamazaki, Noda-shi, 278-8510;*
[4]*Department of Applied Aquabiology, National Fisheries University, Nagatahon-machi,
Shimonoseki-shi, 759-6595, Japan*

Abstract. *Background: Mycobacterium tuberculosis infection affects one-third of the world's population and causes the death of three million people each year. To clarify details of M. tuberculosis survival strategies, it is important to establish a suitable in vitro model that mimics a chronic infection in alveolar macrophages by M. tuberculosis. For this reason, we established a new in vitro model using a rat alveolar macrophage cell line, NR8383. Materials and Methods: Basic characteristics, including phagocytotic ability and production of nitrogen oxide and tumor necrosis factor in response to several stimuli, of NR8383 cells were compared with those of primary alveolar macrophages. The course after phagocytosis of live or killed M. bovis bacilli Calmette-Guérin (BCG) was examined over 21days using NR8383 cells as the host. Results: The characteristics that have been examined to date were nearly the same for both primary alveolar macrophage and NR8383 cells. Live BCG phagocytosed by NR8383 cells had successfully begun to grow in the cells within 7 days, while killed BCG were almost completely destroyed by 21 days. Conclusion: BCG-infected NR8383 cells are potentially a suitable in vitro model that mimics a chronic infection with M. tuberculosis.*

Tuberculosis is one of the most serious infectious diseases designated by the WHO. The bacillus of tuberculosis,

Correspondence to: Gen-Ichiro Soma, Institute for Health Sciences, Tokushima Bunri University, Nishihama, Yamashiro-cho, Tokushima-shi, 770-8514, Japan. Tel: +81-88-622-9611, Fax: +81-88-6223217, e-mail: sma5628@tokushima.bunri-u.ac.jp

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Mycobacterium tuberculosis, is an intracellular pathogen that can survive in host macrophages. Tuberculosis is an old disease and its clinicopathological features are well documented (1). *M. tuberculosis* is inhaled and then invades the lung, where it is phagocytosed by alveolar macrophages (AMs). *M. tuberculosis* escapes the digestive action by AMs and survives in phagosomes until the macrophages die through necrosis or apoptosis. *M. tuberculosis* then repeats the infectious cycle, which leads to consecutive infection. Thus, survival in macrophages is a prerequisite for *M. tuberculosis* to develop tuberculosis. Therefore, it is important to understand the mechanism by which *M. tuberculosis* manages to reside in the phagosomes of macrophages. There are at least two different steps involved in the strategy of *M. tuberculosis* that accounts for its intracellular survival (1). An early step occurs within 24 h after phagocytosis of *M. tuberculosis* and is responsible for the prevention of bactericidal phagolysosome formation. The stage that occurs over the next few days after phagocytosis results in the stable residence of *M. tuberculosis* in the phagosomes. It is not yet clear what mechanisms are involved in each stage and whether or not these mechanisms are independently regulated.

In recent years, there has been a good deal of research that has clarified the intracellular survival strategies of *M. tuberculosis,* especially during the early stage of phagocytosis. Toll-like receptor-2 and -4 (2-5), tumor necrosis factor (TNF) (6, 7), interleukin-12 (IL-12) (8) and interferon-gamma (9) were reported as host factors relevant to the survival of *M. tuberculosis*. Interference with signal transduction has been reported; *M. tuberculosis* inhibited activation of calmodulin and Ca^{2+}/calmodulin kinase II, which are key molecules responsible for forming

bactericidal phagolysosomes (10, 11). Although these results indicate that host factors may be involved in the strategy of *M. tuberculosis* for intracellular survival, this research focused almost entirely on the early stage after phagocytosis. By contrast, little work has been done on the host factors that assures survival of *M. tuberculosis* after phagocytosis. Most of the serious problems caused by tuberculosis are ascribed to the survival of *M. tuberculosis* in host cells and the subsequent chronic infection. To understand the molecular basis of the patho-physiology of *M. tuberculosis*, it is essential to study host factors relevant to the later stages of the infection. This type of study has not been possible because there has not been a suitable *in vitro* experimental system mimicking this stage. Ideally, this type of system should provide certain conditions. Most importantly, the host macrophages that are being tested should also be actual targets of *M. tuberculosis*, namely AMs. Secondly, the test bacteria must survive within the macrophages without loss of viability for more than 7 days. This is because it takes 3-7 days or more for settlement of chronic *M. tuberculosis* infection, according to observations in an *in vivo* model (12).

Monocyte cell lines have often been used as the host when testing for candidate molecules and/or mechanisms relevant to the intracellular survival strategy of *M. tuberculosis*. This is mainly because monocytes are easier to handle than AMs, both for preparation and for long-term cultivation, *in vitro* (13-16). However, there are reports of differences even between different monocyte cell lines. For example, binding of zymosan by THP-1 cells was significantly lower than that by monocyte-derived macrophages (17). In addition, it has been shown that AMs and monocyte cell lines have different cellular responses against *M. tuberculosis*. Hirsch *et al.* reported that *M. tuberculosis* uptake by AMs was mainly mediated through complement receptor 4 (CR4), whereas CR1 and CR3 were dominantly used in monocytes. Also, AMs were more efficient in phagocytosis of *M. tuberculosis* than monocytes (18). Moreover, they showed that *M. tuberculosis*-infected AMs produced higher amounts of TNF than monocytes. Cellular signaling by TNF was mediated through activation of NF-ÎB (19) and MAP-kinases (20). Surewicz *et al.* demonstrated that, when compared with monocytes, p38 MAP kinase was more rapidly induced in AMs by phagocytosis of *M. tuberculosis* (21). Finally, multiplication of *M. tuberculosis* was impaired within phagosomes of J774.1, a mouse monocyte-derived cell line, but not in AMs (22). This also suggests that monocyte cell lines might be inappropriate for use in the study of the strategies of *M. tuberculosis* for intracellular survival, especially in the later stages after phagocytosis. Thus, when studying *M. tuberculosis* infection, it is desirable to choose AMs as the host macrophages.
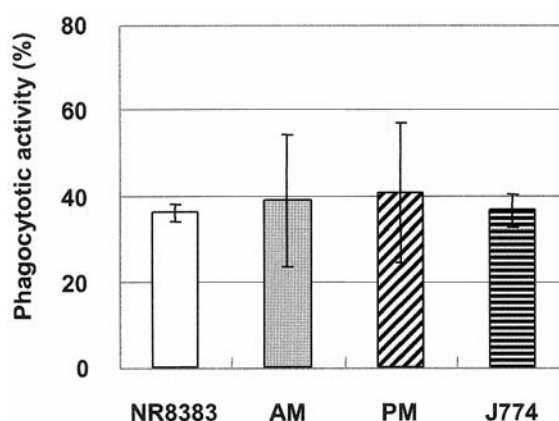

Figure 1. *Phagocytotic ability of NR8383 cells, AMs, PMs and J774.1 cells. FITC-latex beads (2 μM) were added to NR8383 cells, AMs, PMs and J774.1 cells at a ratio of beads to macrophages of 10:1 and incubated for 1 h.*

However, there are several problems with using primary AMs as host cells: i) It is difficult to obtain a sufficient number of cells for multiple experiments. ii) The stages of differentiation of AMs isolated from alveoli are quite heterogeneous, leading to scattered results. iii) The viability of AM cells decreases during cultivation, so they are not suitable for long-term observations of *M. tuberculosis* after phagocytosis. Consequently, established cell lines of AMs would be preferred.

In this research, an *in vitro* model mimicking the later stages after phagocytosis of bacilli (including *M. tuberculosis*) was first established enabling the analysis of the host factors and mechanisms relevant to survival strategies of tubercle bacilli. A comparison of the biological characteristics of NR8383 cells with those of primary AMs appeared to be the same with respect to phagocytosis, and nitric oxide (NO) and TNF production after stimulation by lipopolysaccharide (LPS) or *M. bovis* bacillus Calmette-Guérin (BCG). BCG successfully multiplied in NR8383 cells without the loss of viability for 21 days after phagocytosis. These results demonstrated that this model satisfied the essential requirements for the intracellular survival strategies of tubercle bacilli, especially in the later stages after phagocytosis.

Materials and Methods

Isolation of primary AM and PM. Rat alveolar macrophages (AMs) were recovered from male Sprague-Dawley (SD) rats by intratracheal lavage. The rats were anesthetized with intraperitoneal injection of Nembutal (Dainippon Pharmaceutical Corporation, Osaka, Japan) and completely exsanguinated. The trachea was exposed and cannulated by Teflon sonde. The lung was lavaged using intratracheal instillation of 5 ml of sterile saline. The
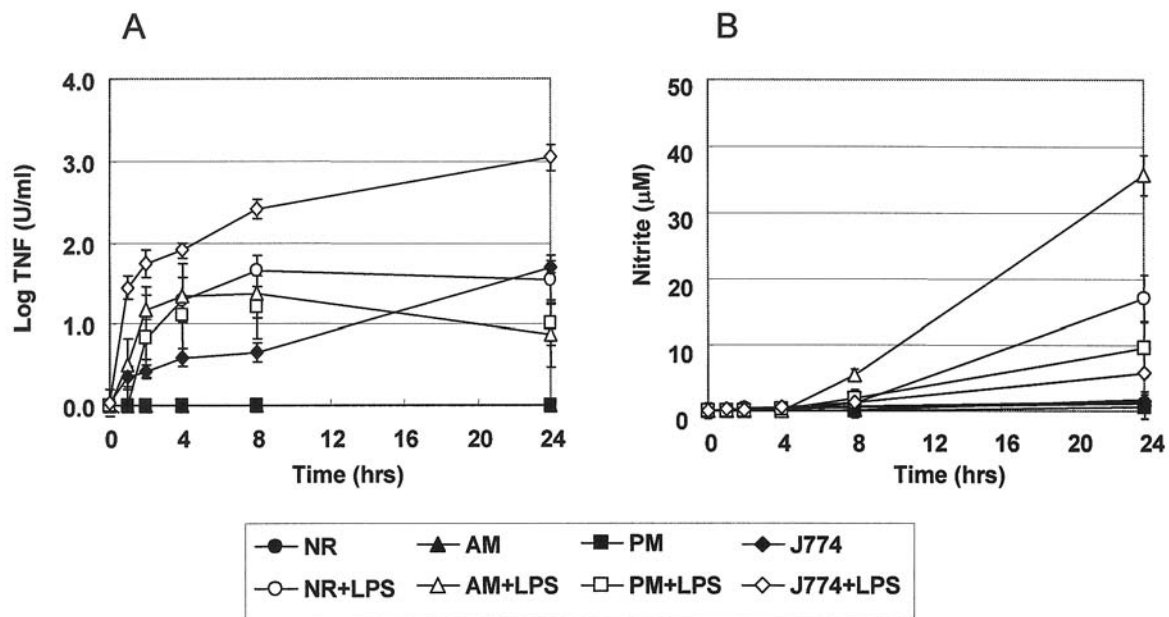
Figure 2. *TNF and NO production by NR8383 cells, AMs, PMs and J774.1 cells after stimulation with LPS. 5x10⁴ cells of NR8383, AM, PM and J774.1 were treated with 1 µg/ml of LPS for 1, 2, 4, 8 and 24 h. (A):TNF activity in the culture medium was measured by cytotoxicity to L929 cells. (B): NO concentration was measured by Griess reagent.*

lavage procedure was repeated 3 times. AMs were isolated from the lung lavage fluid, and pelleted by centrifugation (400 x g, 10 min). After centrifugation, the cells were resuspended in RPMI 1640 medium (Sigma Aldrich Japan K.K., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, Utah, USA) and 100 µg/ml ampicillin (Wako, Osaka, Japan), and counted using a hemocytometer. The isolated cells were mixed with medium and incubated at 37˚C in a 5% CO_2 incubator overnight.

Rat peritoneal macrophages (PMs) were recovered from male SD rats by intraperitoneal lavage. Rats were intraperitoneally injected with 8 ml of 4.05 % (w/v) of thioglycolate medium (Nissui, Tokyo, Japan); 4 days after thioglycolate injection, the rats were anesthetized by subcutaneous injection of Nembutal and intraperitoneally administered 10 ml of sterile saline. Peritoneal cells were collected from the peritoneal cavity. The recovery procedure was repeated 3 times. Peritoneal cells were pelleted by centrifugation (400 x g, 10 min.) and were resuspended in medium. The isolated cells were counted using a hemocytometer, diluted with medium and seeded on a dish, followed by incubation at 37˚C in a 5% CO_2 atmosphere overnight. PMs were isolated as adherent cells on the dish after washing with warm phosphate-buffered saline (PBS) to remove non-adherent cells.

Culture of NR8383 and J774.1. NR8383 is a well-characterized, semi-adherent macrophage cell line that is derived from normal Sprague-Dawley (SD) rat lung (23). The NR8383 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA; ATCC No. 2192) and was maintained in Ham F-12 medium (Invitrogen, Tokyo, Japan) supplemented with 15% FCS (Invitrogen) and 60 µg/ml ampicillin at 37˚C in a 5% CO_2 atmosphere. J774.1, a murine monocyte-macrophage line derived

from BALB/c mouse (Japanese Collection of Research Bioresources [JCRB] 0434), was grown in RPMI 1640 medium supplemented with 10% FCS and 100 µg/ml ampicillin at 37˚C in a 5% CO_2 atmosphere. Cell viability was determined by the trypan blue exclusion method.

Phagocytosis assay. NR8383 cells, AMs, PMs and J774.1 cells were plated in 48-well plates at a density of 5x10⁴ cells/well. After incubation in medium for 2 h at 37˚C in a CO_2 incubator, the cells were exposed with fluorescein isothiocyanate (FITC) latex beads (Polysciences, Inc. 18338, average diameter 2 µm) at a ratio of 1:10 (cells: latex beads) and incubated for 1 h at 37˚C. To remove free beads and surface-bound beads, the cells were transferred to 1.5-ml tubes and centrifuged at 250 x g for 5 min. Pelleted cells were resuspened with 0.25% trypsin in PBS at room temperature for 5 min. After washing twice with PBS, 100 cells were counted with a microscope to distinguish phagocytosing cells from non-phagocytosing cells.

Macrophage stimulation. NR8383 cells, AMs, PMs and J774.1 cells were seeded in 96-well plates at a density of 1x10⁴ cells/well. Each type of macrophage was treated with 1 µg/ml of LPS (*Escherichia coli* 0111: B4, List Biological Laboratories, CA, USA). After specified time-periods (between 1 and 24 h) of incubation, the supernatants were collected and TNF and NO were assayed. Macrophages were also treated with zymosan (zymosan A; Sigma) at a ratio of cells to zymosan of 1:10 and incubated for 4 h at 37˚C. The cells were transferred to 1.5-ml tubes and centrifuged at 250x g for 5 min. Pelleted cells were washed twice with PBS and resuspended in cell culture medium. After incubation for 24 h, the supernatants were collected and TNF and NO were assayed.
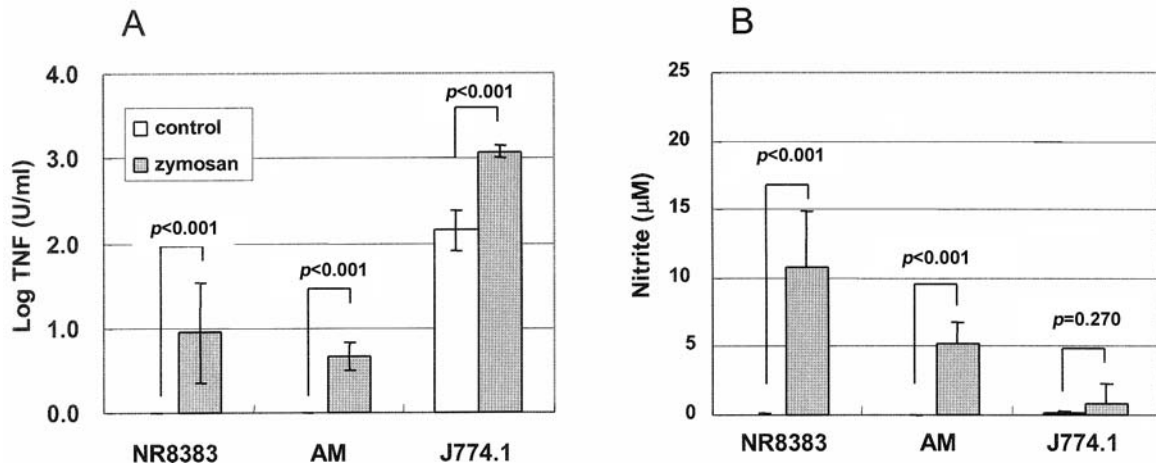
Figure 3. *TNF and NO production by NR8383 cells, AMs and J774.1 cells after phagocytosis of zymosan. $4x10^5$ cells of NR8383, AM and J774.1 were made to phagocytose zymosan for 4 h. After phagocytosis, each type of cell was washed and then incubated for 24 h. The supernatants were then assayed for TNF and NO. (A):TNF activity in the culture medium was measured by cytotoxicity to L929 cells. (B): NO concentration was measured by Griess reagent. Data are expressed as means ± SD of at least two independent experiments, each conducted in duplicate.*
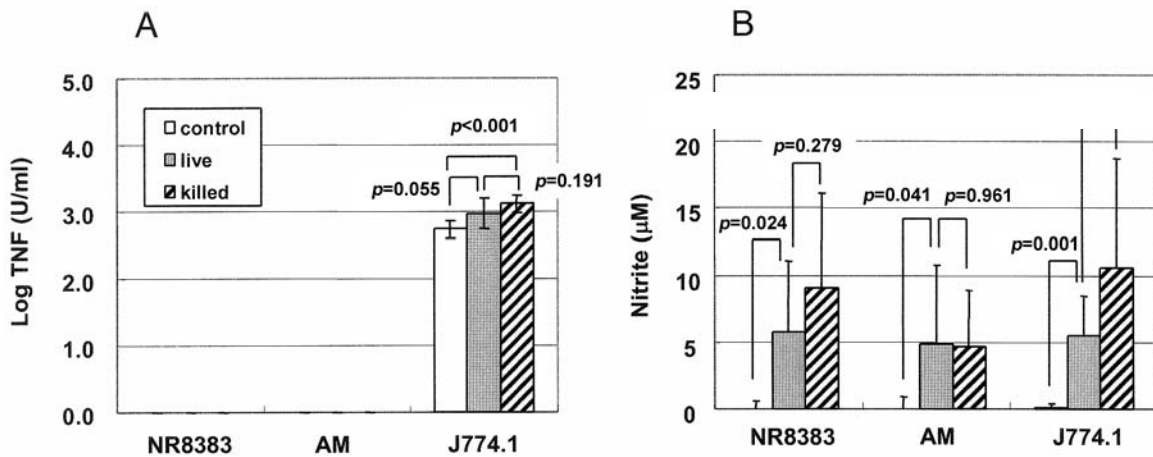

Figure 4. *TNF and NO production by NR8383 cells, AMs and J774.1 cells after phagocytosis of live and killed BCG. NR8383 cells, AMs and J774.1 cells were made to phagocytose live and killed BCG for 4 h. The ratio of bacteria to cells was 10:1. After phagocytosis, cells were washed and further cultured for 24 h. Supernatants were assayed for TNF and NO. (A):TNF activity in the culture medium was measured by cytotoxicity to L929 cells. (B): NO concentration was measured with Griess reagent. Data are expressed as means±SD of at least two independent experiments, each conducted in duplicate.*

BCG culture and infection. **M. bovis** bacillus Calmette-Guérin (BCG) (*M. bovis* BCG, Tokyo, Japan) was purchased from the Japan BCG Group and grown in MycoBroth (Kyokuto 06700) for 2 to 3 days at 37˚C without agitation. Single cell suspensions were prepared by dispersing with gentle shaking for 30 sec with glass beads (SIGMA G-8772, 425-600 microns), resuspended in PBS, and sonicated to disrupt small aggregates of bacteria. These supernatants were collected. The killed BCG were prepared by incubation in an 80˚C water bath for 40 min.

NR8383 cells, AMs and J774.1 cells were plated in 24-well plates at a density of 1x10⁶ cells/ml/well. After incubation in medium for 1 h at 37˚C in a CO_2 incubator, the cells were exposed to live or killed BCG at a ratio of 1:10 (cells: BCG) and incubated for 4 h at 37˚C. To remove free BCG following incubation, the cells were collected and centrifuged at 250 x g for 5 min. Pelleted cells were resuspended with PBS at room temperature. After washing twice with PBS, 100 cells were counted with a microscope to distinguish phagocytosing cells from those not phagocytosing. After incubation for 24 h, the supernatants were measured with TNF and NO assays.

Measurement of TNF activity. The TNF assay was done according to a method described previously (24). In our assay system, the minimum detection limit for TNF was 0.2 U/ml. Briefly, TNF activity was measured by cytotoxicity against L929 cells in the
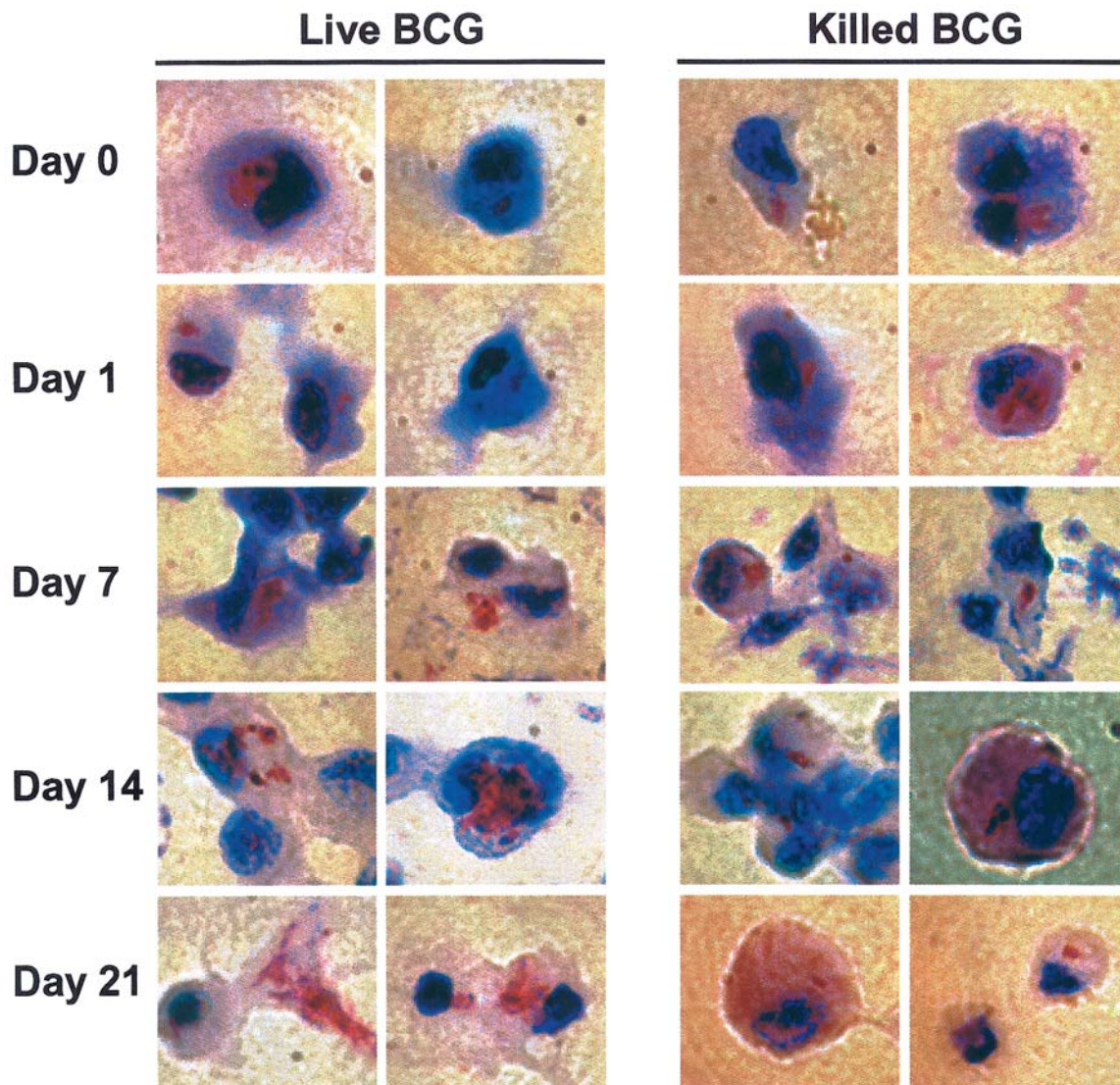
Figure 5. *Course of phagocytosed live and killed BCGs in NR8383 cells. NR8383 cells were made to phagocytose live and killed BCGs for 4 h. The ratio of bacteria to cells was 10:1. The NR8383 cells (blue) were stained with methylene blue, and BCGs (red) were stained with Ziehl Neelsen Carbol-Fuchsin. There are two representative micrographs for each condition at each time-point.*

presence of actinomycin D (1 μg/ml) (Sigma) and using recombinant human TNF-α (AsahiKasei, $2x10^6$ unit/mg protein) as the standard.

Measurement of NO production. Nitrite (NO_2^-), a stable end product of NO, was measured by a microplate assay method with Griess reagent (1% sulfanilamide/0.3% naphthylethylene diamine dihydrochloride/7.5% H_3PO_4) (25, 26). Briefly, culture supernatants (100 μl) were mixed with 100 μl of the Griess reagent, and the nitrite concentration in the culture supernatant was measured 10 min after mixing at an absorbance of 550 nm (BIO-RAD, Model 550, Tokyo, Japan). The nitrite concentration was determined with reference to the standard curve using sodium nitrite.

Results

Phagocytotic ability of NR8383 cells. Because the biological characteristics of macrophages differ depending on the tissues from which they are derived (27), AMs that are physiological target cells of *M. tuberculosis in vivo* should be selected as host cells. In this sense, a cell line, NR8383, which is the only available cell line derived from normal alveolar macrophages, seemed to be suitable as the host. Prior to using the NR8383 cells as the host, the basic characteristics of this cell line were examined in comparison

to those of primary rat AMs, PMs and J774.1 cells. First, we examined the relative phagocytotic abilities of each cell type. As shown in Figure 1, the percent of macrophages phagocytosing latex beads was 36.0% for NR8383 cells, 38.9% for AMs, 40.7% for PMs and 36.5% for J774.1 These results demonstrated that NR8383 cells had the same phagocytotic ability as normal macrophages.

TNF and NO production in NR8383 cells after stimulation with LPS. Besides their phagocytotic ability, the most prominent characteristic of macrophages is their response to LPS (though there are some exceptional anergy responses in intestinal macrophages) (28). Macrophages usually produce several cytokines, reactive oxygen species and nitrogen oxide species in response to LPS (29). However, the timing of these reactions is not necessarily the same for different tissue macrophages or macrophage cell lines (30, 31). It has been suggested that both TNF and NO may be involved during the establishment of the tubercle bacilli in host phagosomes. Thus, the characteristics of TNF or NO production could possibly affect cellular responses during the later stages after phagocytosis of the bacilli (1, 32). We then compared the timing of the production of TNF and NO by NR8383 cells with or without LPS stimulation and compared these values with those for AMs, PMs and J774.1 cells.

As shown in Figure 2A, production of TNF by NR8383 cells, AMs or PMs was not detectable unless there was stimulation by LPS. In contrast, J774.1 cells produced TNF 1 h after incubation. In the first three kinds of cells, TNF appeared in the medium 2 h after stimulation by LPS, and the amount produced reached almost the maximum level (22-48 U/ml) 8 h after incubation. There were no significant differences between NR8383 cells, AMs and PMs in the amounts or time-course of production of TNF in response to LPS. However, J774.1 cells, which have been widely used as host cells for tubercle bacilli, were different from the other three kinds of cells in respect to both the amount and time-course of production of TNF in response to LPS stimulation. In particular, J774.1 cells produced 100 times more TNF than the other types of cells (Figure 2A). In contrast, NR8383 cells produced TNF with and without LPS stimulation, in a way that is characteristic of normal primary rat AMs.

As shown in Figure 2B, without stimulation by LPS, only small amounts of NO were produced by all cell types examined. In contrast, 24 h after LPS stimulation, significant amounts of NO were produced by both AMs and NR8383, and these amounts were larger than for PMs or J774.1 cells. The amount of NO produced by AMs was higher than for NR8383 cells after LPS stimulation. The NO production by NR8383 cells was similar to that of AMs, especially after LPS stimulation. These results show that AMs and NR8383 cells were more responsive to LPS than PMs and J774.1 cells, suggesting again that NR8383 cells have similar characteristics to normal AMs.

TNF and NO production in NR8383 cells after phagocytosis of zymosan. Phagocytosis is an essential first step in assuring the intracellular survival of the tubercle bacilli. Also, phagocytosis later modulates cellular responses including cytokine and NO production. For this reason, we next compared the production of TNF and NO after phagocytosis of zymosan for both NR8383 cells and AMs. Zymosan particles are widely used to examine changes in cellular phagocytotic responses and J774.1 cells are used as the control. In this experiment, PMs were omitted because the main objective of the study was to reveal whether NR8383 cells could be used as target cells for tubercle bacilli instead of AMs. Tests were performed with NR8383 cells, AMs and J774.1 cells. The phagocytotic ratio of zymosan in the three types of cells was nearly the same, and over 90%. Without phagocytosis, the amount of TNF produced by NR8383 cells and AMs was under the detection limit, but the amount was significantly enhanced after phagocytosis of zymosan (Figure 3A). Significant enhancement of TNF production after phagocytosis was also demonstrated by J774.1 cells, though these cells produced TNF spontaneously even without phagocytosis. The amount of TNF produced after phagocytosis was not significantly different for NR8383 cells and AMs. However, TNF production by J774.1 cells was significantly higher than for NR8383 cells and AMs.

The pattern of NO production after phagocytosis of zymosan was the reverse of the TNF production pattern. Both NR8383 cells and AMs produced larger amounts of NO than did J774.1 cells (Figure 3B). No statistically significant NO production was observed by J774.1 cells after phagocytosis.

As with TNF, there were no significant differences in the amount of NO produced after phagocytosis between NR8383 cells and AMs. These observations demonstrate again that NR8383 is similar in character to AMs, and that both behave differently than J774.1 cells.

TNF and NO production in NR8383 cells after phagocytosis of BCG. Based on the similarity of NR8383 cells to AMs in phagocytotic ability, TNF and NO production, both after LPS stimulation and after phagocytosis of zymosan, NR8383 cells are thought to be a more suitable cell line for a model of AMs than are J774.1 cells.

The response of NR8383 cells to BCG was then tested. To test the early response after phagocytosis of BCG, TNF and NO production by NR8383 cells at 24 h after phagocytosis of BCG were measured and compared with values for AMs and J774.1 cells. About 20% of NR8383 cells and AMs phagocytized BCG, whereas only about 10% of the J774.1 cells did so.

As shown in Figure 4A, TNF production was not observed by AMs or NR8383 cells after phagocytosis of either live or killed BCG. Conversely, J774.1 produced high

amounts of TNF. However, the amount of TNF produced by J774.1 cells after phagocytosis of live or killed BCG was not significant when compared with the amount of TNF produced spontaneously without phagocytosis. Interestingly, the production of TNF by J774.1 cells after phagocytosis of killed BCG was significantly higher than that of the control. This was different from the cellular response with killed BCG that occurred with NR8383 cells and AMs.

The production of TNF by NR8383 cells and AMs after phagocytosis of BCG was different than after phagocytosis of zymosan. BCG, either killed or live, did not result in the production of TNF at the early stage after phagocytosis in either of these cell types (see Figures 3A and 4A).

As shown in Figure 4B, the amount of NO produced after phagocytosis of either killed or live BCG did not show significant differences for any of the cell types. These results demonstrated that NR8383 cells showed similar characteristics in terms of production of NO after phagocytosis of either killed or live BCG.

Dynamic state of BCG in NR8383 cells after infection. Since it was our intent to analyze host factors relevant to the survival of intracellular tubercle bacilli after phagocytosis, we tested whether NR8383 cells could survive for more than 7 days while containing live bacilli within the phagosomes, and whether there would be proliferation of live BCG or digestion of killed BCG.

Figure 5 shows the cytological observations of killed or live BCG in NR8383 cells after phagocytosis. In this experiment, the FCS concentration was adjusted to 5% to maintain the viability without proliferation of NR8383 cells. Seven days after phagocytosis, microscopic observations showed clear morphological differences between killed and live BCG. As shown in the left lane of Figure 5, proliferation of live BCG could be observed as soon as 7 days after phagocytosis and, on day 21, BCG growing outside of the NR8383 cells were also observed. Conversely, the right lane of Figure 5 shows that killed BCG were reduced 7 days after phagocytosis. In addition to the morphological differences between killed bacilli and live bacilli in NR8383 cells, there were also differences in the percentage of NR8383 cells containing the BCG bacilli. The percentage of NR8383 cells containing either live or killed BCG on day 0 were 17% and 20%, respectively. This changed to 24% and 6%, respectively, on day 21.

When using J774.1 cells as the host for BCG, the cells tended to go into apoptosis within 7 days of incubation. Thus, comparative analyses of cellular factors involved in the later stages after phagocytosis using J774.1 cells that had phagocytosed either killed or live bacillus were not possible (data not shown). Also, primary AMs could not live 7 days even without BCG phagocytosis.

In this set of tests, it was demonstrated that NR8383 cells phagocytosed both killed or live BCG and remained viable.

These properties allowed us to conduct experiments with BCG that were potentially relevant to understanding the host factors involved in the intracellular survival of *M. tuberculosis,* both during the early and later stages after phagocytosis of the bacilli.

Discussion

A model that mimics the later stages after phagocytosis of bacilli, hereafter called the chronic model, is indispensable for studying the survival strategies of *M. tuberculosis* while in the phagosomes of host macrophages. A chronic model must satisfy a number of criteria. It is well known that certain properties of macrophages vary greatly, depending on the tissue from which they were derived (27). It appears that the macrophages used as the target cells for bacilli should be AMs or cells having similar biological responses after the phagocytosis of bacilli. Also, the cells must allow the survival of bacilli without loss of the own viability for at least 7 days, as it appears that it takes this long for the bacilli to be established in the *in vivo* model. In this study, we were successful in establishing a chronic model with these properties using NR8383 cells as the host, which is the only cell line derived from normal AMs.

The NR8383 cell line was established from AM cells recovered from the fluid from lung lavage of the SD rat (23). Helmke *et al.* reported that NR8383 cells preserved the characteristics of macrophages well in terms of phagocytosis, nonspecific esterase activity, expression of Fc receptors, oxidative burst, IL-1 secretion and response to exogenous growth factor. It was thus claimed that the cell line could serve as an excellent *in vitro* model for studying the biological activity of macrophages (33). In this report, we further examined the biological characteristics of NR8383 cells and focused on their similarity to AMs. One biological difference between macrophages from different tissues is their response to bacterial LPS. For example, intestinal macrophages were reported to be unresponsive to LPS, which is markedly different from the response from other macrophages (28). In addition, it is known that the level of response to LPS is different among tissue macrophages, *i.e.* AMs produce abundant TNF after stimulation with LPS in contrast to the response by monocytes or PMs (31, 34). Therefore, the type of response and the degree of responsiveness to LPS were thought to be good indices for evaluating the biological similarity of NR8383 cells and AMs. As shown in Figure 2A and B, AMs freshly recovered from alveoli produced nearly the same amount of TNF, but a significantly higher amount of NO than NR8383 cells after stimulation with LPS. However, as the time-course for the production of both substances was nearly the same, this suggests that the kinetics of activation of NF-ÎB after stimulation with LPS might be parallel in both NR8383 cells and AMs.

Phagocytosis is known to activate macrophages to secrete TNF and NO through activation of cellular signals *via* receptors such as Fc receptors and complement receptor, that is different from those activated by LPS (35, 36). Thus, the responses of cells after phagocytosis can be used as another index for evaluating the similarity of NR8383 cells to AMs. As shown in Figure 3A and B, phagocytosis of zymosan induced NO and TNF production both by NR8383 cells and by AMs. Conversely, J774.1 cells (a monocytic leukemia cell line that has been widely used as the host for studying early cellular responses to infection with *M. tuberculosis*) produced a significant amount of TNF but lesser amounts of NO after phagocytosis of zymosan. These results again indicated that NR8383 cells preserved the same biological characteristics as AMs, and that these characteristics were quite different from those of monocytic leukemia cell lines.

As NR8383 cells appeared to have the same characteristics in terms of production of TNF and NO after different stimuli, the biological characteristics of NR8383 were further examined after infection with BCG. Soon after *M. tuberculosis* has been phagocytosed by AMs, the bacilli successfully inhibits the formation of phagolyososomes. After creating an impediment to the fusion of phagosomes and lysosomes, the bacilli takes up stable residence in the phagosomes (1, 37). This unique behavior was only observed after infection of live bacilli. Killed bacilli are known to be digested in phagolysosomes, as is usual for foreign substances. In our experiment, BCG was used instead of *M. tuberculosis*. BCG is safe for healthy humans and, thus, can be handled in a P2-level laboratory. Nevertheless, it is considered to have a pathological character and has been used in many studies for tuberculosis (38, 39).

In choosing host cells for a chronic infection with *M. tuberculosis*, resistance against infection by the host cells must be considered. For example, in mice a mutation in Nramp was reported to relate to the susceptibility to infection by *M. tuberculosis* (40). However, for rats and humans the relationship between Nramp and resistance to *M. tuberculosis* is not clear. Although, Nramp of SD rat appears to have a normal and resistant type, BCG successfully resided in the NR8383 cells originated from a SD rat strain.

We confirmed that the similar response pattern of AMs and NR8383 cells continued after phagocytosis of either live or killed BCG. As shown in Figure 4A, after both AMs and NR8383 cells phagocytosed either live or killed BCG, they did not produce TNF. This result was different from that obtained after phagocytosis of zymosan and shows that there is a difference in the intracellular signals induced by zymosan than those induced by BCG. (3, 10, 11, 18, 41). After J774.1 cells phagocytosed killed BCG, they produced a significantly higher amount of TNF compared to control

cells. After AMs and NR8383 cells phagocytosed live or killed BCG, they had the same expression profile for NO production (Figure 4B). All the observations described above indicate that NR8383 cells have the same characteristics as normal AMs, even after phagocytosis of the tubercle bacilli. It is noteworthy that the experiments did not demonstrate significant differences between live and killed BCG on induction of either TNF or NO. It is probable that, during the early phase after phagocytosis, the host function relevant to intracellular survival of BCG may be concealed. Thus, it is important that there be an analysis of host functions at the later stages after the cells phagocytosed either live or killed BCG.

Finally, we examined the fate of live or killed BCG after being phagocytosed. The morphological changes and viability of NR8383 cells and incorporated live and killed BCG were observed over time until the 21st day after phagocytosis. As shown in Figure 5, there was an increase in the number of live BCG in the NR8383 cells 7 days after the BCG had been phagocytosed. In contrast, at 21 days, the lysis of BCG was observed in the NR8383 cells that had phagocytosed killed BCG. Over the period of the experiment, the viability of NR8383 cells did not decline, whether or not the bacilli that had been phagocytosed were live or killed. Unlike the NR8383 cells, the J774.1 cells went to apoptosis after phagocytosis of either live or killed BCG (data not shown). Although the mechanisms for these differences are not clear, they may be due to characteristics of J774.1 cells. Of special interest is the fact that J774.1 cells spontaneously produced TNF without stimulation, and this might be involved in the occurrence of apoptosis after phagocytosis of BCG.

The results of these tests strongly suggest that NR8383 cells are more suitable for use in a model for tuberculosis studies than J774.1 cells. This model is suitable for studying the intercellular survival strategy of BCG not only during the early stages, but also during the chronic stage.

In recent years, some mechanisms and molecules have been proposed to explain the survival strategy of *M. tuberculosis* in host macrophages during the early stages after phagocytosis. Researchers have focused on suppression of phagosome-lysosome fusion (42, 43). However, there has been research that helps explain how the response of the host somehow results in the residence of *M. tuberculosis* in the phagosomes. In our model system, morphological differences of live and killed BCG could be clearly seen 7 days after phagocytosis of the BCG. Thus, it seems reasonable to consider other mechanisms by which *M. tuberculosis* resides stably in the host phagosomes (besides inhibition of the phagolysosome fusion at an early stage after phagocytosis). The model described here provides an opportunity for studying the survival mechanisms during the chronic stage. Moreover, the model might provide novel

insights into the mechanisms even during the early stages, because NR8383 cells seem to more accurately mimic the cellular responses of AMs. Though TNF was reported to be a factor leading to the successful residency of virulent *M. tuberculosis* in phagosomes (44), it was barely expressed by NR8383 cells that contained live or killed BCG.

It is believed that the mechanism of survival of Mycobacterium in the host body is complicated and may be due to multiple factors derived from the bacillus itself, or the host macrophages may be involved. Mycobacterial surface moieties are known as factors which create and maintain chronic infections by forming granulomas in the lung. This phenomenon was identified 4 to 16 h after Mycobacterium infection of bone-marrow-derived macrophages (14). An analysis of granuloma formation after 16 h is probably not optimal for understanding chronic infections by Mycobacterium in host macrophages. The primary immunological event, including production of cytokines, probably occurs during the acute stage ($\sim$ 7 days) (18).

In summary, the advantages of this model are as follows: i) NR8383 cells closely mimic important biological characteristics of normal alveolar macrophages; ii) the NR8383 and BCG model can be used to study host factors relevant to the mechanisms involved in the survival mechanisms of *M. tuberculosis* during phagocytosis and at least as long as 21 days afterwards (both early and late stages). We believe this model will be useful in clarifying the interaction between the host and *M. tuberculosis*, and may contribute to a greater understanding of the survival mechanisms of *M. tuberculosis*.

Acknowledgements

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