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การศึกษาการตอบสนองของเซลล์ phagocytic และ nonphagocytic ด้วยเชื้อ *Burkholderia pseudomallei*

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พฤษภาคม 2547

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ชุดโครงการ Melioidosis

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

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Abstract

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Project Title: Pathogenesis of *Burkholderia pseudomallei*: *in vitro* study of the host cells modulated bacteria.

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Burkholderia pseudomallei is a causative agent of melioidosis, a life threatening disease which affects both humans and animals. We recently reported that mouse macrophages infected with *B. pseudomallei* fail to produce significant level of inducible nitric oxide synthase (iNOS), a crucial enzyme needed for the cells to control intracellular growth of this bacterium. In this study, to demonstrate that, unlike other gram-negative bacteria that have been investigated, *B. pseudomallei* only minimally activate IFN-β production which leads to the low level of interferon regulating factor-1 (IRF-1) in the mouse macrophages cell line (RAW 264.7) and this paralleled with poor iNOS expression. Adding exogenous IFN-β to the system could upregulate IRF-1 production which in turn enhances iNOS expression in the *B. pseudomallei*-infected macrophages leading to suppression of intracellular growth of this bacterium. It appears that the failure of macrophages to successfully control growth and survival of intracellular *B. pseudomallei* is related, at least in part, to the defective production of IFN-β which modulates the ability of macrophages to synthesize iNOS. In this study, we also extended our investigation, analysing the mechanism(s) by which the two types of interferons (IFNs) regulate antimicrobial activity in the *B. pseudomallei*-infected macrophages. The macrophages that were simultaneously exposed to *B. pseudomallei* and type I IFN (IFN-β) expressed high level of iNOS, leading to enhanced intracellular killing of the bacteria. However, neither enhanced iNOS expression nor intracellular bacterial killing was observed when the macrophages were preactivated with IFN-β prior to being infected with *B. pseudomallei*. On the contrary, the timing of exposure was not critical for the type II IFN (IFN-γ) because when the cells were either prestimulated or costimulated with IFN-γ, both iNOS expression and intracellular killing capacity were enhanced. The differences by which these 2 IFNs regulate antimicrobial activity may be related to the fact that IFN-γ was able to induce

more sustained Interferon Regulatory Factor-1 (IRF-1) expression compared with the cells activated with IFN-β.

Besides phagocytic cells, *B. pseudomallei* has ability to invade and multiply inside nonphagocytic cells in various organs. Among them, lung is the most commonly affected organ resulting in abscess formation in the patients with chronic melioidosis. We demonstrated that *B. pseudomallei* was able to stimulate IL-8 production from human alveolar lung epithelium cell line (A549). However, the level of IL-8 production was significantly lower than the cells infected with other gram-negative bacteria such as *Salmonella enterica* serovar typhi (*S. typhi*). The degree of IκBα degradation in the *B. pseudomallei*-infected cells was also lower than that of the *S. typhi*-infected cells, suggesting that *B. pseudomallei* is a poorer cell activator. Inhibition of *B. pseudomallei* invasion by cytochalasin D did not interfere with either the IL-8 production or IκBα degradation, indicating that bacterial uptake is not required for the production of this chemokine. The signaling which initiated by the interaction of *B. pseudomallei* with the epithelial cell surface is sufficient for cells activation. Immunoblotting analysis indicated that *B. pseudomallei* triggered the phosphorylation of p38 of the cells to facilitate the invasion into the lung epithelial cells. Inhibition of p38 protein by specific inhibitor resulted in the inability of the bacteria to invade the cells.

Keywords: *Burkholderia pseudomallei*, melioidosis, phagocytic cells, nonphagocytic cells

บทคัดย่อ

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ชื่อโครงการ: การศึกษาการตอบสนองของเซลล์ phagocytic และ nonphagocytic เมื่อถูกกระตุ้นด้วยเชื้อ *Burkholderia pseudomallei*

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เมลิออยโดซิสเป็นโรคที่เกิดขึ้นจากการได้รับเชื้อ *Burkholderia pseudomallei* โรคนี้มีการระบาดมากบริเวณพื้นที่เขตร้อนเช่นประเทศไทย ผู้ป่วยที่ได้รับเชื้อชนิดนี้เข้าไปจะเสียชีวิตในระยะเวลาอันสั้น จากการศึกษาพบว่าเซลล์แมคโครฟาจที่รับเชื้อนี้เข้าไปจะไม่มีการสร้าง Inducible Nitric Oxide Synthase (iNOS) ซึ่งเป็นเอนไซม์ที่สำคัญในการฆ่าเชื้อแบคทีเรียภายในเซลล์ เพื่อแสดงให้เห็นว่าเชื้อ *B. pseudomallei* มีความสามารถในการกระตุ้นการสร้าง IFN-β และ Interferon regulating factor-1 (IRF-1) ในระดับที่น้อยกว่าเชื้อแบคทีเรีแกรมลบชนิด ในการศึกษาครั้งนี้คณะผู้วิจัยจึงได้ใส่ exogenous IFN-β เข้าไปในระบบ พบว่า IFN-β สามารถ upregulate การสร้าง IRF-1 ซึ่งมีผลทำให้มีการสร้าง iNOS โดยเซลล์แมคโครฟาจที่ได้รับเชื้อ *B. pseudomallei* มีผลไปสู่การยับยั้งการเจริญเติบโตของแบคทีเรียภายในเซลล์แมคโครฟาจ แสดงให้เห็นว่า IFN-β มีความเกี่ยวข้องกับการควบคุมการเจริญเติบโตและความอยู่รอดของเชื้อ *B. pseudomallei* ในเซลล์แมคโครฟาจ นอกจากนี้คณะผู้วิจัยได้ทำการศึกษาความแตกต่างของ type I IFN (IFN-β) และ type II IFN (IFN-γ) ในการควบคุมการทำลายเชื้อแบคทีเรียในเซลล์แมคโครฟาจที่รับเชื้อ *B. pseudomallei* พบว่าเมื่อใส่ IFN-β พร้อมกับการกระตุ้นเซลล์แมคโครฟาจด้วยเชื้อ *B. pseudomallei* ทำให้เกิดการเพิ่มปริมาณการสร้าง iNOS และมีผลทำให้เชื้อแบคทีเรียถูกทำลาย อย่างไรก็ตามการใส่ IFN-β ก่อนการกระตุ้นเซลล์แมคโครฟาจด้วยเชื้อ *B. Pseudomallei* ไม่สามารถทำให้เกิดการสร้าง iNOS และการทำลายเชื้อแบคทีเรียขึ้น ในทางตรงกันข้ามระยะเวลาในการใส่ type II IFN (IFN-γ) ไม่ว่าก่อนหรือพร้อมกับการกระตุ้นเซลล์แมคโครฟาจไม่มีความสำคัญต่อการสร้าง iNOS และการยับยั้งเชื้อแบคทีเรีย สำหรับความแตกต่างของกลไกในการควบคุมการสร้าง iNOS ของ IFN ทั้งสองชนิดนี้อาจเกี่ยวข้องกับการสร้าง IRF-1 เนื่องจาก IFN-γ สามรถรักษาระดับการสร้าง IRF-1 ให้อยู่ในระดับคงที่ได้นานกว่า IFN-β

นอกเซลล์แมคโครฟาจ *B. pseudomallei* ยังมีความสามารถในการเข้าไปอาศัยและเพิ่มจำนวนในเซลล์ nonphagocytic ที่บริเวณอวัยวะต่างๆ โดยเฉพาะที่อย่างยิ่งในปอดซึ่งเป็นบริเวณที่เกิดฝีในผู้ป่วยที่เป็นโรคเมลิออยโดซิสเรื้อรัง คณะผู้วิจัยได้แสดงให้เห็นว่าเชื้อ *B. pseudomallei* มีความสามารถ

ในการกระตุ้นให้เกิดการสร้าง IL-8 โดย human alveolar lung epithelial cell line (A549) อย่างไรก็ตามปริมาณการสร้าง IL-8 ที่สร้างขึ้นจากการกระตุ้นของเชื้อ *B. pseudomallei* มีปริมาณน้อยเมื่อเทียบกับการกระตุ้นด้วยเชื้อแบคทีเรียแกรมลบชนิดอื่น เช่น *Salmanella enterica* serova typhi (*S. typhi*) อย่างมีนัยสำคัญ นอกจากนี้ระดับการสลายตัวของ IκBα ในเซลล์ที่ถูกกระตุ้นด้วยเชื้อ *B. pseudomallei* ยังต่ำกว่าในเซลล์ที่ถูกกระตุ้นด้วยเชื้อแบคทีเรียแกรมลบชนิดอื่น จะเห็นได้ว่าเชื้อ *B. pseudomallei* เป็นตัวกระตุ้นเซลล์ที่มีความสามารถต่ำกว่าเชื้อชนิดอื่น การยับยั้งการเข้าสู่เซลล์ของเชื้อ *B. pseudomallei* โดยการใช้สาร cytochalasin D ไม่มีผลเกี่ยวข้องกับการสร้าง IL-8 หรือระดับการสลายตัวของ IκBα ⟨⊇×♣©⊄⌈◊⊄⌋≠∩\® chemokine เหล่านี้ไม่มีความจำเป็นในกระบวนการการเข้าสู่เซลล์ การสัมผัสระหว่างเชื้อ *B. pseudomallei* กับผิวเซลล์เพียงพอต่อการสร้างสัญญาณเพื่อใช้ในการกระตุ้นเซลล์ จากการทำ Immunoblotting analysis แสดงให้เห็นว่าเชื้อ *B. pseudomallei* กระตุ้นให้เกิดการ phosphorylation ของ p38 ซึ่งช่วยในการเข้าสู่ lung epithelial cells การยับยั้งโปรตีน p38 มีผลทำให้เชื้อ *B. pseudomallei* ไม่สามารถเข้าสู่เซลล์ได้

คำหลัก: *Burkholderia pseudomallei*, melioidosis, phagocytic cells, nonphagocytic cells

Pathogenesis of *Burkholderia pseudomallei*: *in vitro* study of the host cells modulated by bacteria

The results from the study of pathogenesis of *Burkholderia pseudomallei* in this proposal will be divided in to two parts. The first part will focus on the modulation of this bacterium with phagocytic cells, macrophages. In this study, we will demonstrate the mechanism of how *B. pseudomallei* escape the macrophage killing. In the second part of this research, we will demonstrate the modulation of this bacterium with non phagocytic cells using human lung epithelial cell line (A549) as a model. The study will include the modulation of IL-8 production by *B. pseudomallei*. Moreover, the mechanism for bacterial invasion will also include in this study.

In vitro study of the modulation of *Burkholderia pseudomallei* with phagocytic cells (Part I)

Burkholderia pseudomallei is a causative agent of melioidosis, an endemic disease in tropical countries including Southeast Asia and northern Australia (4, 11, 17, 39). This facultative intracellular bacterium is known to survive and multiply inside both phagocytic and nonphagocytic cells (13). After internalization, *B. pseudomallei* can escape from a membrane-bound phagosome into cytoplasm (13). The organism can induce cell fusion, resulting in a multinucleated-giant cell (MNGC) formation (9, 16). This phenomenon may facilitate *B. pseudomallei* to spread from one cell to another.

Although the host cell and *B. pseudomallei* interaction has been extensively studied, the mechanism(s) by which this microorganism escapes macrophage killing is not clearly understood. Recently we reported that *B. pseudomallei* is able to invade mouse macrophages without activating inducible nitric oxide synthase (iNOS) production (35). This enzyme is known to play an essential role in controlling intracellular survival and multiplication of *B. pseudomallei* (25, 35). The expression of iNOS was markedly enhanced when the macrophages were preactivated with IFN-γ, thus leading to the production of NO to a concentration needed to suppress intracellular growth of this bacterium (35). The

enhancement of host defense by IFN-γ was also observed in mice infected with *B. pseudomallei* (30).

Besides IFN-γ, IFN-β (a member of Type I interferon) has been implicated in playing a role in innate immunity against different microbial infections. IFN-β is largely produced by macrophages infected with microbial pathogens (e.g. *Leishmania major*) or exposed to microbial products (e.g. lipopolysaccharide, LPS) (2, 3, 12). The potent antimicrobial functions of IFN-β have been observed in macrophages infected with such microorganisms as *L. major*, *Toxoplasma. gondii* (24, 27). Different lines of evidence suggest that iNOS and NO production could be enhanced by IFN-β (12, 36). For example, a simultaneous exposure of the macrophages to IFN-α/β and *L. major* enhanced iNOS expression resulting in inhibition of intracellular survival of *L. major* (24). Jacob *et al.* recently demonstrated that IFN-β served as an autocrine mediating interferon regulatory factor-1 (IRF-1) production (12). The expression of this transcriptional activator and the presence of a crucial IRF-1 binding site within the promoter of iNOS gene are necessary for the induction of iNOS in murine macrophages (14, 23).

In this study, we have investigated the role of type I interferon on iNOS expression and NO production of the macrophages treated with LPS isolated from *B. pseudomallei* (strain 844), killed *B. pseudomallei* and living *B. pseudomallei*. We will also focus more on the roles of IFN-β on antimicrobial activity of *B. pseudomallei* infected macrophages.

Materials and Methods

Cell line and culture condition

Mouse macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC, Rockville, MD). If not indicated otherwise, the cells were cultured in Dulbeccco's modified Eagles' medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) at 37^{o}C under a 5% CO_2 atmosphere.

Bacterial isolation

B. pseudomallei strain 844 (arabinose-negative strain) used in this study was originally isolated from a patient admitted to Srinagarind Hospital in the melioidosis endemic Khon Kaen province of Thailand. The bacterium was originally identified as *B. pseudomallei* based on its biochemical characteristics, colonial morphology on selective media, antibiotic sensitivity profiles and reaction with polyclonal antibody (1, 15, 39) and used in our previous reports (16, 35). *Salmonella enterica* serovar Typhi (*S. typhi*) used for comparison throughout these experiments was maintained at Ramathibodi Hospital (Mahidol University, Bangkok, Thailand) and kept as stock culture in our laboratory.

Infection of mouse macrophage (RAW 264.7)

Mouse macrophages (1×10^6 cells) were cultured in a 6-well plate overnight before exposure to bacteria at multiplicity of infection (MOI) of 2:1 for 1 h. To remove extracellular bacteria, the cells were washed 3 times with 2 ml of PBS before replacing with DMEM containing 250 μg/ml kanamycin (Gibco Labs). At the time indicated, the cells were lysed before subjecting to immunoblotting while the supernatant was used for IFN-β analysis.

Immunoblotting

Different mouse macrophage preparations were lysed in buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates containing 30 μg of protein were electrophoresed on SDS-PAGE at 10% polyacrylamide and then electrotransferred to nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany). The membrane was blocked with 5% milk for 1 h before incubating overnight with polyclonal antibody to mouse iNOS, IRF-1 or actin (Santa Cruz, Santa Cruz, CA). Blots were then reacted with horseradish peroxidase-conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostic, Mannheim, Germany).

ELISA

The concentrations of IFN-β in the supernatant of infected macrophages were measured using enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN). The sensitivity of the assay system was 50 U/ml.

NO assay

The production of NO was determined by measuring the quantity of nitrite in the supernatant from the cells cultured under different conditions by the Griess method, using a standard curve constructed with nitrite ranging from 5 to 40 μM (8). Under this condition, the sensitivity limit of the method was 5 μM of nitrite.

Standard antibiotic protection assay

Mouse macrophages (5 x 10^5 cells per well) were simultaneously exposed to IFN-β (100 U/ml) (R&D) and *B. pseudomallei* at MOI of 2:1 at 37 ^oC for 1 h and then washed thoroughly with PBS. The cells were there after incubated for an additional 7 h in the medium containing 250 μg/ml kanamycin to eliminate residual extracellular bacteria; intracellular bacteria were then liberated using 0.1% Triton x-100 and then plated on tryptic soy agar. To inhibit NO production, 500 μM of L-NAME (Sigma, St. Louis, MO) was added

to the culture medium and kept throughout the period of infection. The viability of macrophages when cultured in the presence of L-NAME was more than 90% (judged by trypan blue dye staining). This concentration of iNOS inhibitor did not interfere with bacterial growth as judged by viable count from pour plate technique.

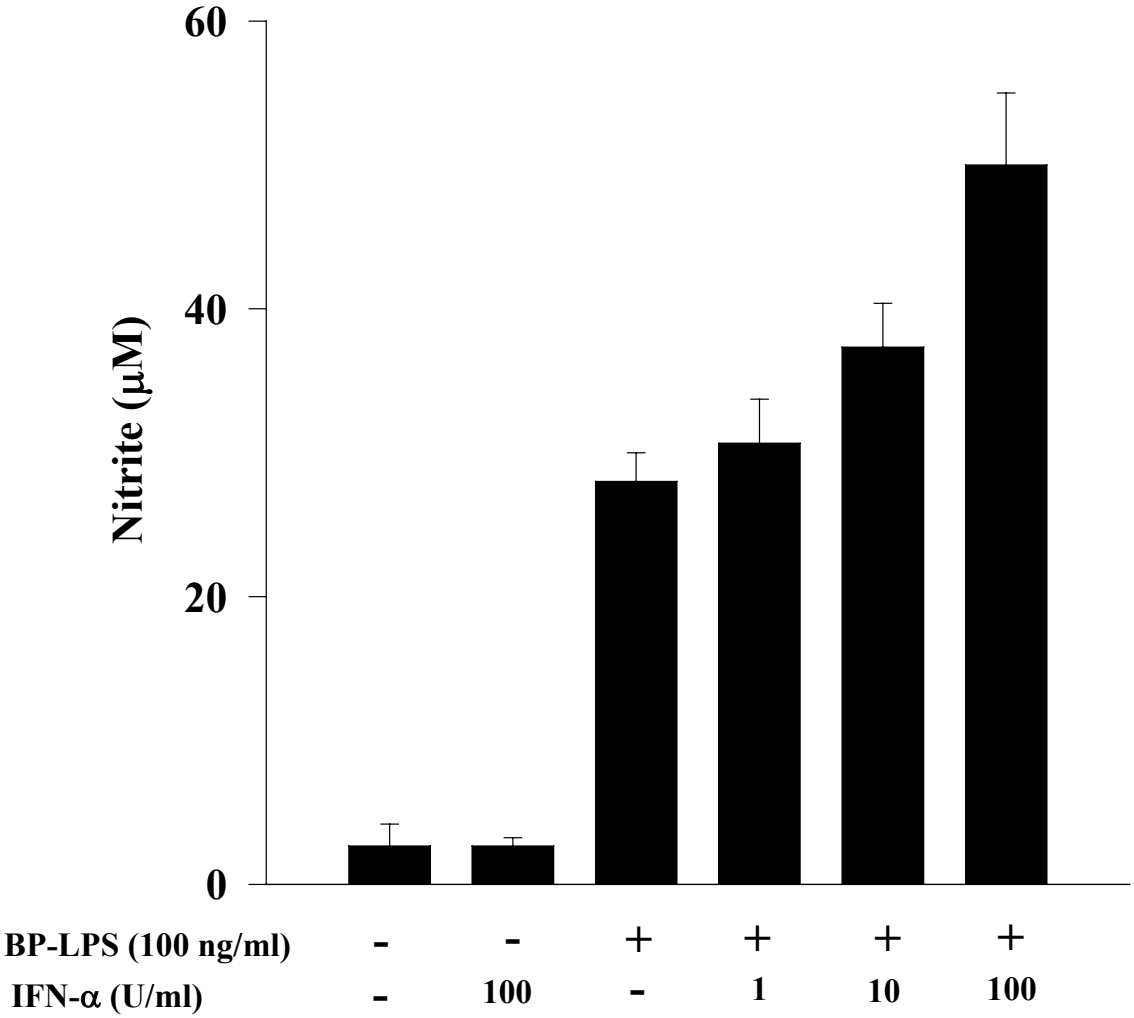
Results

Effect of IFN-α and IFN-β on NO production from the macrophages activated with BP-LPS and heat-killed B. pseudomallei

Mouse macrophages cell lines (RAW 264.7) were preincubated with various concentrations of IFN-α or IFN-β overnight. The samples were washed three times with PBS before incubated in the medium containing BP-LPS (100 ng/ml) (Fig. 1) or heat-killed *B. pseudomallei* (MOI 100:1) (Fig. 2). After incubation overnight, the supernatants were analysed for nitrite by Griess's reaction. As shown in Fig. 1 A, IFN-α was able to enhance NO production from the macrophages activated with BP-LPS. The increase of NO production by IFN-α was a concentration dependent. At IFN-α concentration of 100 U/ml, almost 50 μM of nitrite was detected in the supernatant. IFN-α alone was not able to stimulate NO production. Similar results were observed when the macrophages were stimulated with IFN-β (Fig. 1B). These results suggest that type I interferon can increase NO production from the macrophages activated with BP-LPS.

The effect of type I interferon on NO production from the macrophages activated with heat-killed *B. pseudomallei* was also investigated. The cells were pretreated with various concentrations of IFN-α or IFN-β overnight before washed with PBS then suspended in the medium containing heat-killed *B. pseudomallei* at MOI of 100:1. After incubation overnight, the supernatants were analysed for NO production. As shown in Fig. 2 (A, B), both IFN-α and IFN-β was able to increase NO production from the cells activated with heat-killed bacteria. The increase of NO production by type I interferon was also the concentrations dependence.

In order to investigate the times required for type I interferon to enhance NO production from the cells stimulated with BP-LPS, the macrophages were preincubated with IFN-α (100 U/ml) or IFN-β (100 U/ml) for 0, 1, 2, 3 hr. or overnight. The cells were washed with PBS before suspended in the medium containing BP-LPS (100 ng/ml). After incubation overnight, the supernatants were analysed for NO production.



A

Figure 1 Type I interferon enhances NO production from the macrophages activated with BP-LPS. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was preactivated with IFN-α (A) or IFN-β (B) at concentrations of 1, 10 and 100 U/ml overnight. The cells were washed 3 times with PBS before replaced with medium containing BP-LPS (100 ng/ml). After incubation for 18 hours, the supernatants were analysed for nitrite by Griess reaction.

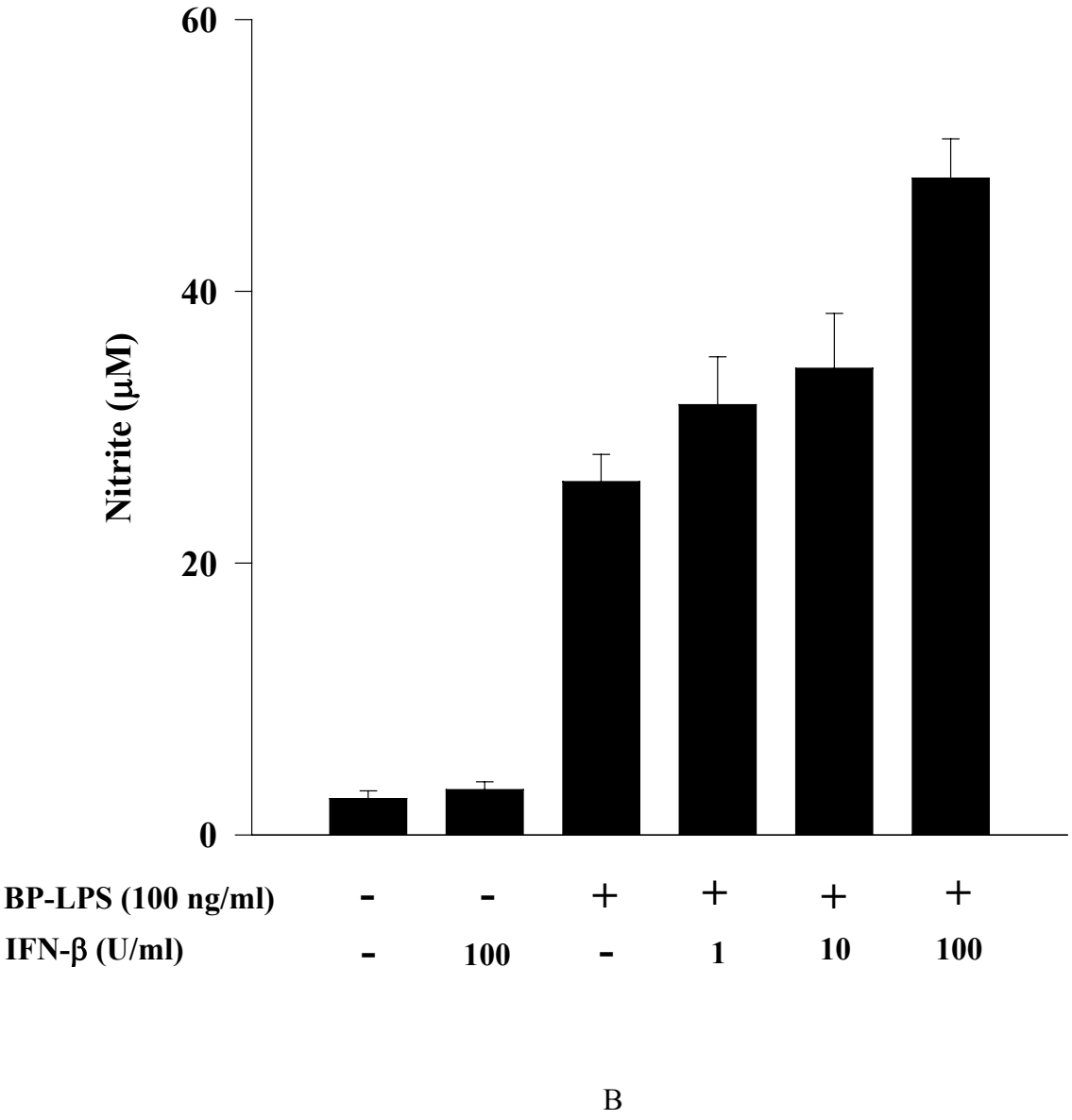


Figure 1 Type I interferon enhances NO production from the macrophages activated with BP-LPS. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was preactivated with IFN-α (A) or IFN-β (B) at concentrations of 1, 10 and 100 U/ml overnight. The cells were washed 3 times with PBS before replaced with medium containing BP-LPS (100 ng/ml). After incubation for 18 hours, the supernatants were analysed for nitrite by Griess reaction.

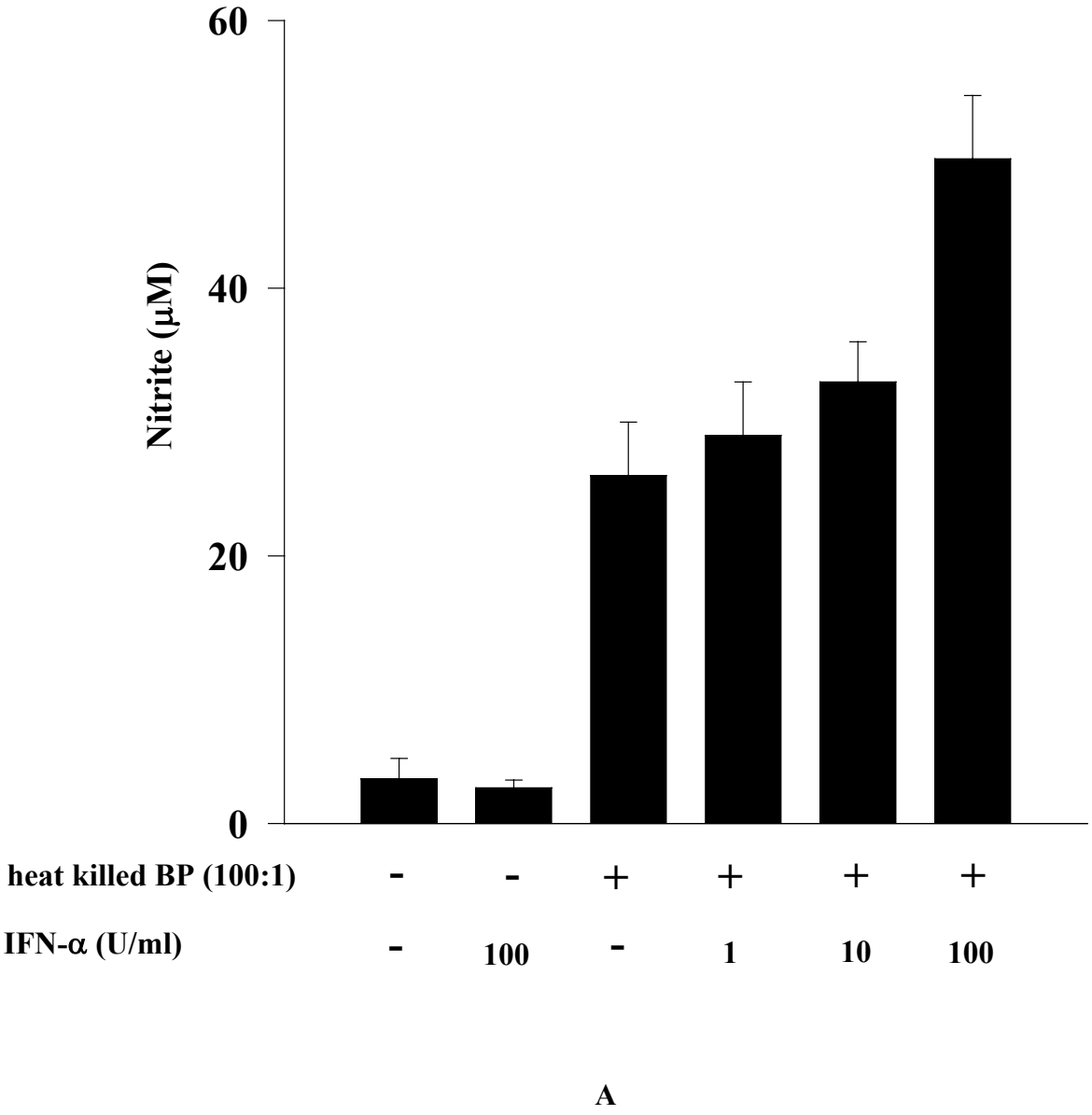


Figure 2 Type I interferon enhances NO production from the macrophages activated with heat-killed *B. pseudomallei*. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was preactivated with IFN-α (A) or IFN-β (B) at concentration of 1, 10 and 100 U/ml overnight. The cells were washed 3 times with PBS before replaced with medium containing heat killed *B. pseudomallei* ($1x10^8$). After incubation for 18 hours, the supernatants were analysed for nitrite by Griess reaction.

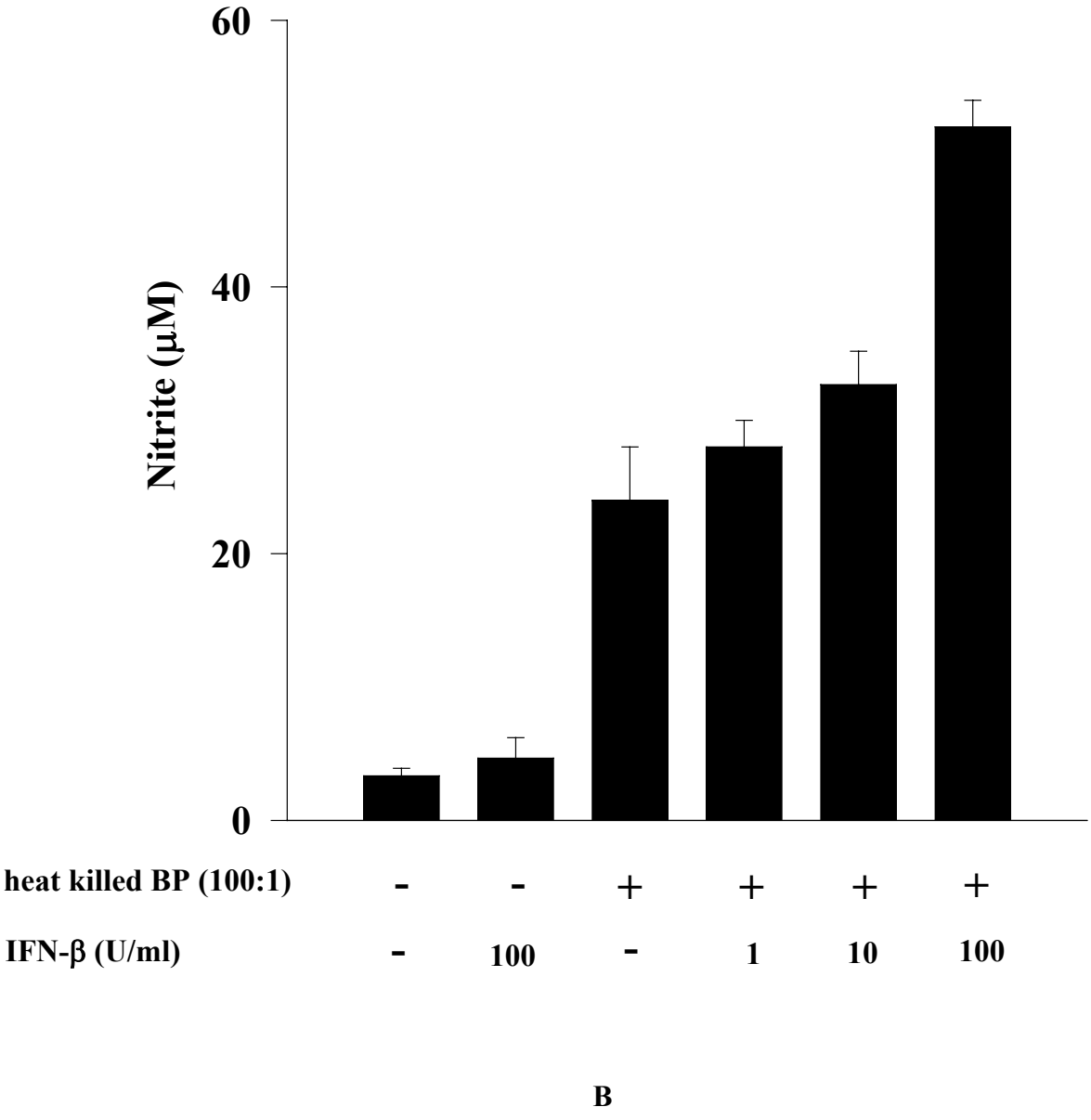


Figure 2 Type I interferon enhances NO production from the macrophages activated with heat-killed *B. pseudomallei*. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was preactivated with IFN-α (A) or IFN-β (B) at concentration of 1, 10 and 100 U/ml overnight. The cells were washed 3 times with PBS before replaced with medium containing heat killed *B. pseudomallei* ($1x10^8$). After incubation for 18 hours, the supernatants were analysed for nitrite by Griess reaction.

As shown in Fig. 3, IFN-α was able to enhance NO production from the cells activated with BP-LPS when it was added at the same time as BP-LPS (0 hr). However, IFN-α increases more the NO production if it was added before BP-LPS. Preincubation of the cells with IFN-α for 3 hours, the macrophages were able to produce maximum NO production. Similar results were observed with the cells pretreated with IFN-β (Fig. 3B).

***Effects of IFN-α and IFN-β on iNOS expression from the macrophages treated with heat-killed* B. pseudomallei**

In order to investigate the effects of type I interferon on iNOS expression from the cells activated with heat-killed *B. pseudomallei*, the macrophages were preactivated with IFN-α (100 U/ml) or IFN-β (100 U/ml) overnight. The cells were treated with killed-bacteria at MOI (100:1) for 1, 2, 4 and 8 hours. The iNOS from the activated macrophages were determined by western blot analysis. Fig. 4 shows that, in the absence of interferon, heat-killed *B. pseudomallei* were able to activate iNOS expression. However, the expression of iNOS was increased when the cells were preactivated with IFN-α (Fig. 4A) or IFN-β (Fig.4B). It should be mention that nitrite production from the cells activated with heat-killed bacteria for 1 to 8 hours in the presence or absence of interferon was not detected, which may probably due to the sensitivity of Griess reaction. These results indicate that type I interferon enhances the upregulation of iNOS expression from the cells treated with heat kill *B. pseudomallei*.

***Effects of IFN-β on iNOS expression from the macrophages infected with living* B. pseudomallei**

The macrophages were infected with *B. pseudomallei* (strain 844) at MOI of 2:1 for 1 hour. The cells were washed three times with PBS and replaced with medium containing 250 μg/ml of kanamycin. After 2 hours, the cells were washed with PBS then replaced with medium containing 20 μg/ml of kanamycin. The cells were harvested at 2, 4 and 8 hours after infection. The iNOS expression was determined by western blot.

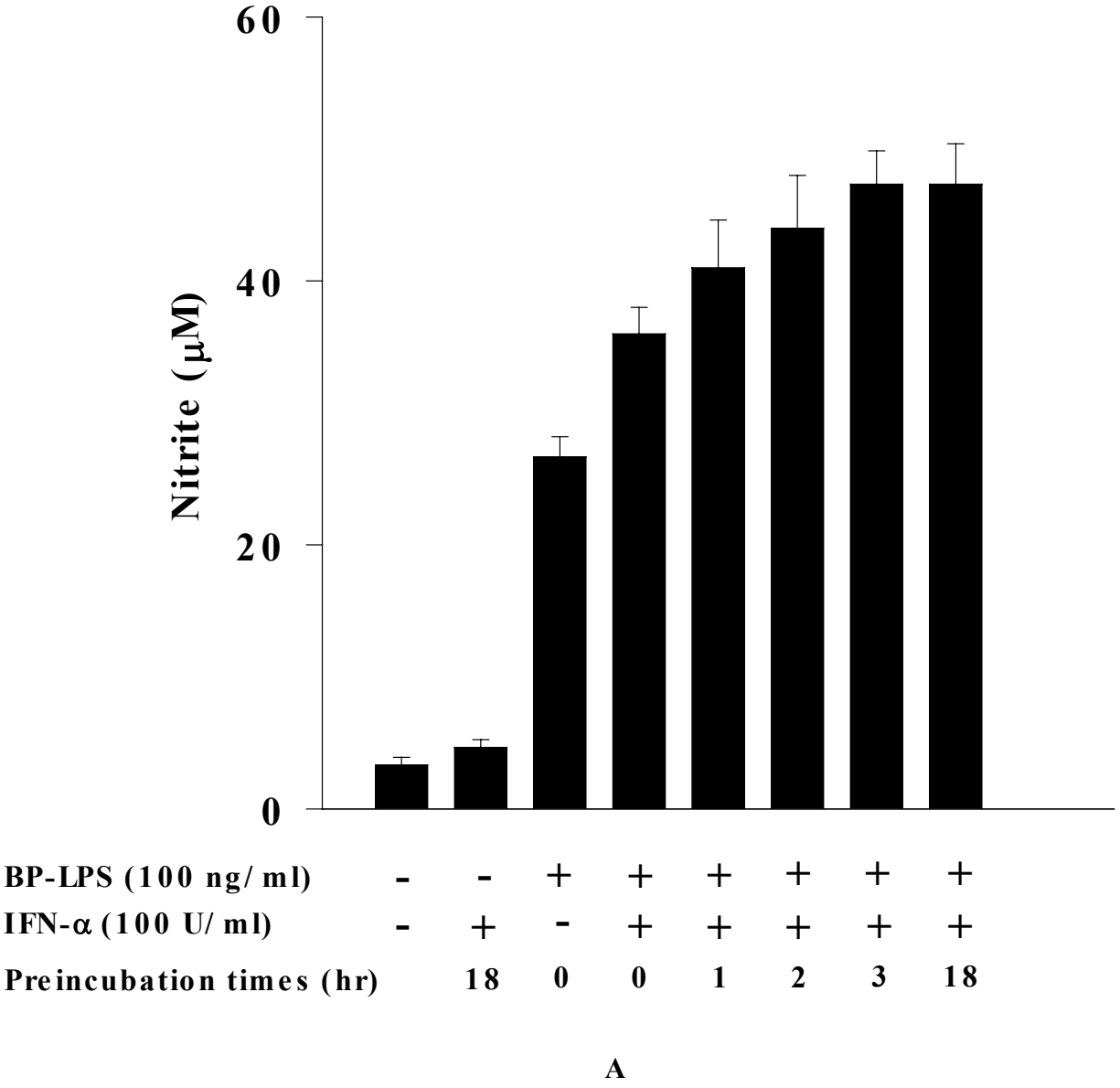


Figure 3 Incubation times required for type I interferon to enhance NO production from the macrophages activated with BP-LPS. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was preactivated with 100 U/ml of IFN-α (A) or IFN-β (B) for 0, 1, 2, 3 and 18 hours. The cells were washed 3 times with PBS before replaced with medium containing BP-LPS (100 ng/ml). After incubation of 18 hours, the supernatant were analysed for nitrite by Griess reaction

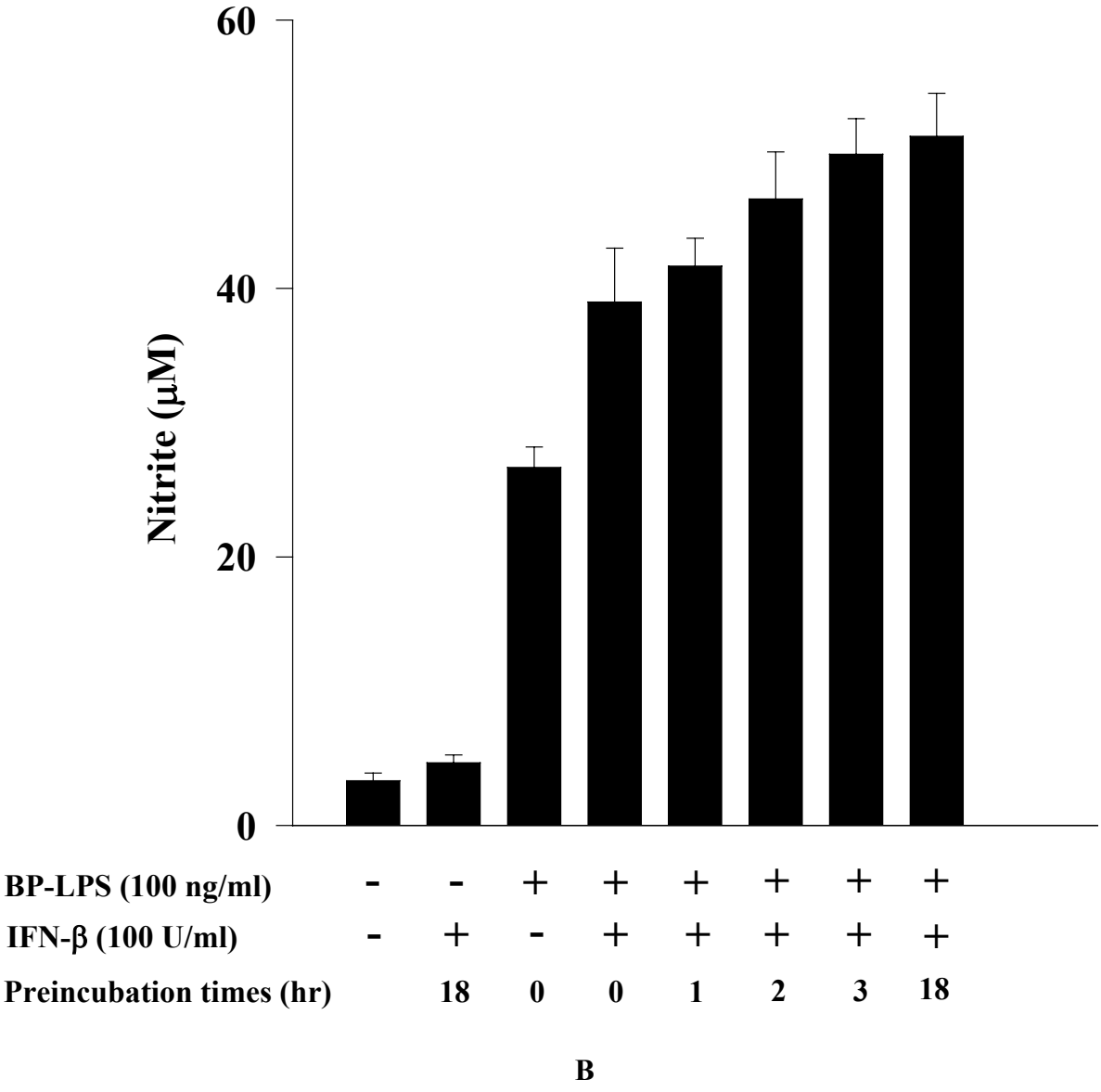
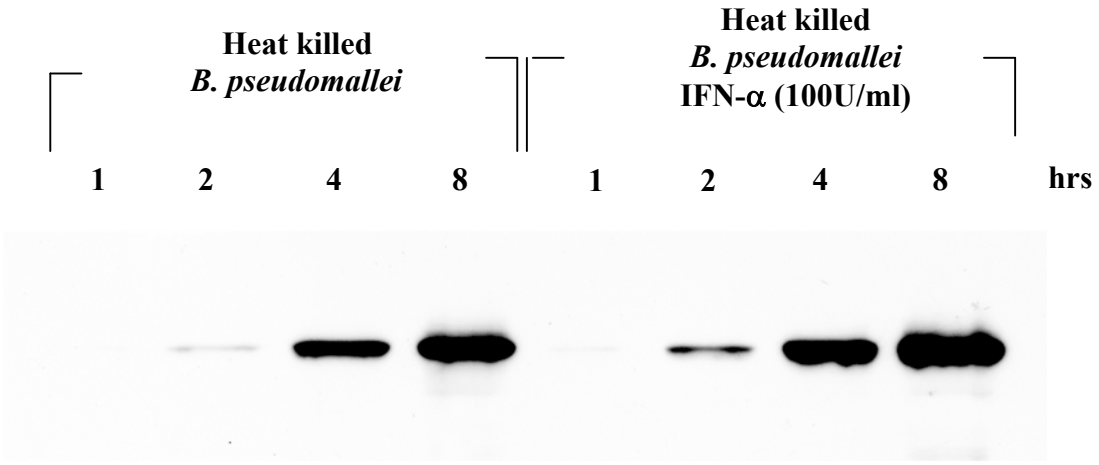
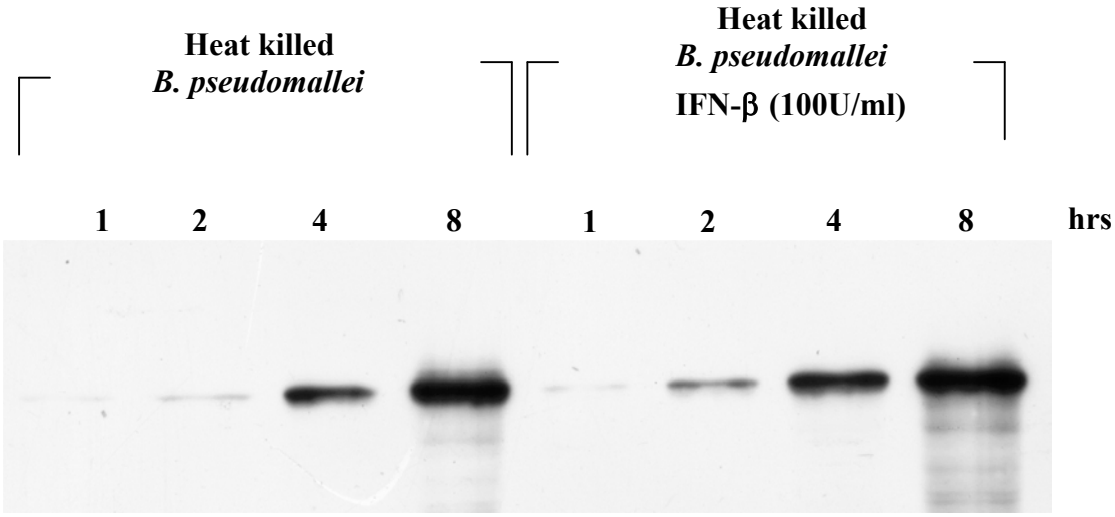


Figure 3 Incubation times required for type I interferon to enhance NO production from the macrophages activated with BP-LPS. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was preactivated with 100 U/ml of IFN-α (A) or IFN-β (B) for 0, 1, 2, 3 and 18 hours. The cells were washed 3 times with PBS before replaced with medium containing BP-LPS (100 ng/ml). After incubation of 18 hours, the supernatant were analysed for nitrite by Griess reaction



A



B

Figure 4 Type I interferon enhances iNOS production from the macrophages activated heat killed *B. pseudomallei*. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was preincubated with 100 U/ml of IFN-α (A) or IFN-β (B) overnight. The cells were washed 3 times with PBS before replaced with medium containing heat-killed bacteria ($1x10^8$). The cells were harvested after 1, 2, 4, and 8 hours and the production of iNOS were determined by western blotting.

As shown in Fig. 5, in the absence of interferon, the macrophages infected with *B. pseudomallei* were unable to express iNOS indicating that this bacterium was not able, by itself to stimulate iNOS expression. However, the IFN-β coactivated macrophages were able to express iNOS at detectable level after 2 hours of infection. It should be mention that the nitrite production was too low to be detected in the supernatant. This probably may due to the limited sensitivity of Griess reaction.

***Effect of IFN-β on multinucleated giant cell (MNGC) formation induced by* B. pseudomallei**

Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was co- or pretreated overnight with IFN-β (100 U/ml) before infected with *B. pseudomallei* (pathogenic strain, 844) at MOI 2:1. After 1 hour, the cells were washed with PBS 3 times before replaced with medium containing 250 μg/ml of kanamycin for 2 hours. The cells were washed again with PBS then replaced with medium containing 20 μg/ml of kanamycin. At 8 hours after infection, the cells were visualised by light microscope. Fig. 6A shows that IFN-β was able to inhibit MNGC induced by *B. pseudomallei* only when the cells were cotreated of IFN-β with bacteria. Surprisingly, IFN-β can not prevent MNGC when the cells were preincubated with this cytokine. In contrast, inhibition of MNGC formation was observed when the macrophages were either co- or preactivated with IFN-γ (Fig. 6B).

In order to quantitate the MNGC, the macrophages were cultured onto the coverslips in the presence and absence of IFN-β (100 U/ml) before infected with *B. pseudomallei* at MOI of 2:1. At 4, 6 and 8 hours after infection with *B. pseudomallei*, the coverslips were washed with PBS, fixed for 15 min with 1% paraformaldehyde and then washed sequentially with 50% and 90% ethanol for 5 min each. The coverslips were air dried before staining with Giemsa stain. For enumeration of MNGC, at least 1,000 nuclei per coverslips were counted and the percentage of MNGC was calculated. The results, as shown in Fig. 7, indicate that after 4 hours of infection the percentage of MNGC from the cells infected with *B. pseudomallei* in the absence or pretreated with IFN-β was increasing to

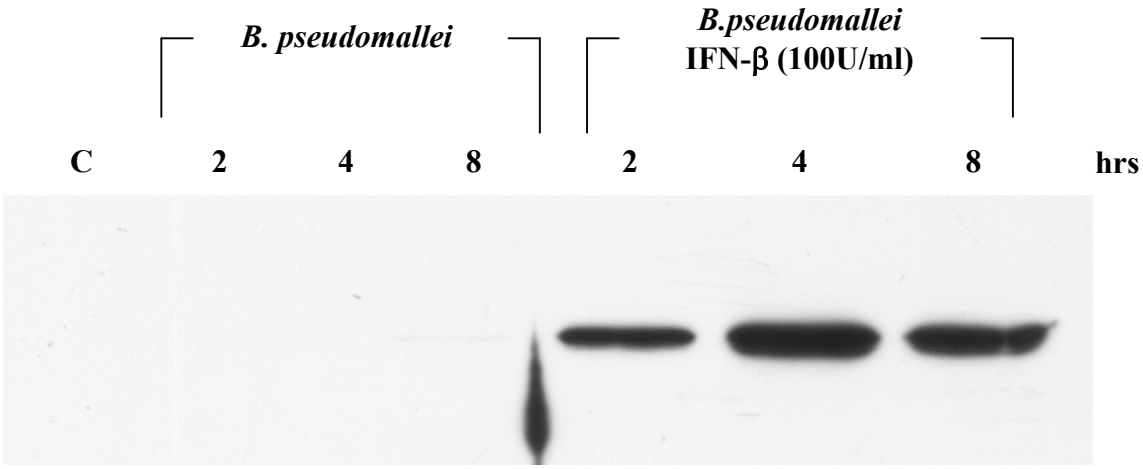


Figure 5 Type I interferon enhances iNOS production from the macrophages infected with living *B. pseudomallei*. Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was simultaneously exposed with IFN-β (100 U/ml) and *B. pseudomallei* for 1 hour at MOI of 2:1. To remove extracellular bacteria, the cells were washed with 3 times PBS before replacing in the medium containing 250 μg/ml of kanamycin. After 2 hours, the medium was replaced with medium containing 20 μg/ml of kanamycin. The cells were harvested after 2, 4 and 8 hours following bacterial infection. The production of iNOS was analysed by western blotting.

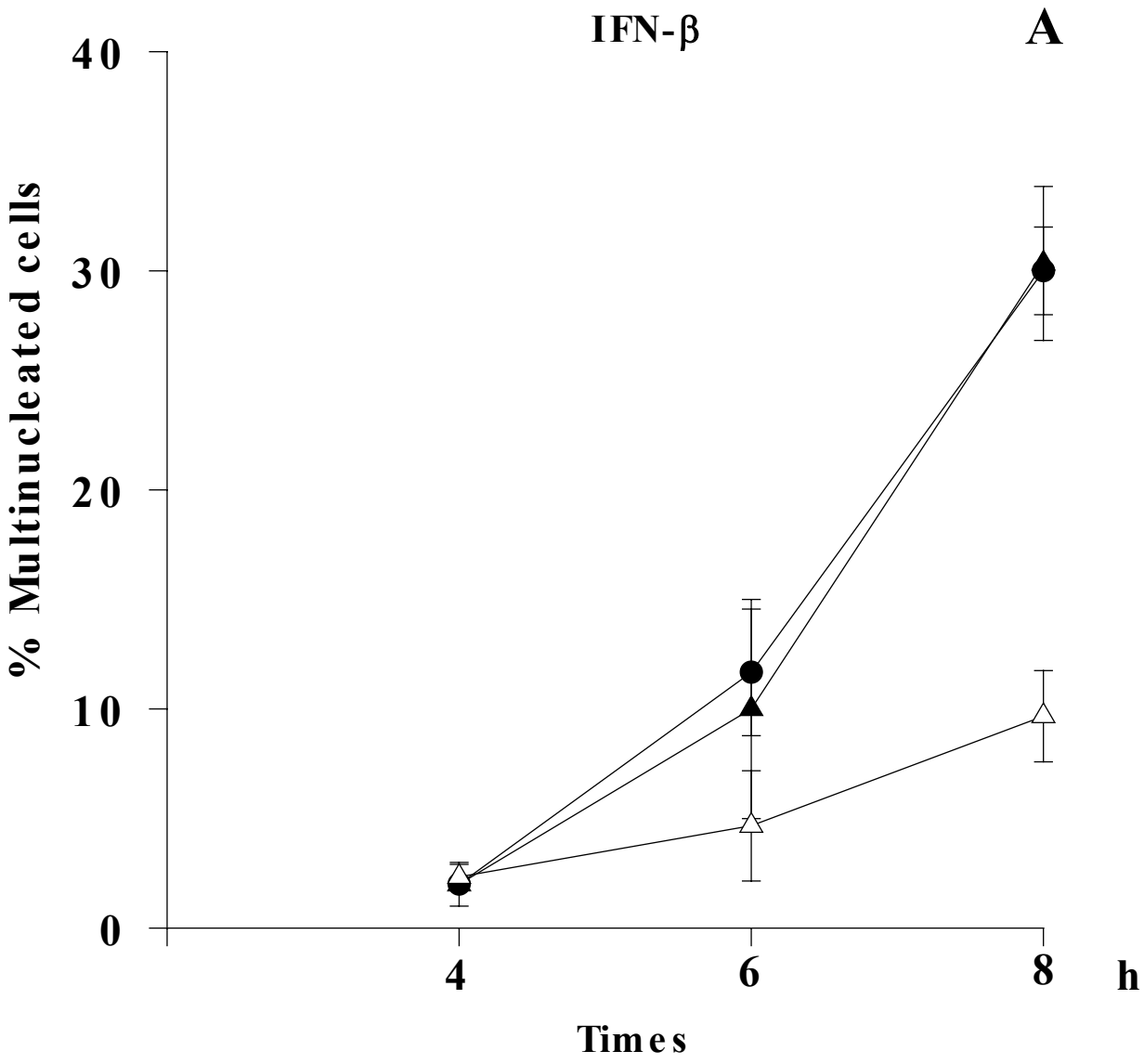


Figure 6 Effect of IFN-β and IFN-γ on MNGC. Mouse macrophages were pretreated overnight (▲) or cotreated (Δ) with 100 U/ml of IFN-β (A) or IFN-γ (B) before being infected with *B. pseudomallei* at MOI of 2:1. After 4, 6 and 8 hours of infection, the cells were fixed and stained with Giemsa stain and visualized under microscope (200x). The percentage of MNGC was calculated. Control (●) is the macrophages infected with *B. pseudomallei* in the absence of IFN. Each value represents mean of triplicate results. Error bar indicates standard deviation.

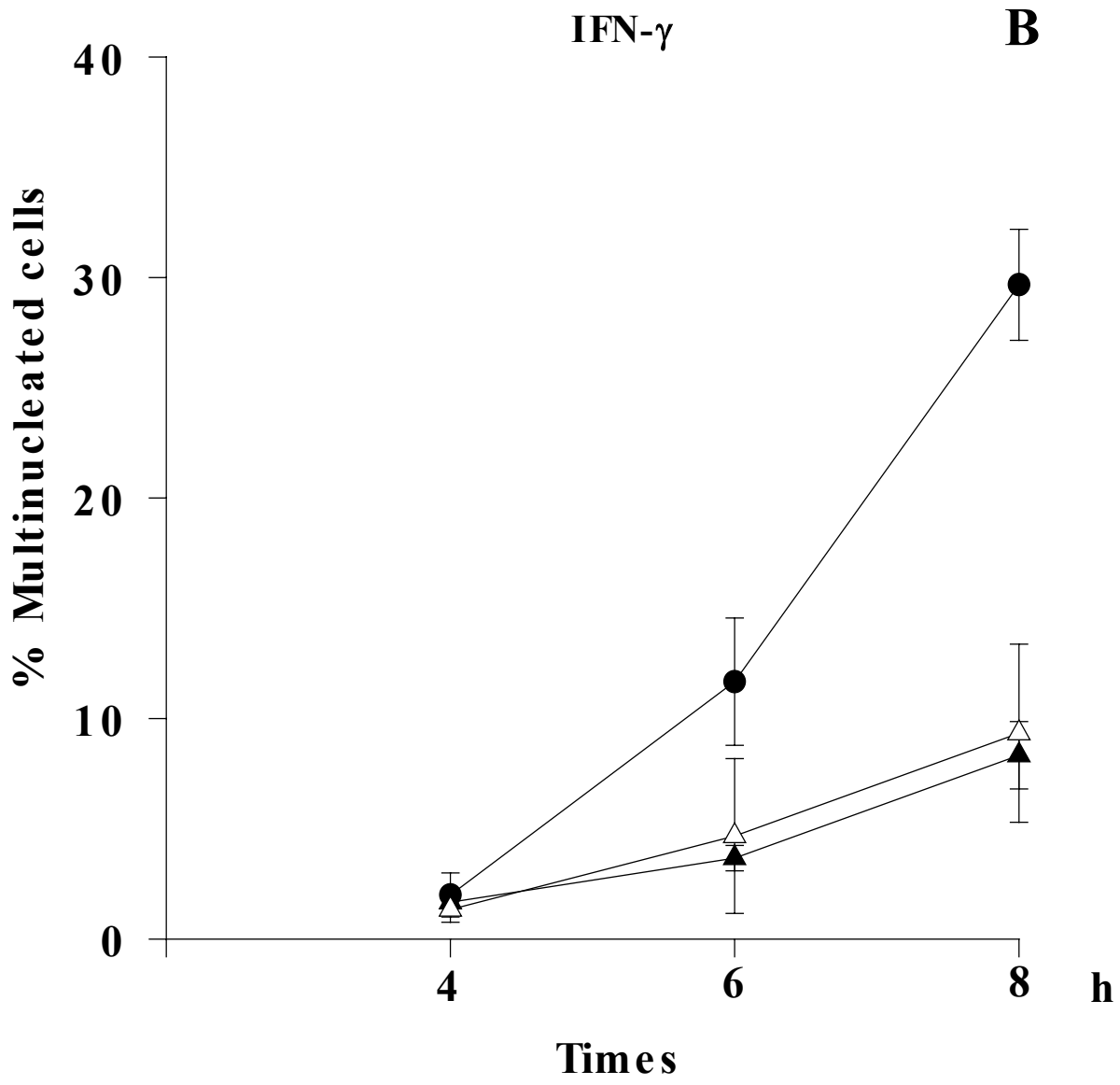


Figure 6 Effect of IFN-β and IFN-γ on MNGC. Mouse macrophages were pretreated overnight (▲) or cotreated (Δ) with 100 U/ml of IFN-β (A) or IFN-γ (B) before being infected with *B. pseudomallei* at MOI of 2:1. After 4, 6 and 8 hours of infection, the cells were fixed and stained with Giemsa stain and visualized under microscope (200x). The percentage of MNGC was calculated. Control (●) is the macrophages infected with *B. pseudomallei* in the absence of IFN. Each value represents mean of triplicate results. Error bar indicates standard deviation.

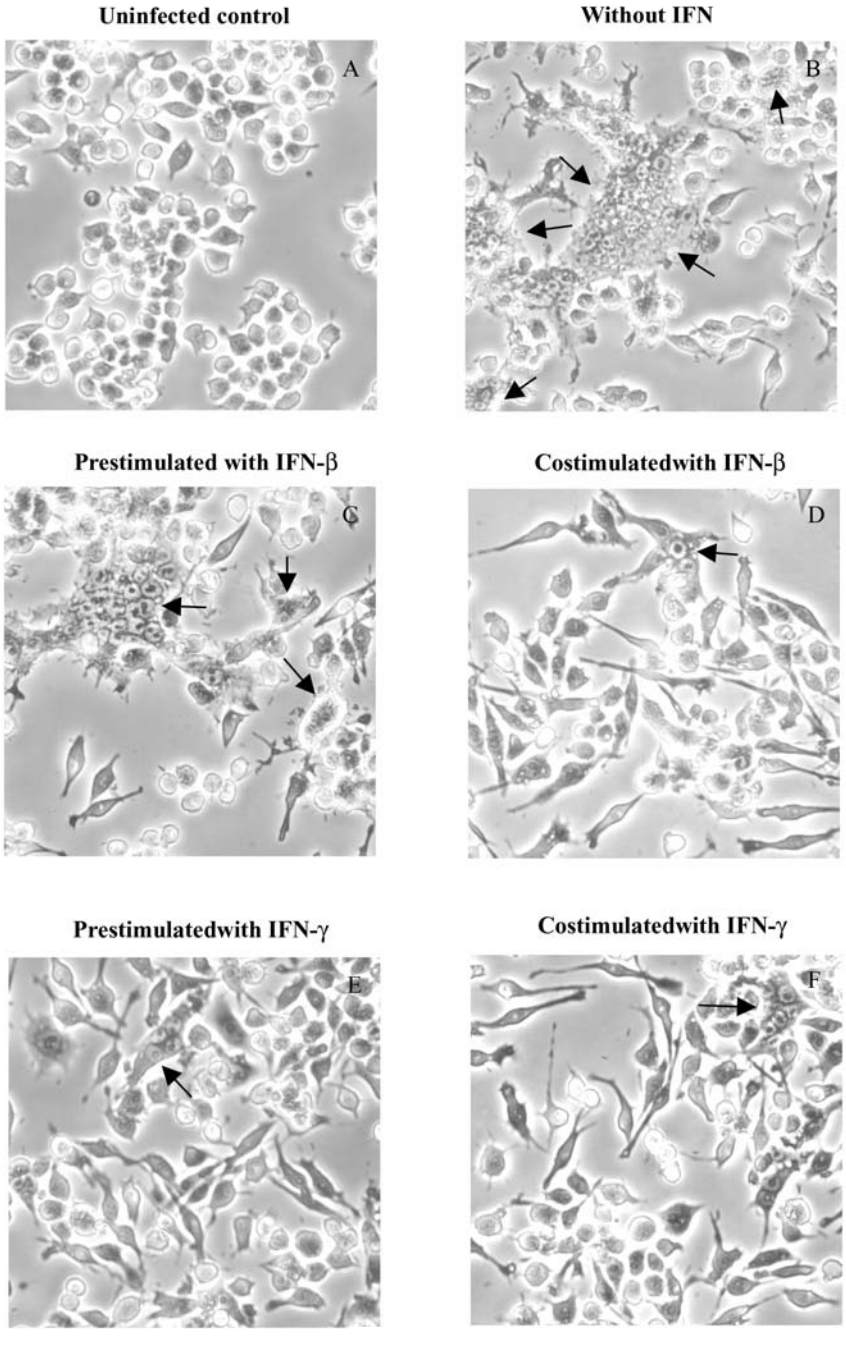


Figure 7 Effect of IFN-β and IFN-γ on MNGC formation induced by *B. pseudomallei*. Mouse macrophage cell line (RAW 264.7) ($1x10^6$ cells/well) was either prestimulated with IFN (100 U/ml of IFN-β or 10 U/ml of IFN-γ) for 18 h (C and E) or simultaneously costimulated (D and F) with *B. pseudomallei* (MOI 2:1). After 8 h, the cells were visualized under inverted microscope (20x). Uninfected cells (A) and *B. pseudomallei*-infected cells alone without IFN (B) were included for comparison. MNGC formation is indicated by arrow.

10% at 6 hours and 30% at 8 hours. However, the macrophages which cotreated with IFN-β exhibit significantly less percentage of MNGC (5% at 6 hours and 8% at 8 hours). This result suggests that IFN-β can inhibit MNGC only when it cotreated with *B. pseudomallei*. In contrast, low percentage of MNGC was observed in the macrophages which were either co- or preactivated with IFN-γ.

Effect of IFN-β on* B. pseudomallei *survival in mouse macrophages cell line

Mouse macrophage cell line (RAW 264.7) ($1x10^6$) was co- or pretreated with IFN-β (100 U/ml) before infected with *B. pseudomallei* at MOI of 2:1. After 8 hours, the intracellular bacteria were liberated by using 0.1% Triton x-100 and plated on tryptic soy agar. As shown in Fig. 8, in the absence or pretreated with IFN-β, the number of intracellular was not statistically significantly different. However, when the macrophages were cotreated of IFN-β with *B. pseudomallei*, the number of intracellular bacteria significantly decreased. This result indicates that IFN-β can suppress intracellular survival of *B. pseudomallei* only when its cotreated with bacteria. However, the macrophages which were preactivated with IFN-β were unable to suppress intracellular bacteria viability. In contrast to IFN-β, the macrophages that were either co- or preactivated with IFN-γ were able to inhibit the bacteria growth inside the macrophages.

In order to prove that the decrease of *B. pseudomallei* survival from the cells cotreated with IFN-β was due to NO produced from the infected cells, the macrophages were pretreated with iNOS inhibitor, L-NAME (500 μM), in the presence of IFN-β (cotreated) and infected with *B. pseudomallei* at MOI of 2:1. At 8 hours after infection, the number of intracellular bacteria was determined as described. As shown in Fig. 8, the number of intracellular *B. pseudomallei* was significantly decreased when the cells were cotreated with IFN-β. However, in the presence of L-NAME, the number on intracellular bacteria was significantly increased.

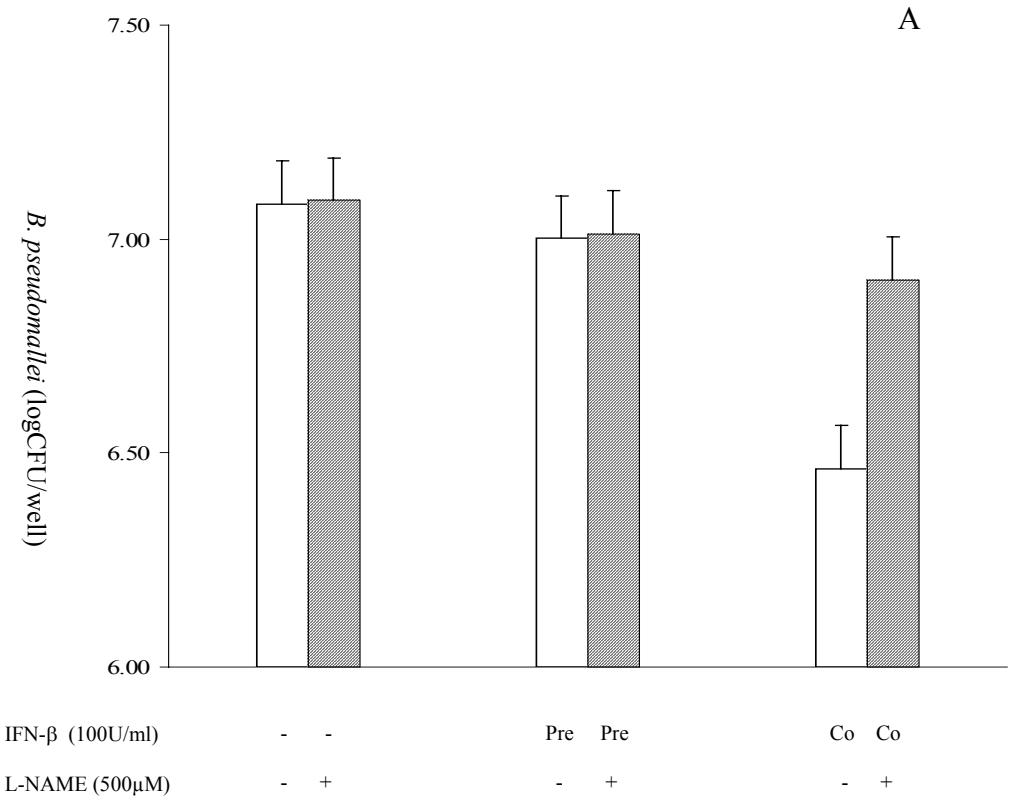


Figure 8 IFNs inhibit intracellular survival of *B. pseudomallei* in mouse macrophages. Macrophages were prestimulated with either 100 U/ml of IFN-β (A) or 10 U/ml of IFN-γ (B) for 18 h or costimulated simultaneously with *B. pseudomallei* (MOI 2:1). The experiment was carried out either without or with L-NAME (500 μM) which was added to the cells 2 h prior to the bacterial infection and kept in the culture medium until the experiment was terminated. At 8 h post infection, the number of intracellular *B. pseudomallei* was determined by standard antibiotic resistant assay as described in Materials and Methods. Data represent mean and s.d. of 3 separate experiments, each carried out in duplicate. *, $p<0.05$ by a Student's *t* test

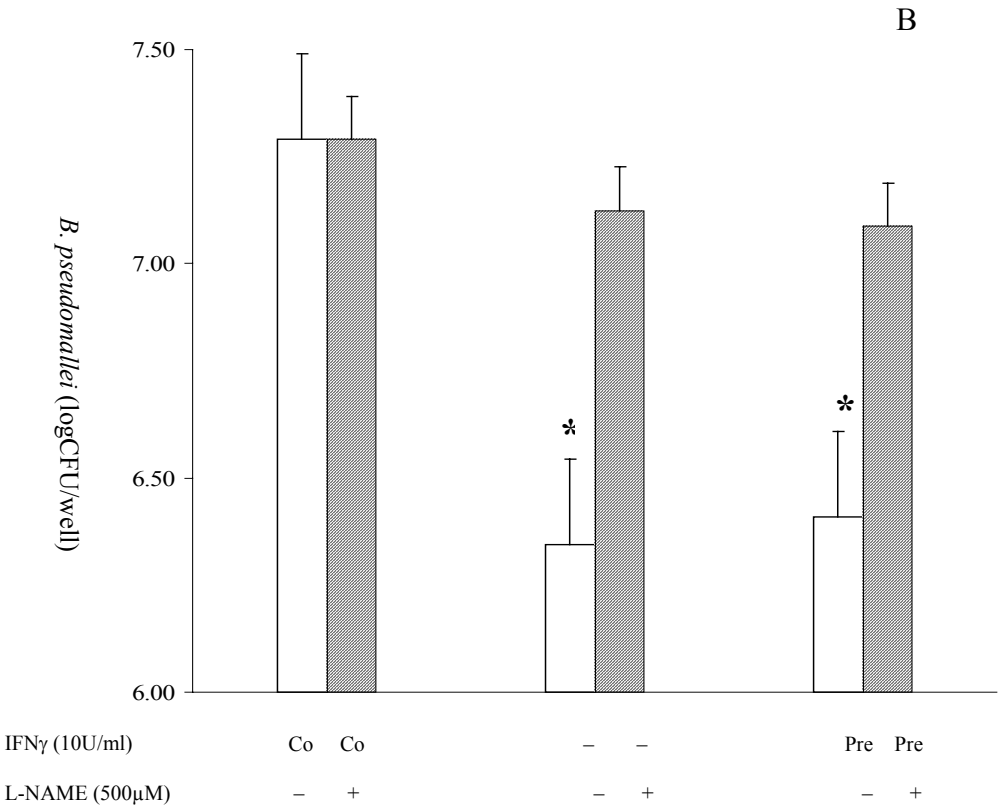


Figure 8 IFNs inhibit intracellular survival of *B. pseudomallei* in mouse macrophages. Macrophages were prestimulated with either 100 U/ml of IFN-β (A) or 10 U/ml of IFN-γ (B) for 18 h or costimulated simultaneously with *B. pseudomallei* (MOI 2:1). The experiment was carried out either without or with L-NAME (500 μM) which was added to the cells 2 h prior to the bacterial infection and kept in the culture medium until the experiment was terminated. At 8 h post infection, the number of intracellular *B. pseudomallei* was determined by standard antibiotic resistant assay as described in Materials and Methods. Data represent mean and s.d. of 3 separate experiments, each carried out in duplicate. *, $p<0.05$ by a Student's *t* test

This result indicates that the decrease of intracellular bacteria survival by cotreated with IFN-β was due to the NO production from the macrophages.

Effect of IFN-β on internalization of B. pseudomallei

In order to investigate the effect of IFN-β on bacterial internalization, the mouse macrophage cell line (RAW 264.7) ($1x10^6$) was prestimulated or simultaneously stimulated with IFN-β (100 U/ml) in the presence of *B. pseudomallei* at MOI of 2:1. The internalized bacteria were determined as described. It should be noted that, in the presence of kanamycin (250 μg/ml) for 1 h, more than 95% of extracellular *B. pseudomallei* was killed by this antibiotic. Fig. 9 shows that the number of intracellular *B. pseudomallei* in the cells treated or untreated with IFN-β was not significantly different which indicated that IFN-β did not interfere with internalization of *B. pseudomallei*.

Comparing iNOS expression from the macrophages co- or pretreated with IFN-β

In order to investigate the effect of IFN-β on iNOS expression, the macrophages were co- or pretreated with IFN-β (100 U/ml) before infected with *B. pseudomallei* at MOI of 2:1. The infected cells were collected at 4, 6 and 8 hours after infection. The iNOS expression was determined by western blotting with monoclonal antibody against iNOS. As shown in Fig. 10, in the absence of IFN-β, *B. pseudomallei* was unable to stimulate iNOS expression even after 8 hours of infection. Similar result was observed in the macrophages pretreated with IFN-β. However, the macrophages which simultaneously exposed IFN-β with *B. pseudomallei* were able to produce iNOS even when the cells were infected for 4 hours. In contrast to IFN-β, the macrophages which were either co- or preactivated with IFN-γ were able to upregulate iNOS expression (Fig. 11)

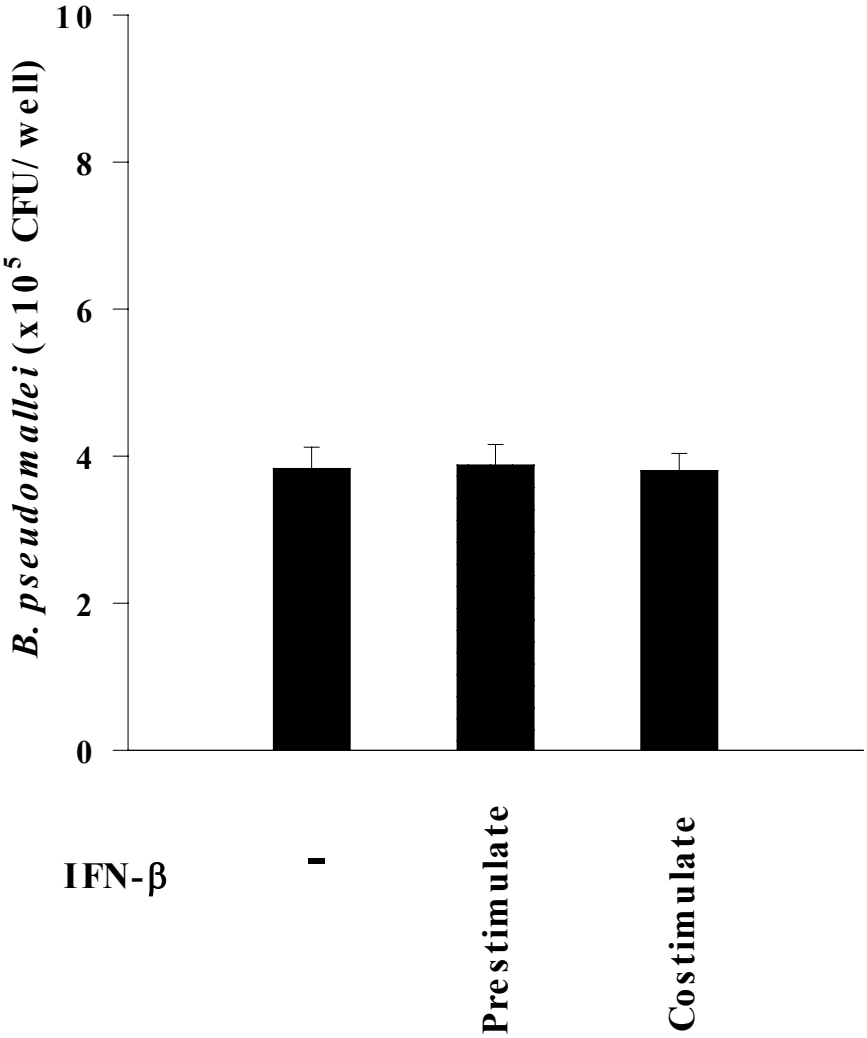


Figure 9 Effect of IFN-β on *B. pseudomallei* survival inside the mouse macrophages. Mouse macrophages were pretreated overnight or cotreated with IFN-β (100 U/ml) before being infected with *B. pseudomallei* at MOI of 2:1. After 8 hours of infection, the intracellular bacteria were liberated and plated on tryptic soy agar. The number of bacterial colony was counted. The value shown represents means and standard deviations of 3 independent experiments. *, $P<0.05$

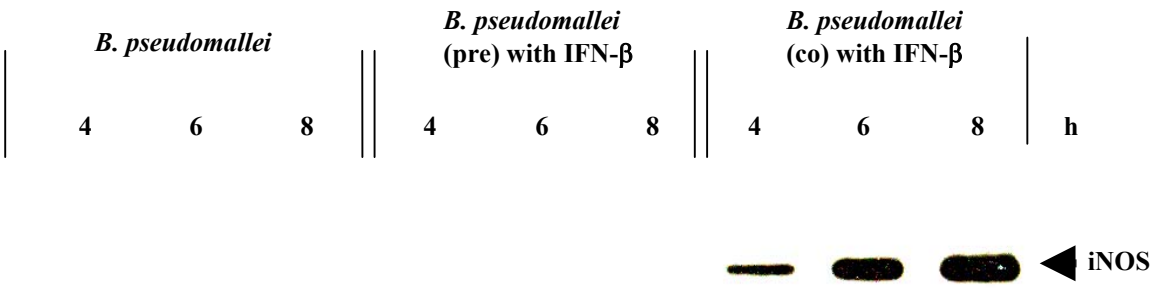


Figure 10 Effect of IFN-β on iNOS expression from the macrophages infected with *B. pseudomallei*. Mouse macrophages were pretreated overnight or cotreated with IFN-β (100 U/ml) before infected with *B. pseudomallei* at MOI of 2:1. After 4, 6 and 8 hours of infection, the cells were lysed and iNOS was determined by western blotting.

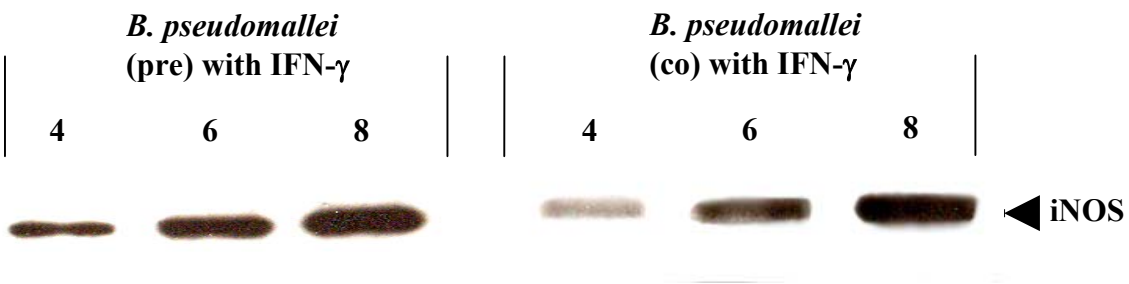


Figure 11 Effect of IFN-γ on iNOS expression from the macrophages infected with *B. pseudomallei*. Mouse macrophages were pretreated overnight or cotreated with IFN-γ (10 U/ml) before infected with *B. pseudomallei* at MOI of 2:1. After 4, 6 and 8 hours of infection, the cells were lysed and iNOS was determined by western blotting.

***Production of IRF-1 and iNOS by macrophages infected with* B. pseudomallei**

Interferon Regulatory Factor 1 (IRF-1) is a transcription factor which known to play essential role in regulating iNOS expression. In order to demonstrate the correlation between IRF-1 and iNOS expression, the macrophages were infected with *B. pseudomallei* (or *S. typhi* used for comparison) at MOI of 2:1. At different times after the infection, the cells were lysed and subjected to immunoblotting using mAbs against IRF-1 and iNOS. The results shown in Fig.12 demonstrated that the macrophages infected with *B. pseudomallei* for as long as 8 h produce neither IRF-1 nor iNOS proteins. However both IRF-1 and iNOS could be readily detected in the macrophages infected with *S. typhi* even after only 4 h of infection and the levels gradually increased with a prolonged period of infection. The production of both IRF-1 and iNOS by these *S. typhi*-infected macrophages paralleled that of IFN-β. These results indicated that *B. pseudomallei* also failed to activate IRF-1 and iNOS expression in the mouse macrophages.

Effect of exogenous IFN-β on IRF-1 expression

The macrophages were either co- or preactivated with IFN-β (100 U/ml) before exposed with *B. pseudomallei* at MOI of 2:1. At time points indicated, the cells were lysed and IRF-1 was determined by Immunoblotting. As shown in Fig. 13, the cells which were simultaneously exposed IFN-β and *B. pseudomallei* was able to express both IRF-1 and iNOS. In contrast, the macrophages that were preactivated with IFN-β prior to bacterial infection were unable to produce IRF-1 and iNOS. Interestingly, the macrophages which either co- or preactivated with IFN-γ were able to upregulate both IRF-1 and iNOS protein. These results suggest that eventhough IFN-β and IFN-γ was able to upregulate iNOS leading to intracellular bacterial killing. The mechanisms between these two cytokines were different.

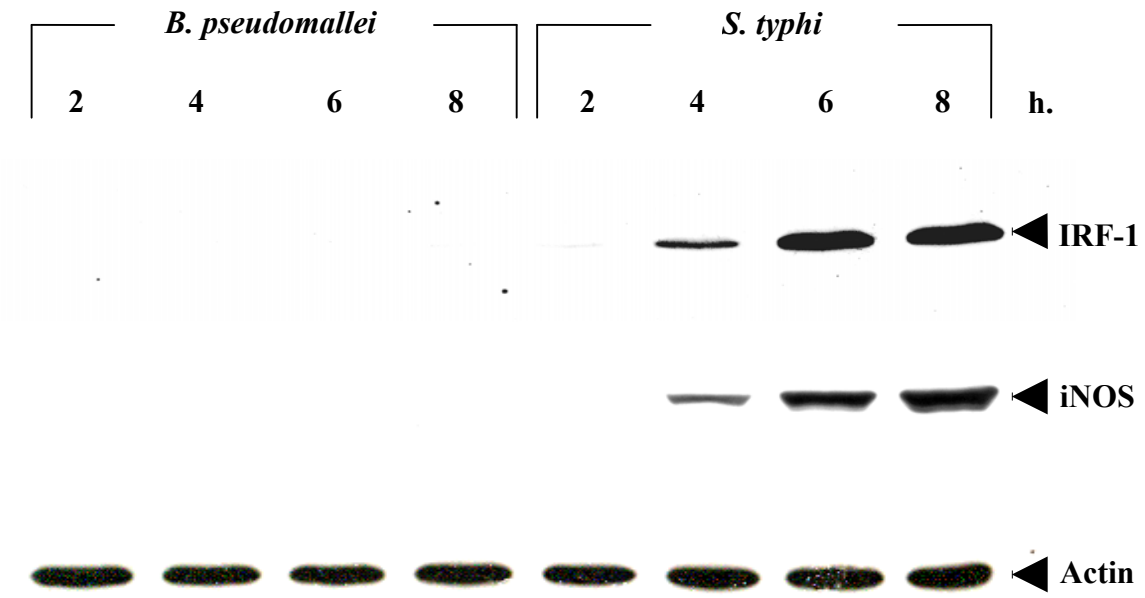


Figure 12 Kinetics of iNOS and IRF-1 expression by infected macrophages. Mouse macrophages were exposed to *B. pseudomallei* or *S. typhi* at MOI of 2:1. At 2, 4, 6 and 8 h after infection, the cells were lysed with lysis buffer and then subjected to immunoblotting using anti-iNOS, anti-IRF-1 and anti-actin (as control).

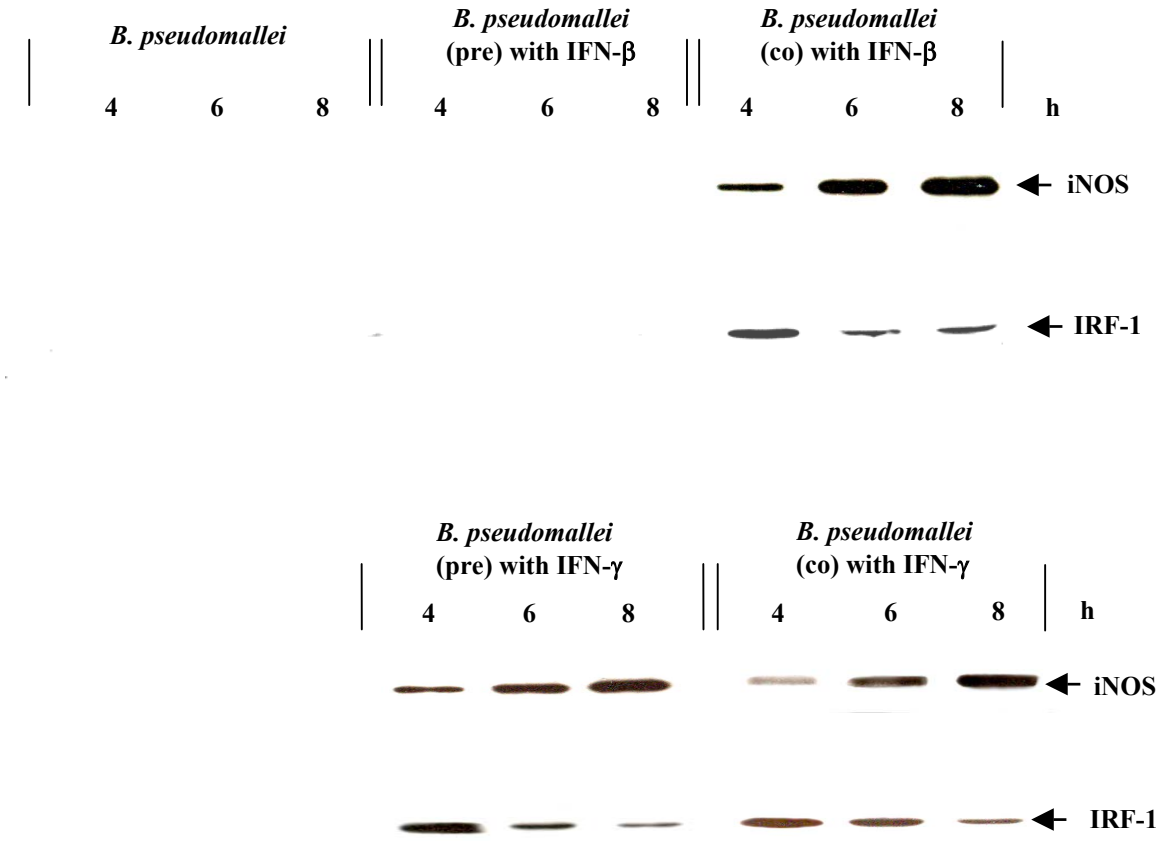


Figure 13 IFNs induce expression of iNOS and IRF-1 in *B. pseudomallei* infected macrophages. Mouse macrophages were prestimulated with either 100 U/ml of IFN-β or 10 U/ml of IFN-γ for 18 h before or costimulated at the time of exposure to *B. pseudomallei* (MOI 2:1). At 4, 6 and 8 h post infection, the cells were lysed with lysis buffer and cell lysate was subjected to immunoblotting using anti-iNOS and anti-IRF-1.

Kinetics of IRF-1 expression by IFN-β and IFN-γ

It is well documented that both IFN-β and IFN-γ, alone by themselves, can stimulate a significant degree of IRF-1 expression. The presence of this transcription factor is required for iNOS gene transcription. We, therefore, analyse the way by which these 2 IFNs differentially regulate the IRF-1 expression by immunoblot. In this experiment, the IFN was added to uninfected macrophages and the levels of IRF-1 expression in the lysate were followed by immunoblotting as described. The results presented in Fig. 14 demonstrated that the expression of IRF-1 in the macrophages stimulated with IFN-β gradually increased and reached a maximum after 2-4 h before being degraded and finally disappeared altogether by 18 h. Unlike the IFN-β, IFN-γ was not only able to stimulate IRF-1 but in addition it was also able to sustain the activation state of IRF-1 for a period as long as 24 h. In fact, a trace of IRF-1 expression could be detected at 36 h when the experiment was terminated.

***Production of IFN-β by macrophages activated with heat killed* B. pseudomallei**

Several stimulators such as bacterial or bacterial components are known to stimulate IFN-β production in macrophages. We, therefore, determined the ability of different killed bacteria to stimulate IFN-β production. Mouse macrophages cell line (RAW 264.7) was treated with heat killed *B. pseudomallei*, *S. typhi* and *E. coli* at MOI of 2:1 for 8 h. The supernatants were analysed for IFN-β production by ELISA (Fig. 15). The results indicate that the macrophages activated with heat killed *S. typhi* were able to produce IFN-β even when it used at MOI as low as 1:1. On the other hand, the production of IFN-β was detected from the cells activated with heat killed *B. pseudomallei* only when they were treated with heat killed *B. pseudomallei* at MOI of 10:1 and 100:1. It should be noted that IFN-β was produced less than 100 U/ml in the unstimulated cells. These results indicated that heat killed *B. pseudomallei* is significantly poor macrophages activator comparing with heat killed *S. typhi* with regard to its ability to activate IFN-β production.

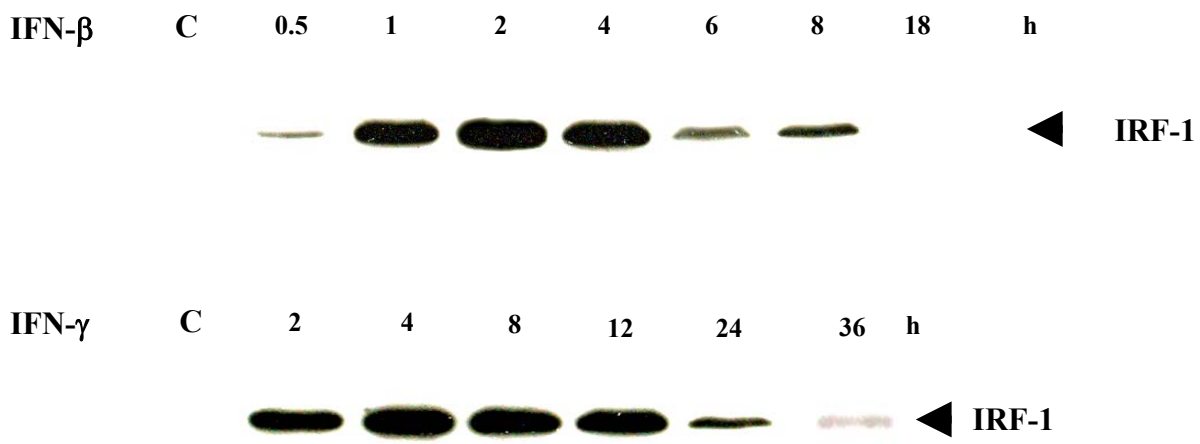


Figure 14 Kinetics of IRF-1 expression from the macrophages activated with IFNs. Mouse macrophages were stimulated with either IFN-β (100 U/ml) or IFN-γ (10 U/ml). At different time intervals, the cells were lysed with lysis buffer and cell lysate was subjected to immunoblotting using anti-IRF-1.

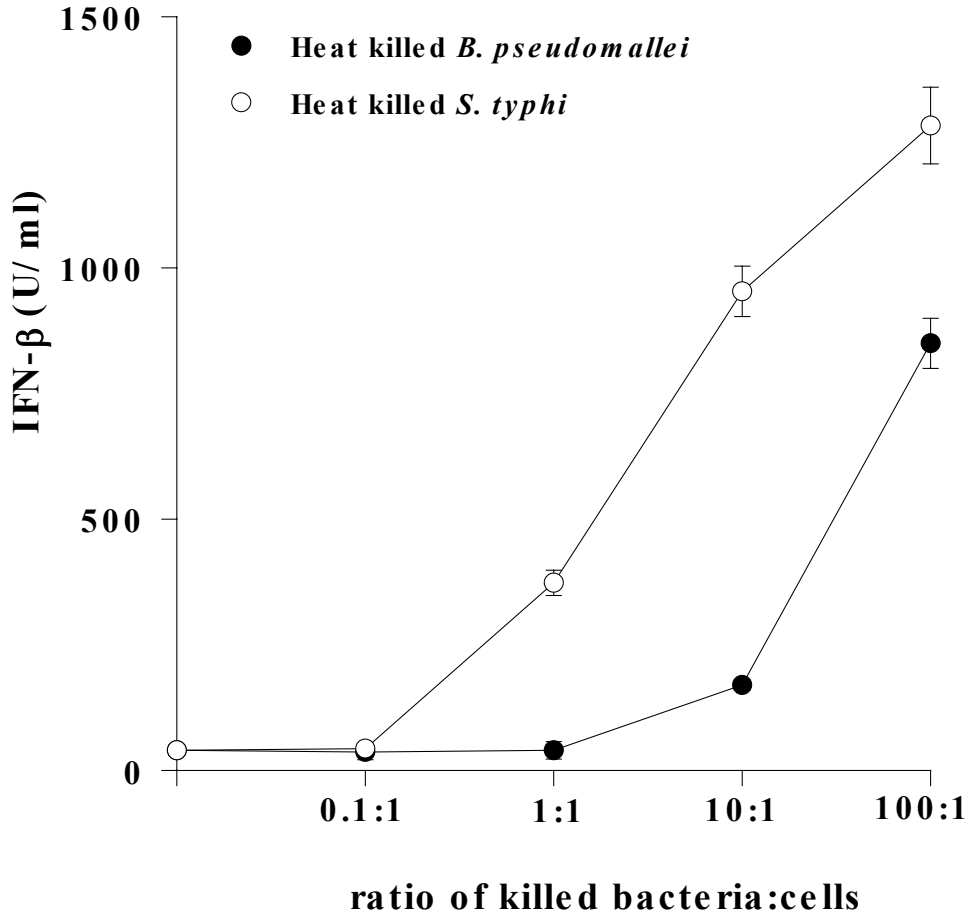


Figure 15 Production of IFN-β by infected-mouse macrophages. Mouse macrophages were exposed to *B. pseudomallei* (●) and *S. typhi* (○) at MOI of 2:1. After 1 h of incubation, the infected cells were washed with PBS before culturing in the medium containing 250 mg/ml of kanamycin to kill residual extracellular bacteria. After 2 hours, the medium was replaced with medium containing 20 μg/ml of kanamycin. At 8 hours after infection, the supernatants were analysed for IFN-β by ELISA. Data represent mean and s.d. of 2 separate experiments with triplicate samples.

***Production of IFN-β by macrophages infected with* B. pseudomallei**

Mouse macrophages were infected with *B. pseudomallei* at MOI of 2:1 for 1 h and washed with PBS before culturing in a medium containing 250 μg/ml of kanamycin. At 4, 6 and 8 hours after infection, the supernatants were analysed for IFN-β production by ELISA. As shown in Fig. 16, the supernatants from the cells infected with *B. pseudomallei* for as long as 8 h contained negligible concentration of IFN-β. In fact, the level of IFN-β produced by the *B. pseudomallei*-infected macrophages was only slightly higher than that of the uninfected macrophages. On the other hand, the levels of IFN-β from cells infected with either *S. typhi* or *E. coli* used for comparison were significantly higher than that of the *B. pseudomallei*-infected cells throughout the period of observation. These results clearly demonstrated that *B. pseudomallei* behaved differently from other bacteria with regard to its ability to stimulate macrophages for IFN-β production.

Effect of IFN-β on iNOS expression in infected macrophages

In order to investigate the possible interrelationship between IFN-β produced by bacterial-infected macrophages and iNOS expression, the mouse macrophages were exposed to *S. typhi* at MOI of 2:1 for 1 h in the presence or absence of neutralizing monoclonal antibody against IFN-β (10 μg/ml). In this experiment, only *S. typhi* was used because under the experimental condition employed, no iNOS was detected in *B. pseudomallei* infected macrophages. The cells were then washed with PBS before culturing in the medium containing 250 μg/ml of kanamycin and Ab to IFN-β. Six hours after the infection, the cells were lysed with lysis buffer and analysed for iNOS and IRF-1 production by immunoblotting. As shown in Fig. 17, mAb against IFN-β totally abolished IRF-1 expression from the macrophages infected with *S. typhi*, indicating that the expression of IRF-1 was mediated by IFN-β. However, under this condition, a very low level of iNOS protein could still be observed. These results implied that IFN-β endogenously produced by the bacterial infected macrophages could enhance iNOS production in an autocrine and/or paracrine fashion.

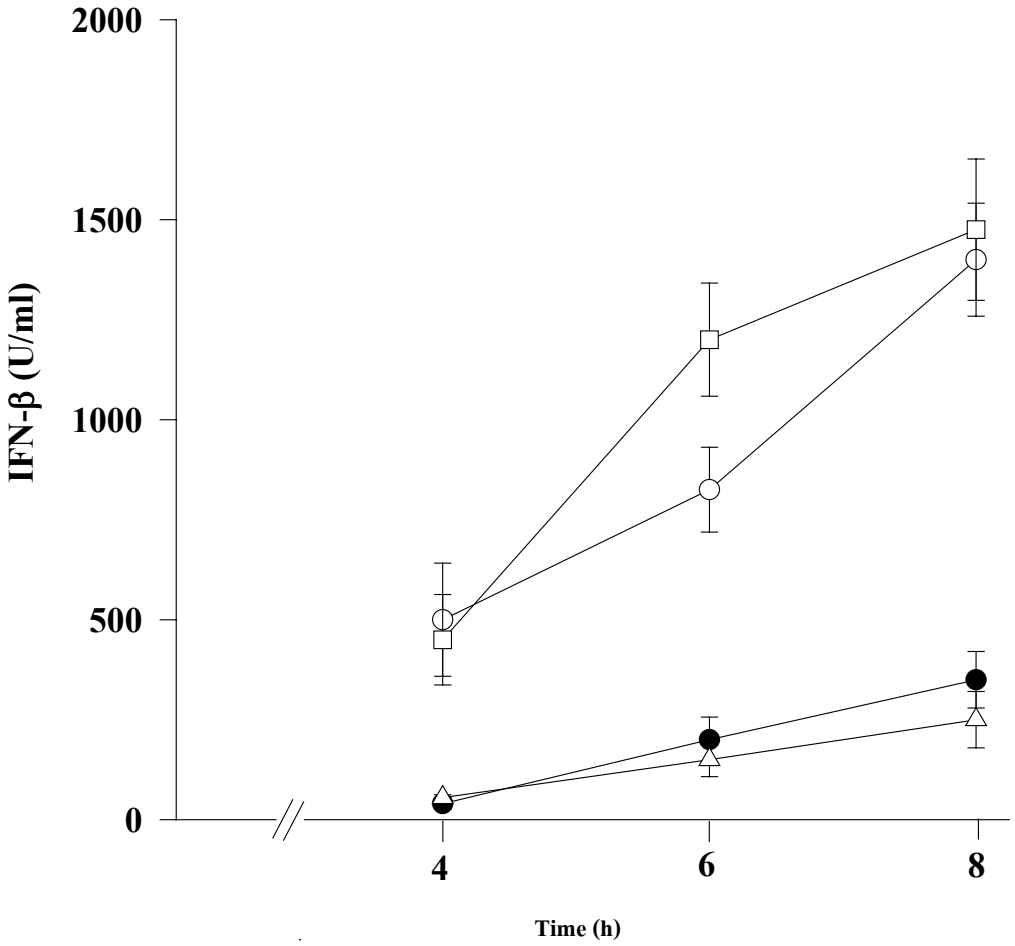


Figure 16 Production of IFN-β by infected-mouse macrophages. Mouse macrophages were exposed to *B. pseudomallei* (•), *S. typhi* (□) or *E. coli* (○) at MOI of 2:1. After 1 h of incubation, the infected cells were washed with PBS before culturing in the medium containing 250 mg/ml of kanamycin to kill residual extracellular bacteria. At 4, 6 and 8 hours after infection, the supernatants were analysed for IFN-β by ELISA. Unstimulated macrophages were used as control (Δ). Data represent mean and s.d. of 2 separate experiments with triplicate samples.

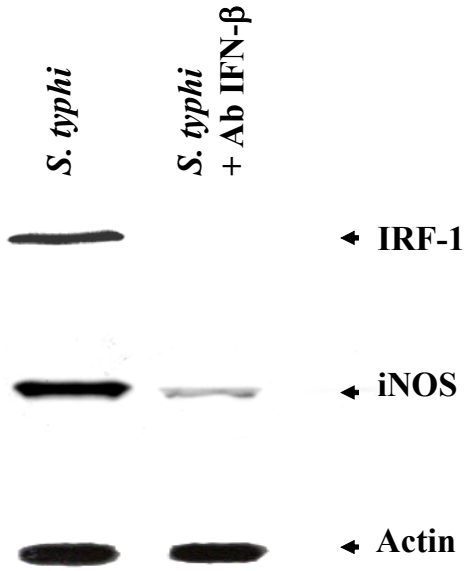


Figure 17 IFN-β neutralizing antibody attenuates the expression of iNOS and IRF-1 in macrophages infected with *S. typhi*. Mouse macrophages were infected with *S. typhi* at MOI of 2:1 in the presence or absence of anti-IFN-β (10 mg/ml). After 1 h, the cells were washed with PBS before culturing in the medium containing 250 mg/ml of kanamycin. It should be noted that this antibody was kept in the culture medium throughout the experiment. At 8 h after infection, iNOS, IRF-1 and actin from the infected cells were determined by immunoblotting.

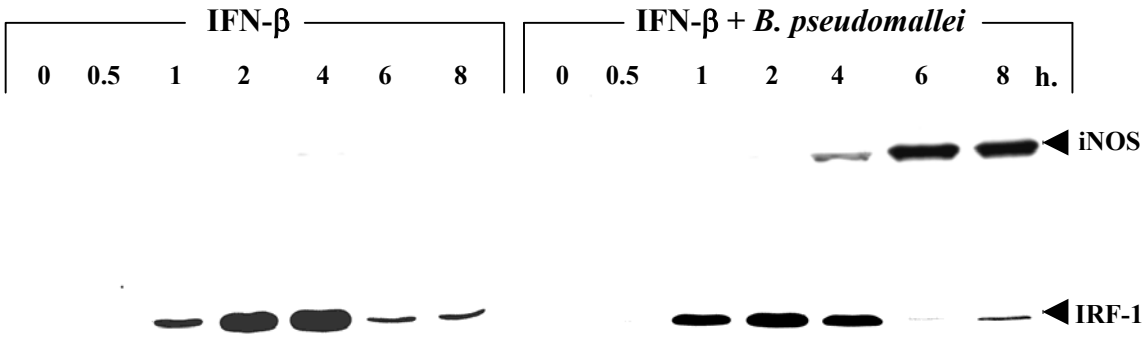


Figure 18 Exogenous IFN-β enhances the expression of iNOS in *B. pseudomallei*-infected mouse macrophages. Macrophages were activated with IFN-β (100 U/ml) in the absence or presence of *B. pseudomallei* (MOI 2:1) for 1 h, then washed with PBS before culturing in the medium containing 250 mg/ml of kanamycin. At different time intervals, the cells were lysed and subjected to immunoblotting using anti-iNOS and anti IRF-1.

Discussion

Inducible nitric oxide synthase is known to play a significant role in host defense against a number of microbial infections. Intracellular microbial killing is often associated with the expression of iNOS and NO (22). When *Leishmania major* promastigotes were injected into the peritoneal cavity of wild type ($iNOS^{+/+}$) and iNOS-knockout ($iNOS^{-/-}$) mice, 40 fold more parasites was recovered from the knockout mice 24 h after the parasite injection (6). In other models, the survival rate of $iNOS^{-/-}$ mice infected with *Listeria monocytogenes* and *Mycobacterium tuberculosis* was also reduced compared with the wild type counterpart, indicating that iNOS is an essential enzyme in protective immunity against these bacterial infections (20, 21). The expression of iNOS could be differentially regulated by different microbial products. In addition to live bacteria, bacterial components like endotoxins, lipoproteins or exotoxins could also effectively stimulate macrophages to express iNOS (9). Previously, we have demonstrated that, unlike other gram negative bacteria, *B. pseudomallei* failed to stimulate iNOS expression in mouse macrophages therefore facilitate this bacterium to survive inside the macrophages (25). However, preactivation the macrophages with IFN-γ were able to enhance the infected cells to upregulate iNOS expression as well as increase the antimicrobial activity of the cells (25).

The roles of IFN-β in innate immunity against microbial infection have been reported (2). Recombinant IFN-β has been shown to protect mice against an infection with *L. monocytogenes*. A similar protective effect of IFN-β was also observed in mice infected with *Trypanosoma cruzi* or *Toxoplasma gondii*. The possible mechanism(s) by which IFN-β enhances antimicrobial activity of the macrophages may be related to the fact that it enhances iNOS and NO production by the macrophages infected with the microorganism. In the *in vitro* study, a costimulation of the cells with IFN-β and *L. major* activates iNOS expression and NO production, leading to the inhibition of intracellular survival of parasites. In the present study, we demonstrated that Type I IFN, IFN-β in particular, was able to synergistically enhance iNOS expression as well as enhancing NO production in the macrophages treated with *B. pseudomallei*-LPS or heat killed bacteria (Fig.1-4). The level of NO production was directly correlated with the concentration of IFN (Fig. 1 and Fig. 2). In this study we show that simultaneously exposed IFN-β with the bacteria, the macrophages were able to upregulate iNOS expression (Fig. 5). Moreover, exogenous IFN-β was also able

to suppress intracellular viability of bacteria inside the macrophages which may lead to the significantly reduction of MNGC formation (Fig 7). Interestingly, the ability of IFN-β to enhance iNOS expression and antibactericidal was not observed if the macrophages were with IFN-β. On the contrary, the timing of exposure was not critical for the IFN-γ because when the cells were either prestimulated or costimulated with IFN-γ, both iNOS expression and intracellular killing capacity were enhanced (Fig 11).

Interferon Regulatory Factor-1 (IRF-1) is a transcriptional activator which binds to sites within the promoters of a number of genes including the iNOS gene. Typically, IRF-1 is not expressed at a detectable level in unstimulated murine macrophages. However, IRF-1 expression can be induced by a variety of activators including IFN-β. The expression of IRF-1 has been reported to be an important factor for the induction of iNOS gene. In the present study, we demonstrated that a gram-negative bacterium like *S. typhi* can stimulate IRF-1 expression and that this correlates with iNOS production (Fig. 12). In contrast, the macrophages infected with *B. pseudomallei* failed to upregulate either IRF-1 or iNOS (Fig 12). However, simultaneously exposed the macrophages with *B. pseudomallei* and IFN-β were able to upregulate IRF-1 and iNOS protein (Fig. 13). It should be mentioned that exogenous IFN-β alone was able to activate IRF-1 expression but not iNOS (Fig. 18). Interestingly, prestimulated the macrophages with IFN-β prior to *B. pseudomallei* infection fail to upregulate IRF-1 which may lead to the inability of IFN-β to enhance iNOS expression and antibactericidal (Fig. 13). On the contrary, upregulation of IRF-1 was observed when the macrophages were either co- or prestimulated with IFN-γ (Fig. 13). The upregulation of IRF-1 was directly correlated with the iNOS expression, which then lead to the inhibition of MNGC formation and intracellular viability of *B. pseudomallei* (Fig. 7). These results indicate that, eventhough, both IFN-β and IFN-γ exhibit ability to increase antibactericidal vial upregulation of IRF-1 and iNOS, the mechanism of these two cytokines were different. This may be due to the fact that both IFN were able to activate IRF-1 differently. IFN-β can stimulate IRF-1 expression and sustains it for only a short period. In contrast, the macrophages stimulated with IFN-γ were able to maintain the IRF-1 level for more than 36 h after activation (Fig. 14). The difference in IRF-1 expression may be due to the fact that IFN-β and IFN-γ use different molecule to transduce the signal (5, 31). The difference in signaling pathways between IFN-β and IFN-γ may result in different kinetics of IRF-1 expression.

Macrophages are known to be one of the major cellular sources of IFN-β produced by the immune system. The production of IFN-β by macrophages is upregulated by microbial infection (e.g. *E. coli* or *L. major*) or by exposure to microbial products (e.g. LPS) (2, 3, 32). Among bacteria, only gram-negative ones can stimulate IFN-β production from mouse macrophages. *Listeria monocytogenes*, *Staphylococcus aureus* or *Lactobacillus bulgaricus* and other gram-positive bacteria failed to activate IFN-β production (32). These results strongly suggested that LPS is the only bacterial component capable of inducing IFN-β in mouse macrophages. Recent evidence indicated that LPS induced expression of gene encoding IFN-β through Toll-like Receptor 4 (TLR4) but not TLR2 agonist (33). However, the LPS isolated from *Porphyromonas gingivalis*, which differs from enterobacterial LPS structurally and functionally, activated proinflammatory cytokine via TLR2 instead of TLR4 (10). The *P. gingivalis* LPS did not stimulate IFN-β production from murine macrophages (33). In the present study, we demonstrated that, unlike other *S. typhi*, living *B. pseudomallei* failed to stimulate IFN-β production in mouse macrophages (Fig. 16). Moreover, heat killed *B. pseudomallei* was able to stimulate significantly less IFN-β when comparing with heat killed *S. typhi* (Fig. 15). Moreover, by neutralizing secreted IFN-β from the *S. typhi* infected cells, the macrophages significantly lose the ability to upregulate IRF-1 which then lead to the reduction of iNOS (Fig. 17). This result indicated that IFN-β acted as paracrine to autoregulate the expression of IRF-1 in the macrophages infected with *S. typhi* which led to enhanced iNOS expression. In contrast, the macrophages infected with *B. pseudomallei* did not produce IRF-1 at a level detectable by the immunoblotting used in the present study (Fig. 13). The failure of IRF-1 production is most likely associated with the inability of the *B. pseudomallei*-infected cells to produce IFN-β because addition of exogenous IFN-β to the system did not only induce IRF-1 expression but also stimulated iNOS expression in these *B. pseudomallei* infected macrophages.

The pathogenesis of *B. pseudomallei* has been extensively investigated in a murine system but very limited information on the mechanism(s) of intracellular bacterial survival is currently available. Previously, we demonstrated that *B. pseudomallei* could invade macrophages without significantly stimulating iNOS expression but could be enhanced by IFN-γ. In the present study, we showed that the inability of this bacterium to stimulate iNOS was related to the failure of the *B. pseudomallei*-infected macrophages to produce IFN-β and

IRF-1. However, exogenous IFN-β could reverse this defect and reinstall the antimicrobial activity of *B. pseudomallei*-infected macrophages. Furthermore, we also demonstrated that eventhough, both IFN-β and IFN-γ increase ability of infected macrophages to upregulate iNOS expression, the mechanism(s) of these two cytokines were different. These may suggest the different role of IFN-β and IFN-γ in macrophages defending against bacterial infection. It is possible that under *in vivo* animal model, IFN-β in conjunction with IFN-γ produced from other sources can synergistically enhance resistance and reduce severity in this *Burkholderia pseudomallei* infection.

In vitro study of the modulation of *Burkholderia pseudomallei* with nonphagocytic cells (Part II)

In the present study, we had investigated the ability of living *B. pseudomallei* and heat killed *B. pseudomallei* to stimulate IL-8 production from human lung epithelial cell line (A549). This study had been performed using *S. typhi* as comparison bacteria. The study will also determine the expression of IL-8 mRNA from the cells infected with *B. pseudomallei*.

Material and Methods

Reagents and cell culture

Human lung epithelial cell line (A549) obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured in Ham's F-12 (HyClone, Logan, UT) supplemented with 10% FBS (HyClone, Logan, UT) at 37^{o}C and 5% CO_2.

Infection of epithelial cells

Human epithelial cells (A549) (2.5×10^5 cells) were cultured in a 6-well plate overnight before exposure to bacteria for 1 h. To remove extracellular bacteria, the cells were washed 3 times with 2 ml of PBS before replacing with Ham's F12 containing 250 μg/ml kanamycin (Gibco Labs). At the time indicated, the supernatant were collected for IL-8 analysis while the cells were lysed and mRNA was isolated.

IL-8 production

The concentrations of IL-8 in the supernatant of infected cells were measured using enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN).

Reverse transcriptase-polymerase chain reaction

Human lung epithelial cell line (A549) was infected with bacteria at MOI of 10:1 for 1 hour. At specific times, the cells were extracted for mRNA isolated as described by manufacture (eppendrof, Hamburg, German). The extracted mRNA was subsequently used for cDNA synthesis (eppendrof). The PCR reaction was conducted by using cDNA as template for IL-8. The primers for IL-8 were: sense 5'ATG ACT TCC AAG CTG GCC GTG GCT 3', antisense 5'TCT CAG CCC TCT TCA AAA ACT TCT C 3'. The amplified products were electrophoresed on 1.8% agarose gel before being transferred to Hybond-N^+ membrane (Amersham, Aylesbury, UK). The membranes were prehybridized in buffer containing 1% bovine serum albumin (BSA), 7% SDS, 1 mM EDTA, 0.5 M phosphate buffer at 60^{o}C overnight with radiolabelling ^{32}P-ATP oligonucleotide probes of IL-8 (5' CTG CGC CAA CAC AGA AAT TA 3'). Thereafter, the membranes were washed and subjected to autoradiography.

Immunoblotting

The cells (A549) infected with bacteria were lysed in buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates containing 30 μg of protein were electrophoresed on SDS-PAGE at 10% polyacrylamide and then electrotransferred to nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany). The membrane was blocked with 5% milk for 1 h before incubating overnight with polyclonal antibody to human IκBα (Santa Cruz, Santa Cruz, CA). Blots were then reacted with horseradish peroxidase-conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostic, Mannheim, Germany).

Quantitation of multinucleated giant cells (MNGCs) in B. pseudomallei-infected macrophages

In order to quantitate the degree of MNGC formation, the human lung epithelium cell lines (A549)(5x105) were first cultured overnight on a coverslip. After 2 h of incubation with *B. pseudomallei* at MOI of 10:1, the cells were washed 3 times with PBS and then incubated in DMEM containing 250 μg/ml of kanamycin for 2 h to kill residual extracellular bacteria. At 10 h after infection, the coverslips were washed with PBS, fixed for 15 min with 1% paraformaldehyde and then washed sequentially with 50% and 90% ethanol for 5 min each. The coverslips were air dried before staining with Giemsa. For enumeration of the MNGC formation, at least 1,000 nuclei per coverslip were counted using light microscope at a magnification of 40x and the percentage of multinucleated cells was calculated. The MNGC was defined as the cell possessing more than one nuclei within the same cell boundary.

ELISA

Lung epithelial cells (1.5×10^4cells) were cultured in a 96-well plate overnight before exposure to bacteria at multiplicity of infection (MOI) of 10:1. The measurement of p38 was done by using FACE^{TM} p38 ELISA kit (Active Motif, Carlsbad, CA).

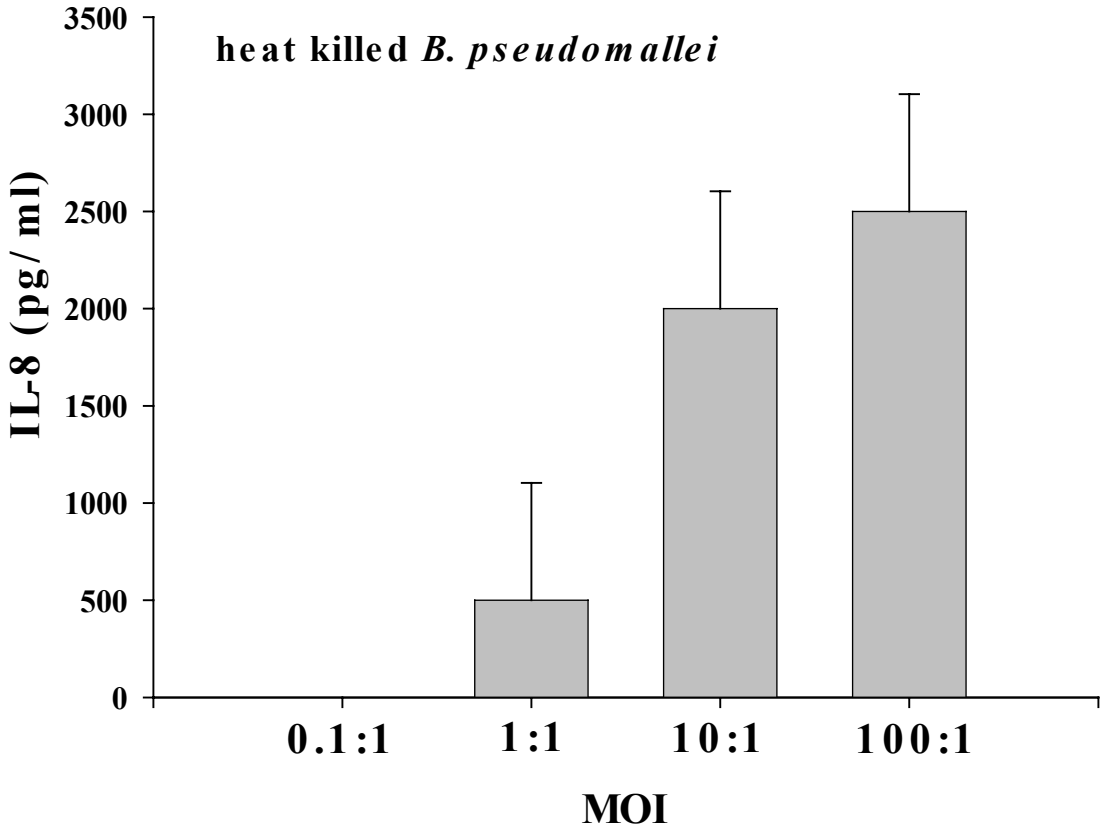
Results

IL-8 production from human lung epithelial cell line (A549) activated with heat killed bacteria

Human lung epithelial cell line (A549) were treated with heat killed *B. pseudomallei* or *S. typhi* at various MOI for 8 hours. The supernatants were determined for IL-8 production by ELISA. As shown in Fig. 19 (A), heat killed *B. pseudomallei* was able to stimulate IL-8 production from the cells at detectable level at MOI higher than 0.1:1. The level of IL-8 produced from the stimulated cells was also depending on the number of kill bacteria. However, the level of IL-8 from the cells stimulated with heat killed *S. typhi* was detected at MOI as low as 0.1:1 (Fig. 19B). Moreover, at the same MOI, the cells stimulated with *S. typhi* were able to produce IL-8 at significantly higher level than the cells treated with heat killed *B. pseudomallei*. These results indicated that even though heat killed *B. pseudomallei* was able to stimulate, this bacterium is a poor activator comparing with *S. typhi*.

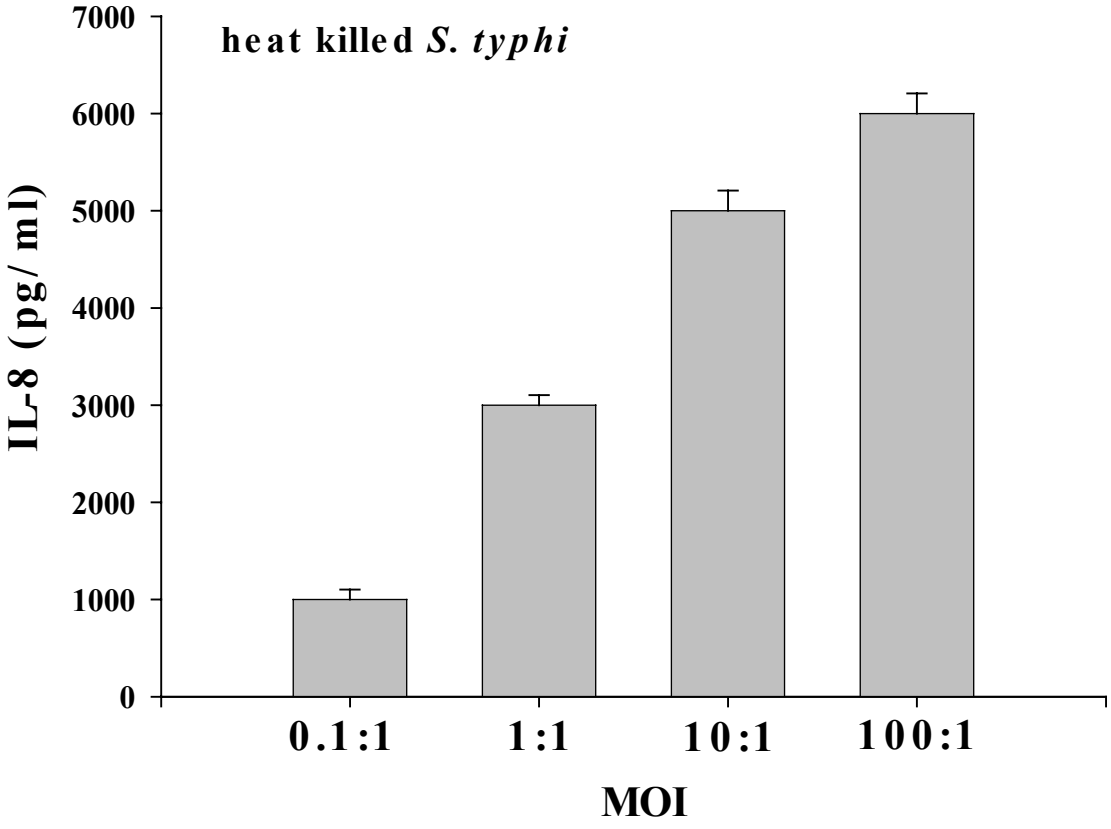
IL-8 production from human lung epithelial cell line (A549) infected with bacteria

In order to investigate the production of IL-8 from the bacterial infected cells, the cells (A549) were infected with living *B. pseudomallei* or *S. typhi* at various MOI. After 1 h, the cells were washed 3 times with PBS before cultured in the medium containing 250 μg/ml of kanamycin. At 8 hours after infection, the supernatants were analysed for IL-8 by ELISA. As shown in Fig. 20, *B. pseudomallei* was able to stimulate IL-8 production from the epithelial cells even at MOI as low as 0.1:1. The level of IL-8 was also depending on the number of bacteria. However, the level of IL-8 produced from *B. pseudomallei* infected cells was significantly lower than the cells infected with *S. typhi* at the similar MOI. Interesting, both living bacteria can stimulate IL-8 production at higher level comparing with the heat killed bacteria.



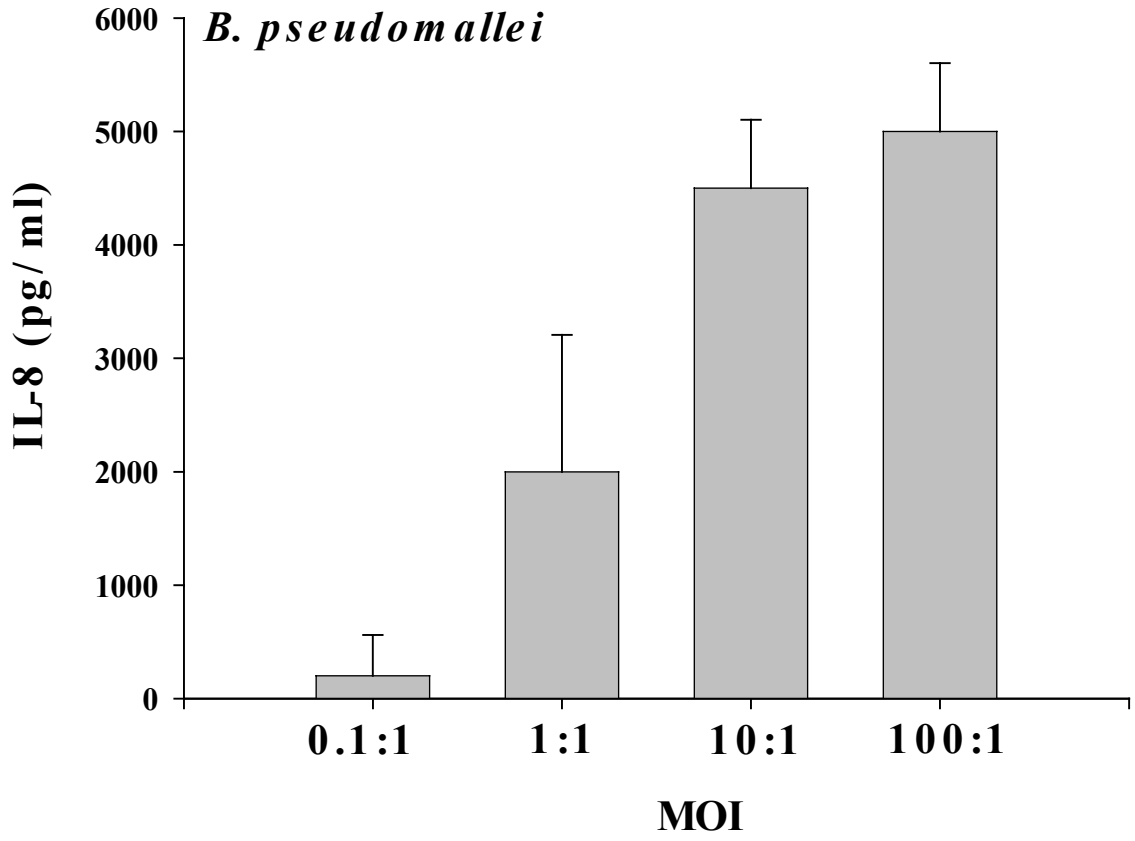
A

Figure 19. Production of IL-8 from human lung epithelial cell line (A549) activated with heat killed bacteria. The cells ($2.5x10^5$ cells) were activated with various MOI of heat killed *B. pseudomallei* (A) or *S. typhi* (B). After 8 h, the supernatants were determined for IL-8 by ELISA. Data represent mean and s.d. of 2 separate experiments with triplicate samples.



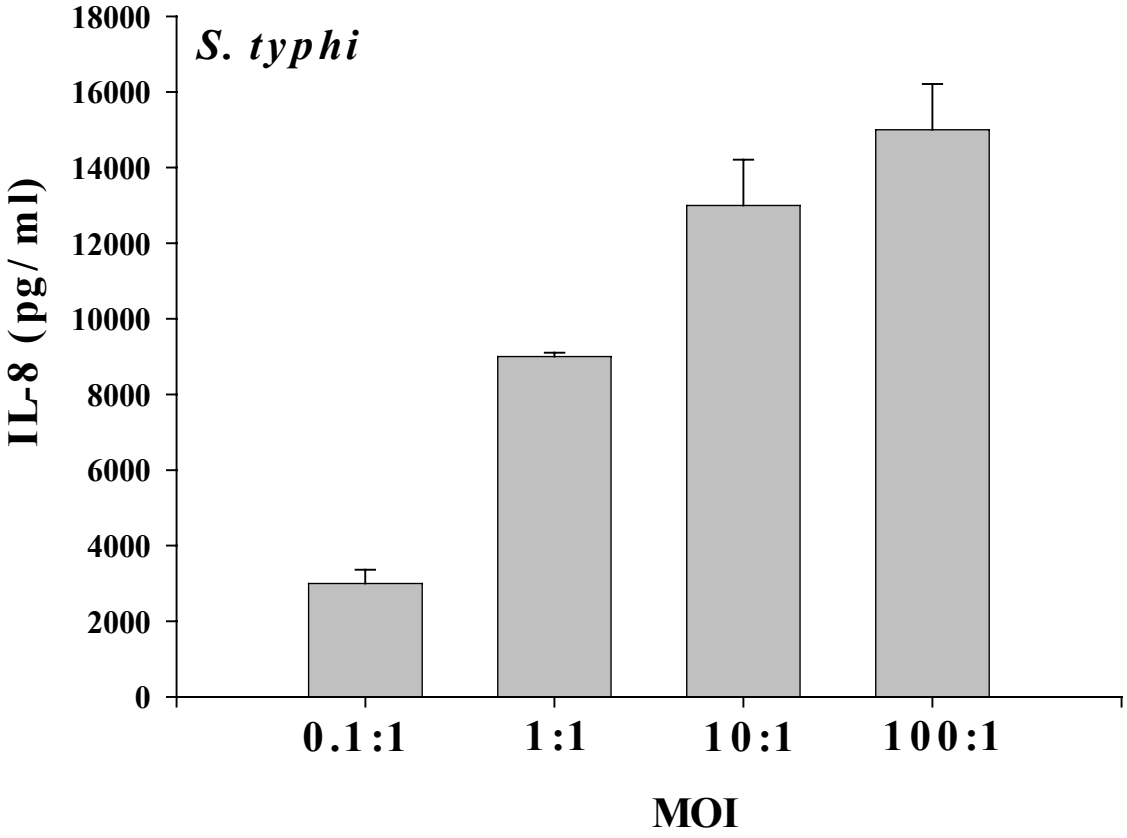
B

Figure 19. Production of IL-8 from human lung epithelial cell line (A549) activated with heat killed bacteria. The cells ($2.5x10^5$ cells) were activated with various MOI of heat killed *B. pseudomallei* (A) or *S. typhi* (B). After 8 h, the supernatants were determined for IL-8 by ELISA. Data represent mean and s.d. of 2 separate experiments with triplicate samples.



A

Figure 20 Production of IL-8 from human lung epithelial cell line (A549) infected with bacteria. The cells ($2.5x10^5$ cells) were infected with *B. pseudomallei* (A) or *S. typhi* (B) at MOI of 0.1:1, 1:1, 10:1 and 100:1. After 1 h, the cells were washed 3 times with 2 ml of PBS before replacing with media containing 250 μg/ml of kanamycin. At 8 h after infection, the supernatants were collected for IL-8 analysis. Data represent mean and s.d. of 2 separate experiments with triplicate samples.



B

Figure 20 Production of IL-8 from human lung epithelial cell line (A549) infected with bacteria. The cells ($2.5x10^5$ cells) were infected with *B. pseudomallei* (A) or *S. typhi* (B) at MOI of 0.1:1, 1:1, 10:1 and 100:1. After 1 h, the cells were washed 3 times with 2 ml of PBS before replacing with media containing 250 μg/ml of kanamycin. At 8 h after infection, the supernatants were collected for IL-8 analysis. Data represent mean and s.d. of 2 separate experiments with triplicate samples.

Kinetics study of IL-8 production from the cells (A549) infected with bacteria

The cells (A549) were infected with *B. pseudomallei* or *S. typhi* at MOI of 10:1. After 1 hour, the cells were washed 3 times with PBS before cultured in the medium containing 250 μg/ml of kanamycin. The supernatants were collected at 0.5, 1, 2, 4, 6 and 8 hours and analysed for IL-8 production. It should be mentioned that the supernatants at time 0.5 an 1 hour were not subjected to kanamycin, the samples were centrifuged (to removed extracellular bacteria) and the supernatants were used to determined IL-8 production. As shown in Fig. 21, the cells infected with *B. pseudomallei* were able to produce IL-8 at detectable level after 2 hours of infection. The level of IL-8 was gradually increased and reached the maximum after 6 hours of infection. Similar results were observed from the cells infected with *S. typhi*. However, comparing with the *S. typhi* infected cells, the level of IL-8 produced from the cells infected with *B. pseudomallei* was significantly lower.

Expression of IL-8 mRNA in cells infected with bacteria

Expression of IL-8 mRNA was determined by PCR and hybridization with specific probe for IL-8. As shown in Fig. 22, the cells infected with *B. pseudomallei* expressed at detectable level at 30 min after infection. The level of IL-8 mRNA was then gradually increased. Similar result was observed form the cells infected with *S. typhi*. However, the level of IL-8 mRNA from the *S. typhi* infected cells was significantly higher than the cells infected with *B. pseudomallei* at the same time point. It should be mentioned that the uninfected cells (served as a control) did not express IL-8 mRNA. These results indicated that even though *B. pseudomallei* can stimulate expression of IL-8 mRNA, this bacterium is a poor activator comparing with other gram negative bacteria such as *S. typhi*.

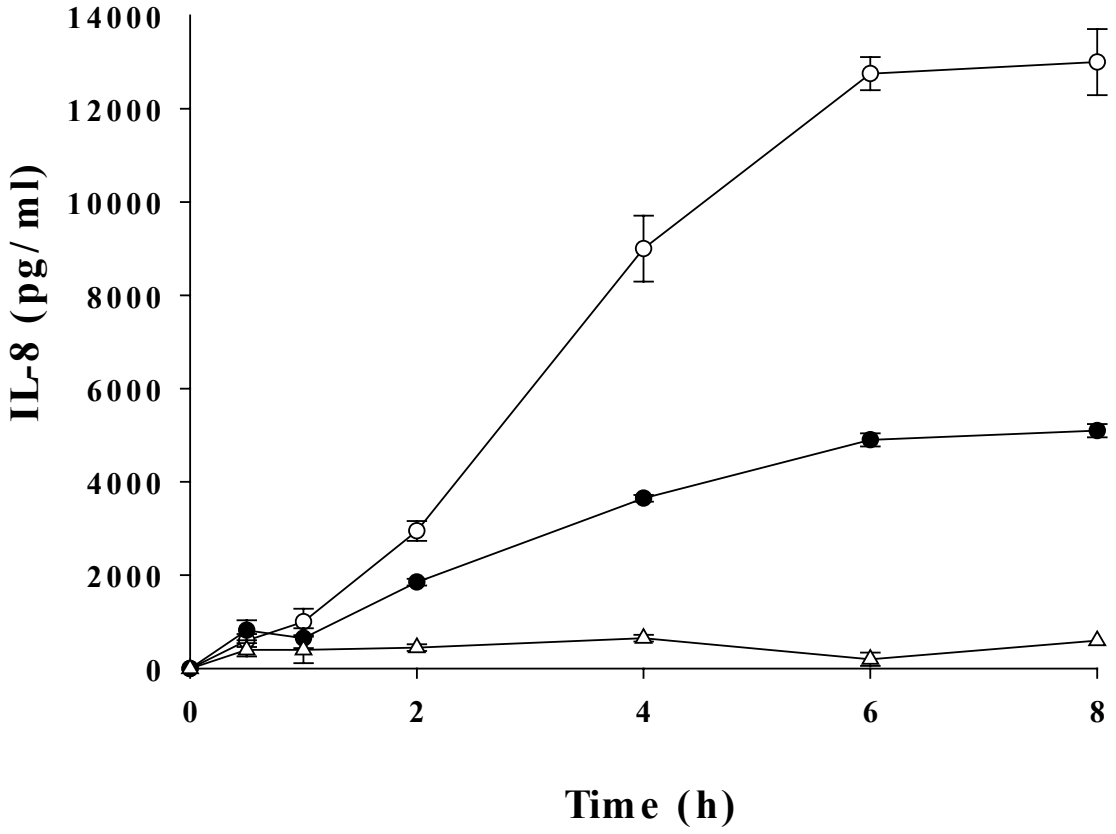


Figure 21 Kinetics of IL-8 production from human lung epithelial cell line (A549) infected with bacteria. The cells ($2.5x10^5$ cells) were infected with *B. pseudomallei* (●) or *S. typhi* (○) at MOI of 10:1. After 1 h, the cells were washed 3 times with 2 ml of PBS before replacing with media containing 250 μg/ml of kanamycin. At different times interval, the supernatants were collected for IL-8 analysis. The uninfected cells (Δ) were served as control. Data represent mean and s.d. of 2 separate experiments with triplicate samples.

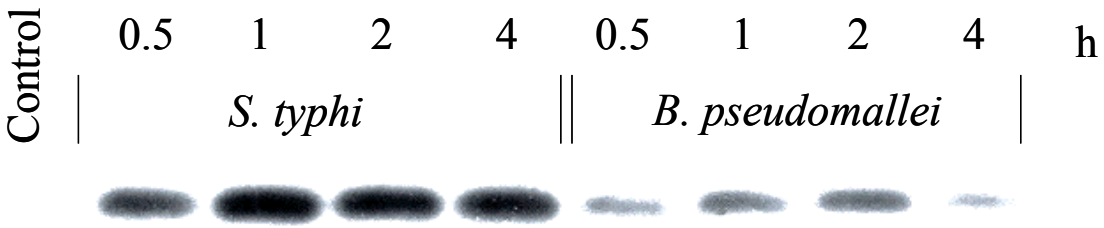
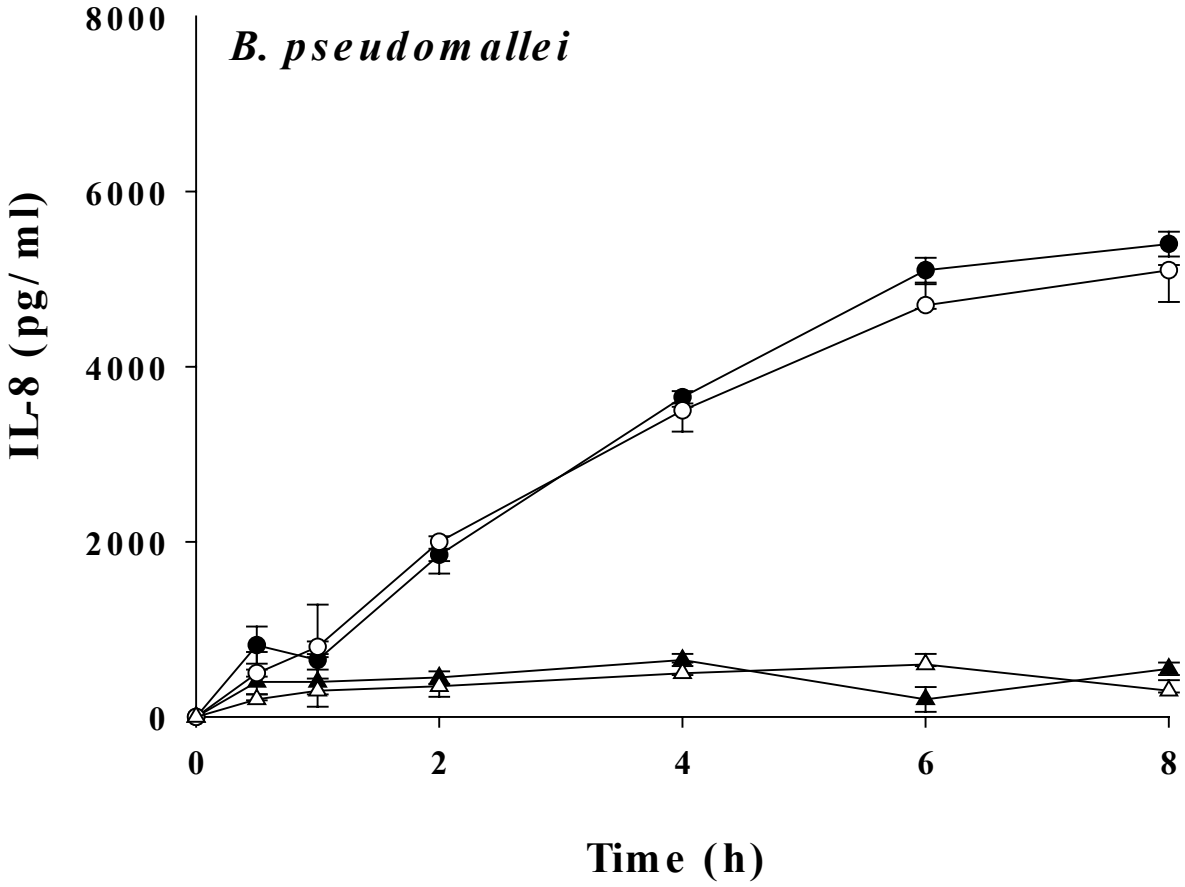


Figure 22 Expression of IL-8 mRNA on the cells infected with bacteria. The human epithelial cell line (A549) ($2.5x10^5$ cells) were infected with *B. pseudomallei* or *S. typhi* at MOI of 10:1. The cells were lysed and the mRNA was isolated and subsequently used for cDNA synthesis. The expression of IL-8 mRNA was determined by PCR followed by hybridization with specific probe for IL-8.

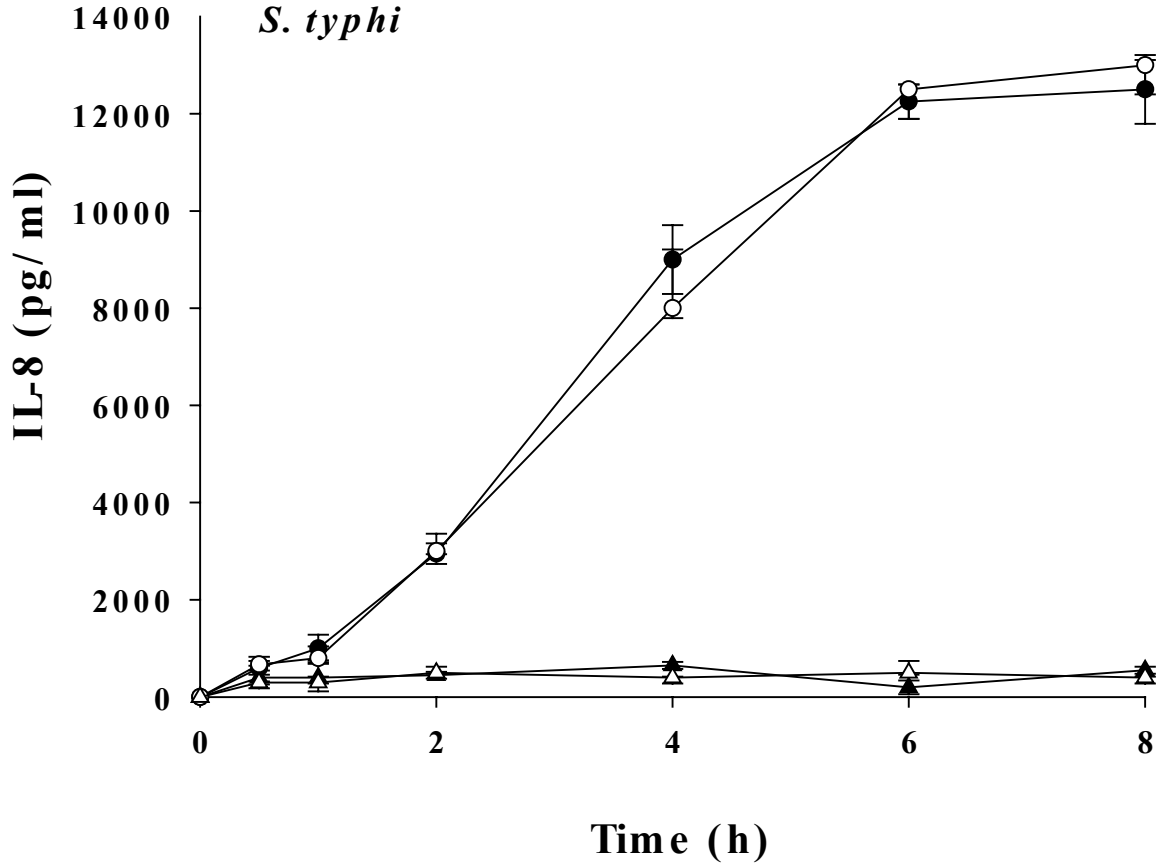
Effect of cytochalasin D on IL-8 production of the cells infected with bacteria

Cytochalasin D is an inhibitor for actin polymerization. This inhibitor is known to prevent the internalization of bacteria into the host cells. Therefore we used this inhibitor to investigate that the signal for IL-8 production from the cells infected with bacteria was triggered after the bacteria interact to the cell surface or after the bacteria were internalized into the cells. In order to investigate this phenomenon, the cells (A549) were pretreated with cytochalasin D (2.5 μg/ml) for 2 hours. The cells were then infected with *B. pseudomallei* or *S. typhi* at MOI of 10:1. After 1 hour, the cells were washed 3 times with PBS before cultured in the medium containing 250 μg/ml of kanamycin. At specific times, the supernatants were collected and the IL-8 was determined by ELISA. As shown in Fig. 23, in the present of cytochalasin D, the cells infected with either *B. pseudomallei* or *S. typhi* were able to stimulate IL-8 production similar to the cells which were not treated with this inhibitor. This result indicated that the signal for IL-8 production was trigger after the bacteria interact to the cell surface. The production of IL-8 does not require internalization of the bacteria.



A

Figure 23 Effect of cytochalasin D on IL-8 production from the cells infected with bacteria. The cells (A549) were pretreated with (●) or without (○) cytochalasin D (2.5 μg/ml) for 2 h before infected with *B. pseudomallei* (A) or *S. typhi* (B) at MOI of 10:1. After 1 h, the cells were washed with PBS before cultured in the media containing 250 μg/ml of kanamycin. At various times as indicated the supernatants were determined for IL-8 by ELISA. The uninfected cells, which were treated (▲) or untreated with cytochalasin D (Δ), were served as control. Data represent mean and s.d. of 2 separate experiments with triplicate samples.



B

Figure 23 Effect of cytochalasin D on IL-8 production from the cells infected with bacteria. The cells (A549) were pretreated with (●) or without (○) cytochalasin D (2.5 μg/ml) for 2 h before infected with *B. pseudomallei* (A) or *S. typhi* (B) at MOI of 10:1. After 1 h, the cells were washed with PBS before cultured in the media containing 250 μg/ml of kanamycin. At various times as indicated the supernatants were determined for IL-8 by ELISA. The uninfected cells, which were treated (▲) or untreated with cytochalasin D (Δ), were served as control. Data represent mean and s.d. of 2 separate experiments with triplicate samples.

Degradation of IκBα from the cells infected with bacteria

In order for the cells to initiate transcription of IL-8, the cells require the transcription factors to bind to the promoter regions upstream of IL-8 gene. Among them NF-κB is known to be a crucial transcription factor which will bind to the KB site upstream of the IL-8. In the unstimulated cells, NF-κB forms the complex with IκB in the cytoplasmic of the cells. After the cells were stimulated, IκB was degraded by specific proteolytic enzyme in the cytoplasm resulting in the free NF-κB which then will translocate into the nuclease. Therefore, the degradation of IκB from the stimulated cells could be used as an indicator for NF-κB translocation. In order to investigate IκB degradation, the cells were infected with bacteria at MOI 10:1. At specific times, the cells were lysed and the lysed were analysed for IκB by immunoblotting. As shown in Fig. 24, IκB was slightly degraded from the cells infected with *B. pseudomallei* after 30 min of infection. However, in the cells infected with *S. typhi*, the IκB was totally degraded after 30 min of infection before reappeared after 2 hours of infection. These results indicated that the lower level of IL-8 production from the cells infected with *B. pseudomallei* may be due to the fact that this bacterium triggers a poor signal transduction comparing with the cells infected with *S. typhi*.

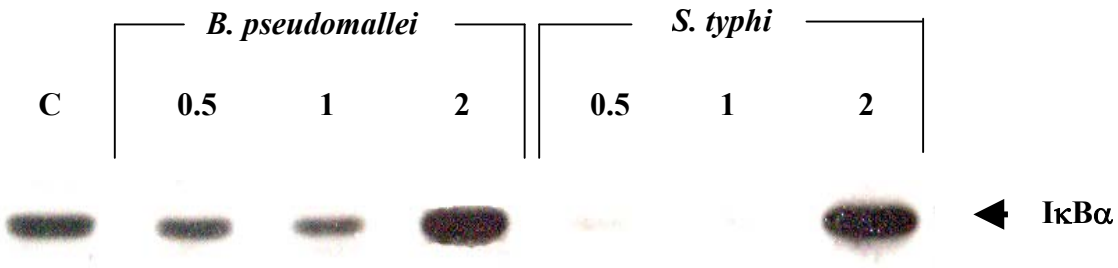
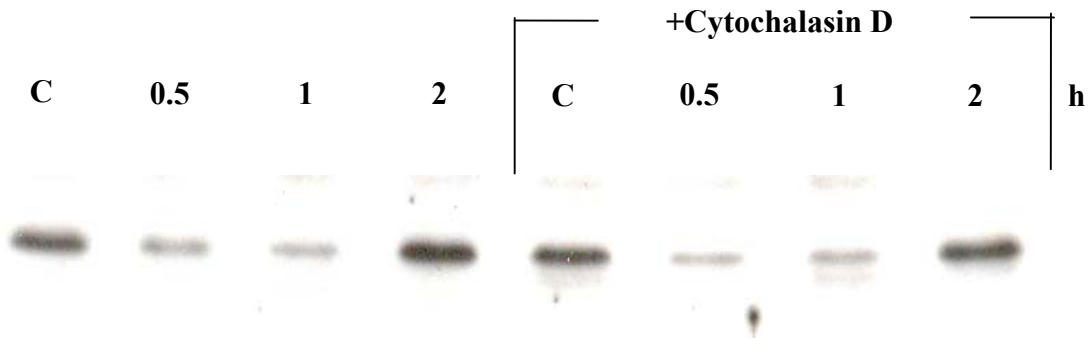


Figure 24 Degradation of IκBα from the cells infected with bacteria. Human lung epithelial cell line (A549) ($2.5x10^5$ cells) were infected with *B. pseudomallei* or *S. typhi* at MOI of 10:1. At different times interval, the cells were lysed and IκBα was determined by Immunoblotting.

A

Figure 25 Effects of cytochalasin D on the cells infected with *B. pseudomallei*. The cells ($5x10^5$ cells/well) were pretreated with cytochalasin D (2 μg/ml) for 2 h before being infected with *B. pseudomallei* at an MOI of 10:1. Expression of IL-8 mRNA was determined by RT-PCR after 2 h of infection (A) and the degradation of IκB was determined by immunoblotting (B).



B

Figure 25 Effects of cytochalasin D on the cells infected with *B. pseudomallei*. The cells ($5x10^5$ cells/well) were pretreated with cytochalasin D (2 μg/ml) for 2 h before being infected with *B. pseudomallei* at an MOI of 10:1. Expression of IL-8 mRNA was determined by RT-PCR after 2 h of infection (A) and the degradation of IκB was determined by immunoblotting (B).

Table I. Effect of cytochalasin D on bacterial internalisation and IL-8 production

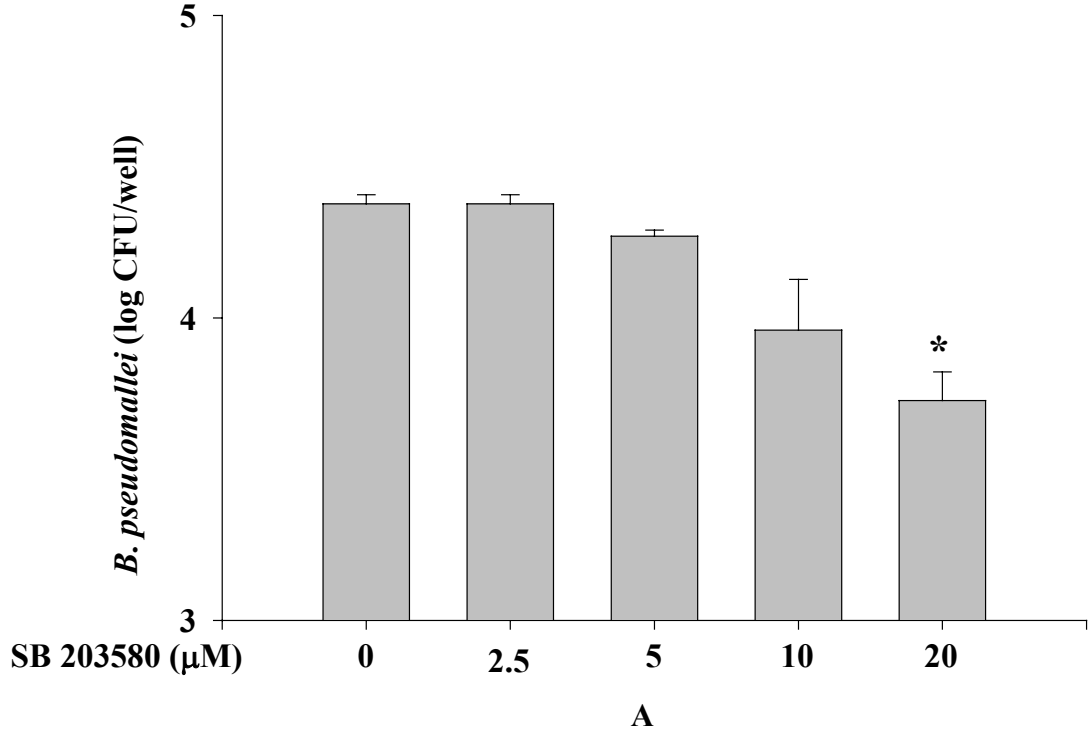
	number of intracellular bacteria	IL-8 pg/ml
B. pseudomallei	100%	4950
B. pseudomallei + cytochalasin D	3.4%	5100

Effect of several signaling protein inhibitors for invasion of B. pseudomallei

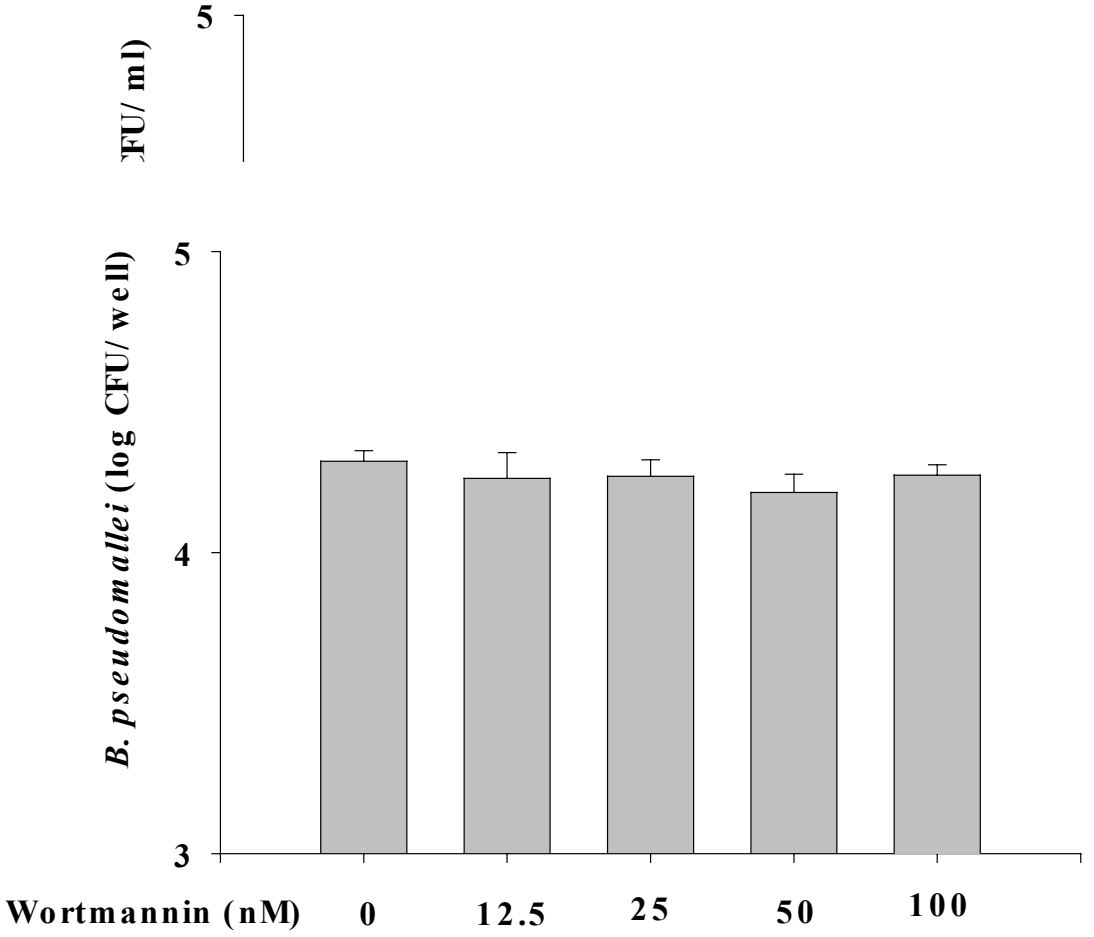
Various inhibitors for cell signaling proteins were used for investigating the host signaling for internalization of *B. pseudomallei*. SB203580 and genistein are the inhibitors for p38 and tyrosin kinase respectively. Wortmannin is a inhibitor for PI3-kinase while PD98059 and U0126 are inhibitor for MEK kinase and MEK1,2 respectively. The human lung epithelium cell lines (A549) were pretreated with these inhibitors at various concentrations for 1 h before infected with *B. pseudomallei*. The effects of these inhibitors for internalization of bacteria were determined by standard antibiotic protection assay. As shown in Fig. 26, SB203580 can significantly inhibit internalization of *B. pseudomallei*. The inhibition effect was a concentration dependent. However, genistein slightly prevent internalization of *B. pseudomallei* when used at 500 μM. In contrast, wortmannin, PD98059 and U0126 did not interfere with bacterial internalization at any concentration used in these experiments. It should be mention that genistein is a general inhibitor for general tyrosin kinase including p38 molecule. These results indicated that internalization of *B. pseudomallei* required activation of p38 protein.

***Effect of inhibitors on the MNGC formation induced by* B. pseudomallei-infected cells**

The ability of *B. pseudomallei* to induce multinucleated giant cell (MNGC) formation has been documented as one of a unique characteristic of this bacterium. The number of MNGC is directly correlated with the number of internalized *B. pseudomallei*. In this experiment, we investigated the effects of host cell signaling protein inhibitor on the MNGC formation induced by *B. pseudomallei*. As shown in Fig. 27, only SB203580 (p38 inhibitor) show significantly inhibition of MNGC formation induced by *B. pseudomallei*. The number of MNGC was also quantitated by Geimsa staining as shown in Fig. 28. The result shown here indicated that only p38 inhibitor significantly reduced MNGC formation induced by *B. pseudomallei* from 22% to 10%, while genistein and PD98059 showed only slightly inhibition of MNGC formation. In contrast, wortmannin and U0126 have no effect on MNGC formation.


5
4
3
B. pseudomallei (log CFU/well)
*
SB 203580 (μM)
0
2.5
5
10
20

A


5
FU/ ml)
5
4
3
B. pseudomallei (log CFU/ well)
Wortmannin (nM)
0
12.5
25
50
100

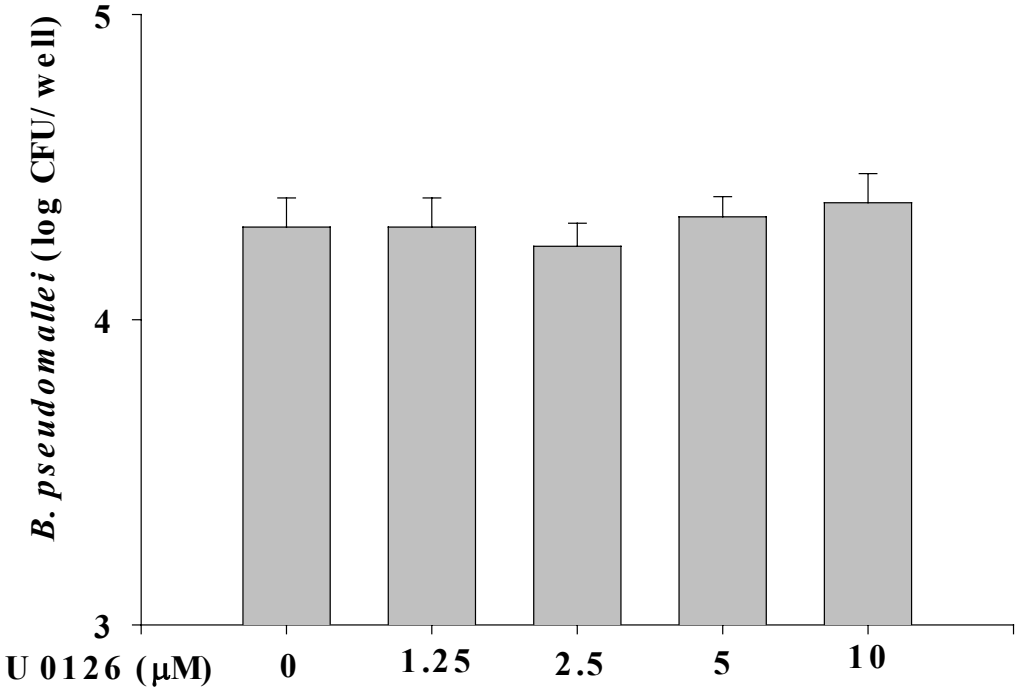


Figure 26 Effect of signaling protein inhibitors on invasion of *B. pseudomallei*. Human lung epithelium cell line (A549) ($5x10^5$ cells/well) were pretreated with various concentration of SB203580 (A), genistein (B), PD98059 (C), wortmannin (D) and U0126 (E) for 1 h before infected with *B. pseudomallei* at MOI of 10:1. The internalized bacteria were determined as described. *, $p<0.05$ by a Student's *t* test

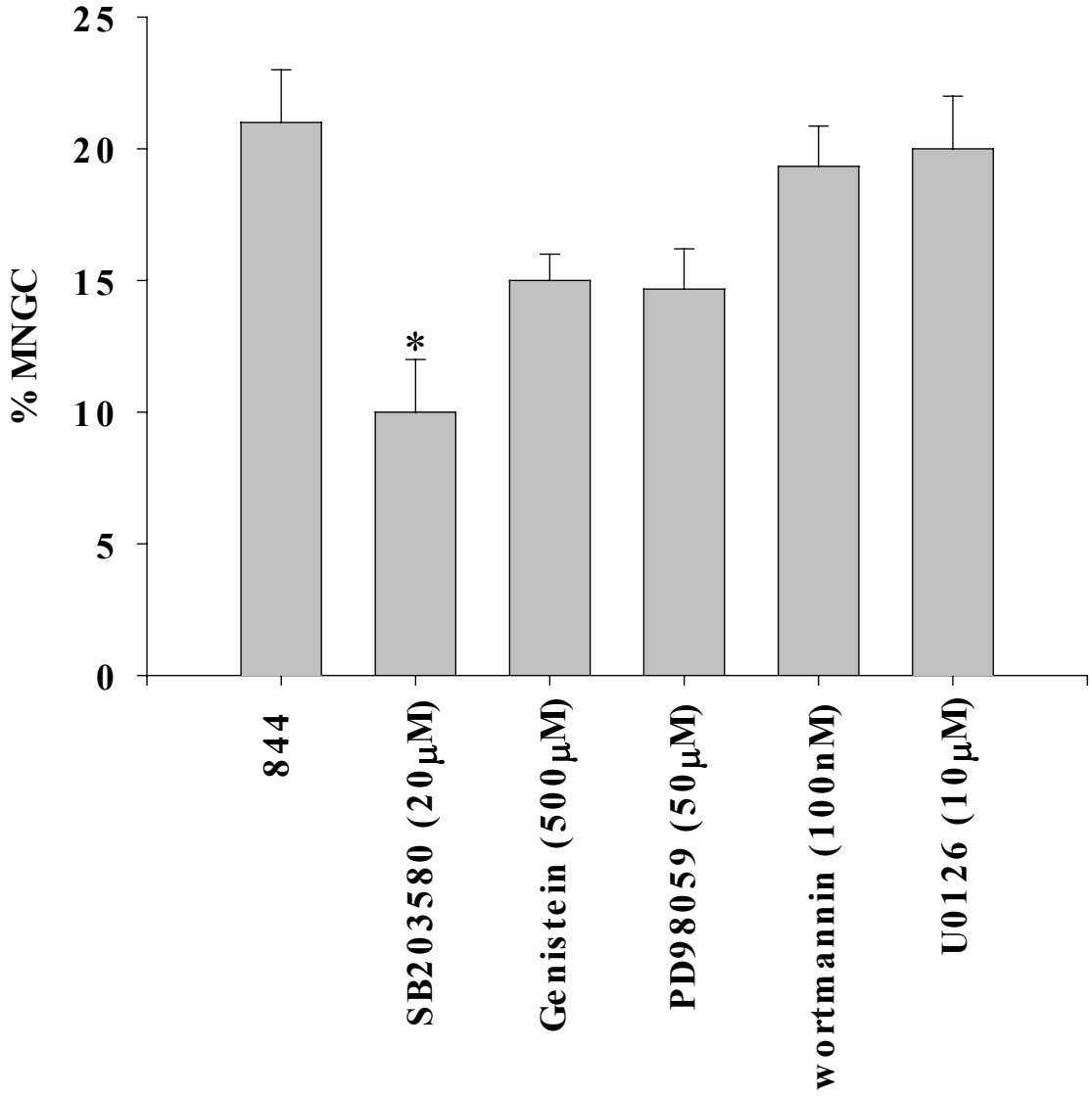


Figure 27 Effect of signaling protein inhibitors on MNGC formation induced by *B. pseudomallei*. Human lung epithelium cell line (A549) ($5x10^5$) were pretreated with 20 μM SB203580, 500 μM genistein, 50 μM PD98059, 100 nM wortmannin and 10 μM U0126 for 1 h before infected with *B. pseudomallei* at MOI of 10:1 as described. The cells were visualized under inverted microscope (40x).

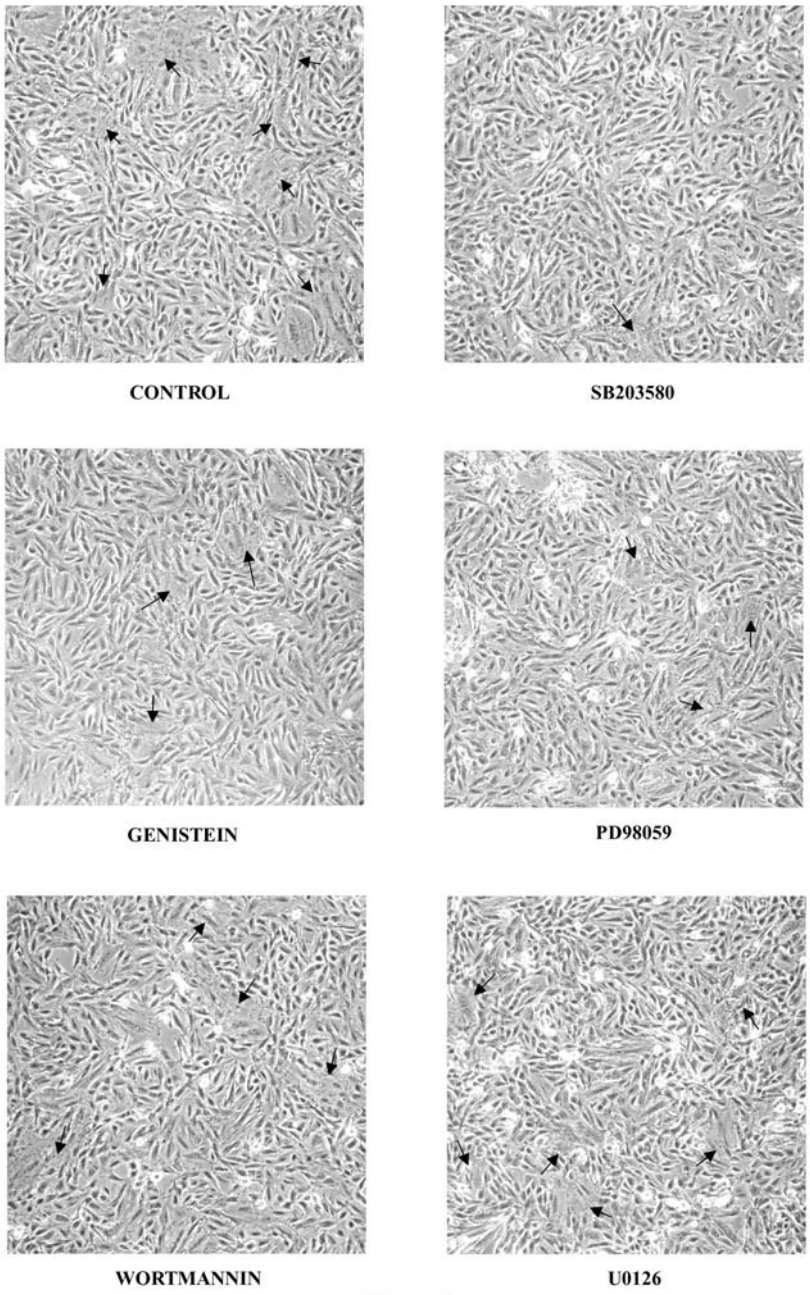


Figure 28 Quantitation of MNGC formation induced by *B. pseudomallei*. Human lung epithelium cell line (A549) were cultured on coverslips and pretreated with signaling protein inhibitors for 1 h before infected with *B. pseudomallei* at MOI of 10:1. The infected cells were washed with PBS before cultured in the medium containing 250 μg/ml of kanamycin. At 10 h post infection, the cells were fixed, stained with Giemsa, and the number of MNGC was determined by microscopic examination (40x). Data represent mean and s.d. of 3 separate experiments. *, $p<0.05$ by a Student's *t* test (arrow indicated the MNGC formation)

Kinetics of the phosphorylation of p38 in human lung epithelial cell line (A549) infected with bacteria

The cells (A549 $1.5x10^4$ cells) were infected with *B. pseudomallei* at MOI of 10:1 for 15 min, 30 min, 1 h and 2 hours. After the infection the cells were washed with PBS to remove extracellular bacteria. The phosphorylation of p38 was determined by using FACE™ p38 ELISA kit. As shown in Fig. 29, the cell infected with *B. pseudomallei* was able to activated phosphorylation of p38 at detectable level within 15 minute after infection. The level of p38 was gradually increased and reached the maximum within 1 hour of infection before decreasing. The result was also consistent with immunoblotting using mAb against p-p38. As shown in Fig. 30, p38 was phosphorylated after 15 min of infection before reaching the maximum at 1 hr after infection. This result indicated that *B. pseudomallei* was able to activated phosphorylation of p38 of the lung epithelial cells.

***Effect of inhibitors on the phosphorylation of p38 activated by* B. pseudomallei**

SB203580, an inhibitor for p38, and Cytochalasin D, an inhibitor for actin polymerization were used for investigating the molecular mechanism of the signaling proteins which required for *B. pseudomallei* invasion. The cells (A549) were pretreated with these inhibitors for 1 hour before infected with *B. pseudomallei* at MOI of 10:1 for 1 hour. After the infection the cells were washed with PBS to remove extracellular bacteria and analysed for p38 production by using FACE™ p38 ELISA kit (Fig. 31) and immunoblotting (Fig. 32). In the present of SB203580, the level of phosphorylation p38 was significantly decreased which indicated that the inhibition of invasion by *B. pseudomallei* observed by standard antibiotic protection assay was due to the phosphorylation of p38. Interestingly inhibition of invasion by Cytochalasin D did not inhibit phosphorylation of p38. This result suggested that activation of phosphorylation of p38 was triggered by the interaction of *B. pseudomallei* to the cell surface.

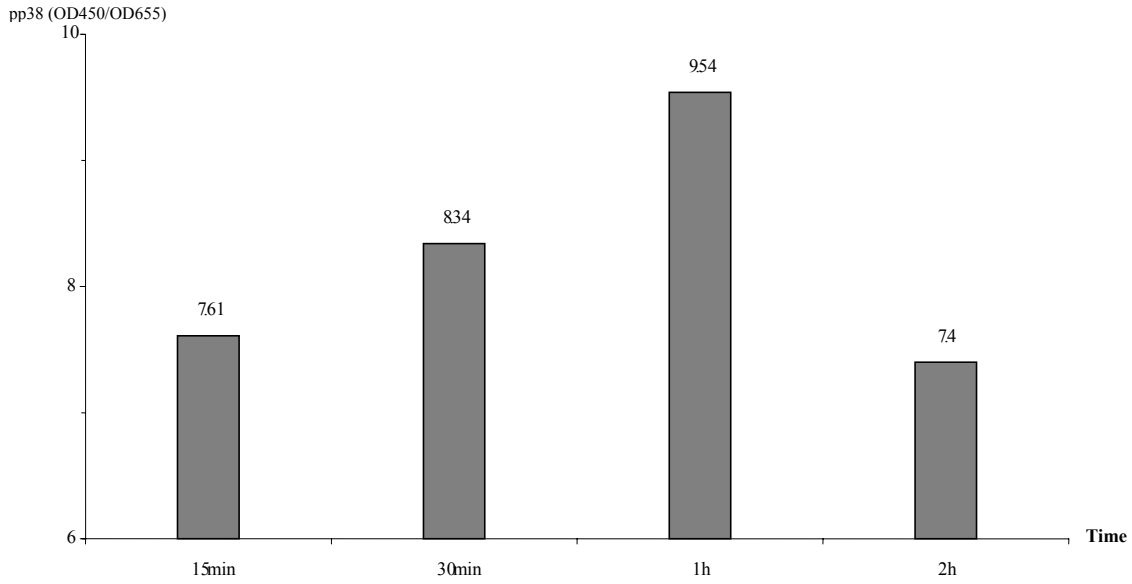


Figure 29 Kinetics of the phosphorylation of p38 in human lung epithelial cell line (A549) infected with bacteria. The cells ($1.5x10^4$ cells) were infected with *B. pseudomallei* at MOI of 10:1. The cells were collected at 15 min, 30 min, 1 h and 2 hours. After the infection, the cells were washed with PBS to remove extracellular bacteria. The phosphorylation of p38 was determined by using FACE™ p38 ELISA kit.

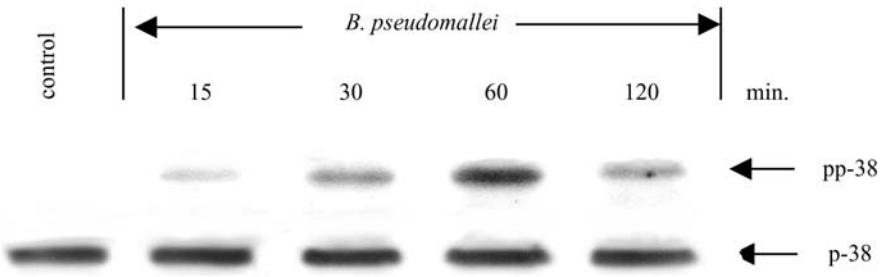


Figure 30 Kinetics of the phosphorylation of p38 in human lung epithelial cell line (A549) infected with bacteria. The cells ($2.5x10^5$ cells) were infected with *B. pseudomallei* at MOI of 10:1. The cells were collected at 15 min, 30 min, 1 h and 2 hours. After the infection, the cells were washed with PBS to remove extracellular bacteria. The cells were lysed and p-p38 was determined by Immunoblotting.

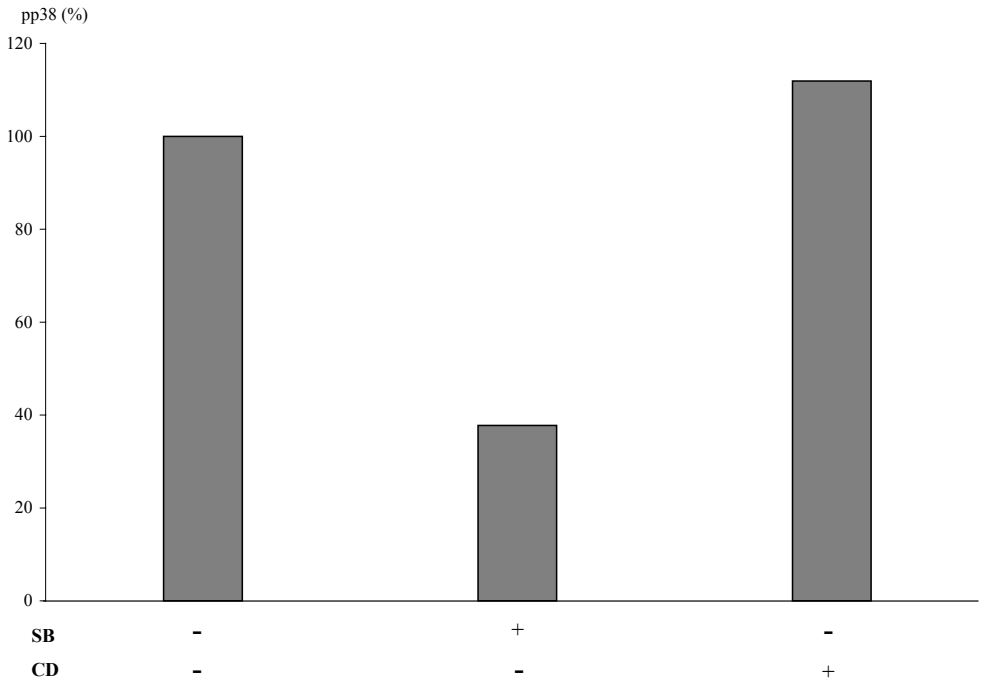


Figure 31 Effect of inhibitors on the phosphorylation of p38 activated by *B. pseudomallei*. The cells ($1.5x10^4$) were pretreated with 20 μM of SB203580 and 2 μg/ml of Cytochalasin D for 1 h before infected with *B. pseudomallei* at MOI of 10:1 for 1 hour. After the infection, the cells were washed with PBS to remove extracellular bacteria. The phosphorylation of p38 was determined by using FACE™ p38 ELISA kit.

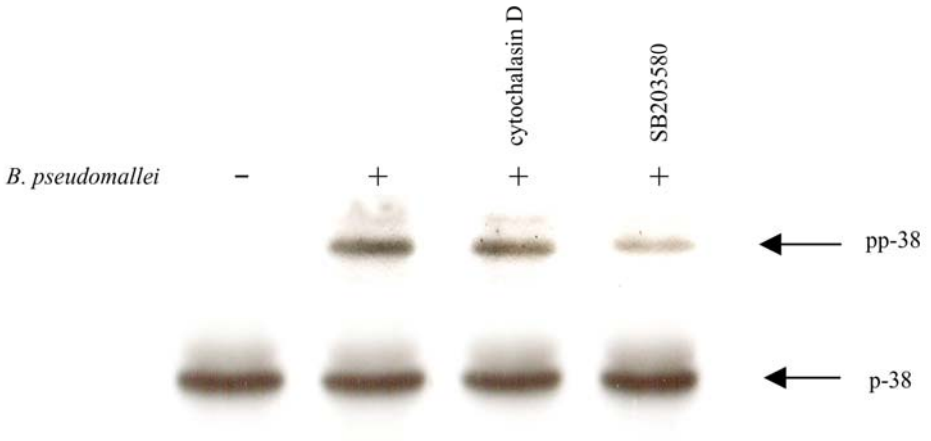


Figure 32 Effect of inhibitors on the phosphorylation of p38 activated by *B. pseudomallei*. The cells ($2.5x10^5$) were pretreated with 20 μM of SB203580 and 2 μg/ml of Cytochalasin D for 1 h before infected with *B. pseudomallei* at MOI of 10:1 for 1 hour. After the infection, the cells were washed with PBS to remove extracellular bacteria. The cells were lysed and p-p38 was determined by Immunoblotting.

Discussion

Since lung is the most commonly affected organ found in the patients with melioidosis, in the present communication, we turned our attention to the interaction of this bacterium with human alveolar lung epithelium cell. During the infection by certain pathogen, epithelium cell were able to produce chemokine such as IL-8 which is a potent chemoattractant for polymorphonuclear cells and can direct recruitment of these cells into the infected sites (7). In this study, we demonstrated that although *B. pseudomallei* could induce the human lung epithelium to produce IL-8 (Fig.20A), the level of the cytokine production and gene expression from *B. pseudomallei* infected cells was significantly lower than the cells infected with other gram-negative bacteria such as *S. typhi* (Fig. 20 and 21). Although the cells infected with *B. pseudomallei* produced significantly less IL-8 than *S. typhi* at the same MOI, the kinetics of IL-8 production from the cells infected with both bacteria was similar. IL-8 was produced at detectable level after 2 hours of infection and gradually increased before reaching the maximum at 6 hours after infection. The production of this cytokine was also produced from the cells activated with heat killed bacteria (Fig. 19). This result indirectly suggests that extracellular component of the bacteria such as LPS may involve, at least in some part, in IL-8 production. One cellular response to bacterial infection is activation of the transcription factor, NF-κB which is also an important regulator of IL-8 gene expression (26). It is well documented that degradation of IκBαregulate NF-κB activation (37).The data presented herein that the IκBκfrom the *B. pseudomallei*-infected cells was degraded to a lesser extent than the cells infected with *S. typhi* which indicated that *B. pseudomallei* was not able to stimulate NF-κB translocation as well as *S. typhi* (Fig. 22). This result may lead to lower level of IL-8 production.

Several factors have been reported to play critical roles in the activation of epithelial cells. Infection of HeLa cells with invasive *Shigella flexneri* induced NF-κB translocation leading to the upregulation of IL-8 mRNA (28). In contrast, non-invasive *S. flexneri* was unable to activate NF-κB and expression of IL-8 gene. Similarly, intracellular growth of *Mycobacterium tuberculosis* was necessary to elicit IL-8 production by alveolar epithelial cell (18). However, inhibition of *B. pseudomallei* by cytochalasin D was not interfered with IL-8 production and gene expression (Fig. 25A and Table I). Moreover, the level of IκBα degradation was similar to the cells which were not treated with this drug (Fig. 25B). These results suggested that the activation of lung epithelial cell by *B. pseudomallei* did not require

bacterial entry into the cells, implying that with *B. pseudomallei*, adherence to the cell surface is sufficient to initiate a signal in epithelial cells. Several components on the surface of gram-negative bacteria e.g. lipopolysaccharide (LPS) have the ability to stimulate epithelial cells which lead to NF-κB activation and IL-8 production (29). The low epithelial cell activation by *B. pseudomallei* could be related to its unusual LPS structure which has been reported by several groups of investigators to be a poor macrophage activator (15, 34). Moreover, another bacterial product, the flagellin, could also elicit IL-8 production in lung epithelial cells via TLR5 (19). The role of *B. pseudomallei* flagellin in lung epithelial cell activation remained to be investigated.

In this study, we are trying to investigate the host signaling proteins which require for *B. pseudomallei* invasion by using various signaling protein inhibitors. The result from this study indicated that p38 (one member of MEK kinase) may play a significant role in invasion of this bacterium. Pretreated the cells with p38 inhibitor (SB203580) showed significantly inhibition of *B. pseudomallei* invasion. In contrast, the inhibitor for other MEK kinase such as U0126 (inhibitor for MEK1 and MEK2) and PD98059 (inhibitor for MAPK kinase) showed no effect on invasion of this bacterium. As to be expected, genistein, which can inhibit all tyrosin kinase including p38 showed slightly inhibitory effect on invasion of *B. pseudomallei*. On the other hands, wortmannin (an inhibitor for PI3 kinase) which has been shown to involve in invasion of *Listeria monocytogenes*, also did not prevent internalization of *B. pseudomallei*.

The effect of these inhibitors on MNGC formation was also investigated. Only inhibitor for p38 (SB203580) can significantly reduce MNGC formation induced by *B. pseudomallei*. Since it has been reported that the number of MNGC formation directly correlated with the number of internalized *B. pseudomallei*. The decrease of MNGC formation by p38 inhibitor, therefore, may be due to the fact that this inhibitor reduces the number of internalized bacteria into the cell. Other inhibitors such as wortmannin and U0126 have no effect on MNGC formation.

We were also further investigated the molecular mechanism of the signaling proteins which were required for *B. pseudomallei* invasion. As shown in Fig 29, invasion of *B. pseudomallei* activated phosphorylation of p38 within 15 min after infection. The phosphorylation reached the maximum within 1h before decreasing. However, in the present of SB, an inhibitor specific for p38, there was no phosphorylation of p38 was observed (Fig.

31). These results also consistent with the experiment from Fig. 30 and Fig. 32. Moreover, inhibition of invasion by cytochalasin D did not prevent phosphorylation of p38 which indicated that the signaling was triggering by the contact of the bacteria to the surface of the cells. Invasion of *B. pseudomallei* was not required to activate phosphorylation of p38.

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Publications

1. **Involvement of Beta Interferon in Enhancing Inducible Nitric Oxide Synthase Production and A**
2. **ntimicrobial Activity of *Burkholderia pseudomallei*-Infected Macrophages**
   Infection and Immunity 2003, Vol. 71(6), p. 3053-3057

3. **Induction of iNOS expression and antimicrobial activity by interferon (IFN)-β is distinct from IFN-γ in *Burkholderia pseudomallei*-infected mouse macrophages.**
   Clinical and Experimental Immunology 2004, Vol. 136, 277-283

4. ***Burkholderia pseudomallei* stimulates low IL-8 production in human lung epithelial cell line (A549)**
   (Under the reviewing process; Clinical and Experimental Immunology)

5. **Mechanism of Burkholderia pseudomallei invasion to human lung epithelial cell line (A549)**
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Involvement of Beta Interferon in Enhancing Inducible Nitric Oxide Synthase Production and Antimicrobial Activity of *Burkholderia pseudomallei*-Infected Macrophages

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***Burkholderia pseudomallei* is the causative agent of melioidosis, a life-threatening disease that affects both humans and animals. This bacterium is able to survive and multiply inside both phagocytic and nonphagocytic cells. We recently reported that mouse macrophages infected with *B. pseudomallei* fail to produce a significant level of inducible nitric oxide synthase (iNOS), a crucial enzyme needed for the cells to control the intracellular growth of this bacterium. In the present study, we extended our investigation to demonstrate that, unlike other gram-negative bacteria that have been investigated, *B. pseudomallei* only minimally activates beta interferon (IFN-β) production; this minimal activation leads to a low level of interferon regulating factor 1 (IRF-1) in the macrophages, in parallel with poor iNOS expression. Adding exogenous IFN-β to the system could upregulate IRF-1 production, which in turn could enhance iNOS expression in the *B. pseudomallei*-infected macrophages and lead to suppression of the intracellular growth of this bacterium. Taken together, these results imply that the failure of macrophages to successfully control the growth and survival of intracellular *B. pseudomallei* is related, at least in part, to the defective production of IFN-β, which modulates the ability of macrophages to synthesize iNOS.**

Burkholderia pseudomallei is the causative agent of melioidosis, an endemic disease in tropical countries, including Southeast Asia and northern Australia (4, 11, 18, 30). This facultative intracellular bacterium is known to survive and multiply inside both phagocytic and nonphagocytic cells (13). After internalization, *B. pseudomallei* can escape from a membrane-bound phagosome into the cytoplasm (13). The organism can induce cell fusion, resulting in a multinucleated giant cell formation (8, 16). This phenomenon may facilitate the spread of *B. pseudomallei* from one cell to another.

Although the interaction of the host cell and *B. pseudomallei* has been extensively studied, the mechanism(s) by which this microorganism escapes macrophage killing is not clearly understood. Recently, Utaisincharoen et al. reported that *B. pseudomallei* is able to invade mouse macrophages without activating inducible nitric oxide synthase (iNOS) production (27). This enzyme is known to play an essential role in controlling the intracellular survival and multiplication of *B. pseudomallei* (21, 27). The expression of iNOS was markedly enhanced when macrophages were preactivated with gamma interferon (IFN-γ), leading to the production of NO to a concentration needed to suppress the intracellular growth of this bacterium (27). The enhancement of host defense by IFN-γ was also observed in mice infected with *B. pseudomallei* (23).

Besides IFN-γ, IFN-β (a type I interferon) has been implicated in playing a role in innate immunity against various microbial infections. IFN-β is largely produced by macrophages infected with microbial pathogens (e.g., *Leishmania major*) or exposed to microbial products (e.g., lipopolysaccharide [LPS]) (2, 3, 9, 12). The potent antimicrobial functions of IFN-β have been observed in macrophages infected with microorganisms such as *L. major* and *Toxoplasma gondii* (20, 22). Different lines of evidence suggest that iNOS and NO production could be enhanced by IFN-β (12, 28). For example, simultaneous exposure of macrophages to IFN-α/β and *L. major* enhanced iNOS expression, resulting in inhibition of the intracellular survival of *L. major* (20). Jacobs and Ignarro recently demonstrated that IFN-β served in an autocrine fashion, mediating interferon regulatory factor 1 (IRF-1) production (12). The expression of this transcriptional activator and the presence of a crucial IRF-1 binding site within the promoter of the iNOS gene are necessary for the induction of iNOS in murine macrophages (14, 19). In the present study, we demonstrated that *B. pseudomallei* failed to stimulate IFN-β production in mouse macrophages. The inability of *B. pseudomallei*-infected macrophages to produce IFN-β resulted in depressed iNOS production, thus allowing the bacteria to survive inside the cells.

MATERIALS AND METHODS

Cell line and culture condition. Mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (ATCC, Manassas, Va.). If not indicated otherwise, the cells were cultured in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (HyClone, Logan, Utah) at 37°C under a 5% CO_2 atmosphere.

Bacterial isolation. *B. pseudomallei* strain 844 (arabinose-negative strain) was originally isolated from a patient admitted to Srinagarind Hospital in the Khon Kaen province of Thailand, in which melioidosis is endemic. The bacterium was originally identified as *B. pseudomallei* based on its biochemical characteristics, colonial morphology on selective media, antibiotic sensitivity profiles, and reaction with polyclonal antibody (1, 15, 29) and was used in previous studies (16, 27). *Salmonella enterica* serovar Typhi, used for comparison throughout these exper-

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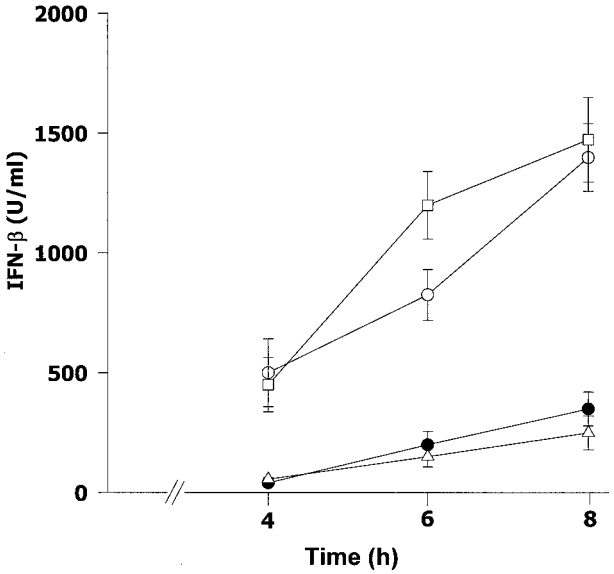

FIG. 1. Production of IFN-β by infected mouse macrophages. Mouse macrophages were exposed to *B. pseudomallei* (●), *Salmonella* serovar Typhi (□), or *E. coli* (○) at an MOI of 2:1. After 1 h of incubation, the infected cells were washed with PBS before being cultured in medium containing 250 μg of kanamycin/ml to kill residual extracellular bacteria. At 4, 6, and 8 h after infection, the supernatants were analyzed for IFN-β by an ELISA. Unstimulated macrophages were used as a control (△). Data represent the means and standard deviations from two separate experiments with triplicate samples.

iments, was maintained at Ramathibodi Hospital (Mahidol University, Bangkok, Thailand) and kept as a stock culture in our laboratory.

Infection of mouse macrophages (RAW 264.7). Mouse macrophages (10^6 cells) were cultured in a six-well plate overnight before being exposed to bacteria at a multiplicity of infection (MOI) of 2:1 for 1 h. To remove extracellular bacteria, the cells were washed three times with 2 ml of phosphate-buffered saline (PBS) before Dulbecco's modified Eagle's medium containing 250 μg of kanamycin (Gibco)/ml was added. At various times, the cells were lysed before being subjected to immunoblotting, and the supernatant was used for IFN-β analysis.

Immunoblotting. Various mouse macrophage preparations were lysed in a buffer containing 20 mM Tris, 100 mM NaCl, and 1% NP-40. Lysates containing 30 μg of protein were electrophoresed by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis and then electrotransferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% milk for 1 h before being incubated overnight with polyclonal antibody to mouse iNOS, IRF-1, or actin (Santa Cruz, Santa Cruz, Calif.). Blots were then reacted with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin G (Dako, Glostrup, Denmark). Protein bands were detected by use of an enhanced chemiluminescence kit as recommended by the manufacturer (Roche Diagnostic, Mannheim, Germany).

ELISA. The concentrations of IFN-β in the supernatants of infected macrophages were measured by using an enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, Minn.). The sensitivity of the assay system was 50 U/ml.

Standard antibiotic protection assay. Mouse macrophages (5×10^5 cells per well) were simultaneously exposed to IFN-β (100 U/ml) (R&D) and *B. pseudomallei* at an MOI of 2:1 at 37°C for 1 h and then washed thoroughly with PBS. The cells were incubated for an additional 7 h in medium containing 250 μg of kanamycin/ml to eliminate residual extracellular bacteria; intracellular bacteria were liberated by using 0.1% Triton X-100 and then were plated on tryptic soy agar. To inhibit NO production, 500 μM L-NAME (Sigma, St. Louis, Mo.) was added to the culture medium and maintained throughout the infection period. The viability of macrophages cultured in the presence of L-NAME was judged by trypan blue dye staining to be more than 90%. This concentration of the iNOS inhibitor did not interfere with bacterial growth, as judged by viable counting with the pour plate technique.

RESULTS

Production of IFN-β by macrophages infected with *B. pseudomallei*. Mouse macrophages were infected with *B. pseudomallei* at an MOI of 2:1 for 1 h, washed with PBS, and cultured in a medium containing 250 μg of kanamycin/ml. At 4, 6, and 8 h after infection, the supernatants were analyzed for IFN-β production by an ELISA. As shown in Fig. 1, the supernatants from the cells infected with *B. pseudomallei* for as long as 8 h contained a negligible concentration of IFN-β. In fact, the level of IFN-β produced by the *B. pseudomallei*-infected macrophages was only slightly higher than that produced by the uninfected macrophages. On the other hand, the levels of IFN-β in cells infected with either *Salmonella* serovar Typhi or *Escherichia coli*, used for comparison, were significantly higher than those in *B. pseudomallei*-infected cells throughout the period of observation. These results clearly demonstrated that *B. pseudomallei* behaved in a manner different from that of other bacteria with regard to its ability to stimulate macrophages for IFN-β production.

Production of IRF-1 and iNOS by macrophages infected with *B. pseudomallei*. Macrophages were infected with *B. pseudomallei* (or *Salmonella* serovar Typhi, used for comparison) at an MOI of 2:1. At various times after infection, the cells were lysed and subjected to immunoblotting with monoclonal antibodies (MAbs) against IRF-1 and iNOS. The results shown in Fig. 2 demonstrated that macrophages infected with *B. pseudomallei* for as long as 8 h produced neither IRF-1 nor iNOS protein. However, both IRF-1 and iNOS could be readily detected in macrophages infected with *Salmonella* serovar Typhi even after only 4 h of infection, and the levels gradually increased with prolonged infection. The production of both IRF-1 and iNOS by *Salmonella* serovar Typhi-infected macrophages paralleled that of IFN-β. These results indicated that *B. pseudomallei* also failed to activate IRF-1 and iNOS expression in mouse macrophages.

Effect of IFN-β on iNOS expression in infected macrophages. In order to investigate the possible relationship between IFN-β produced by bacterium-infected macrophages and iNOS expression, mouse macrophages were exposed to

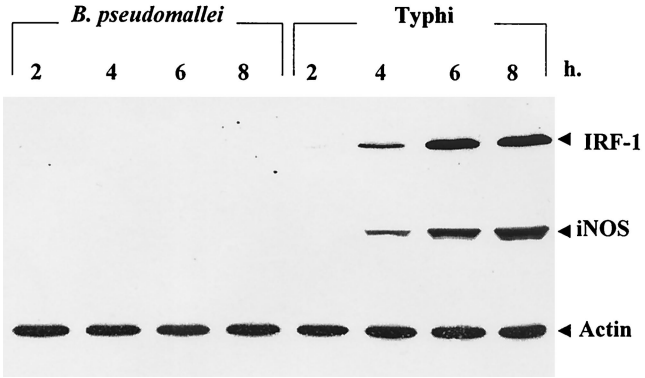

FIG. 2. Kinetics of iNOS and IRF-1 expression by infected macrophages. Mouse macrophages were exposed to *B. pseudomallei* or *Salmonella* serovar Typhi at an MOI of 2:1 as described in the legend to Fig. 1. At 2, 4, 6, and 8 h after infection, the cells were lysed with lysis buffer and then subjected to immunoblotting with anti-iNOS, anti-IRF-1, and antiactin (as a control).

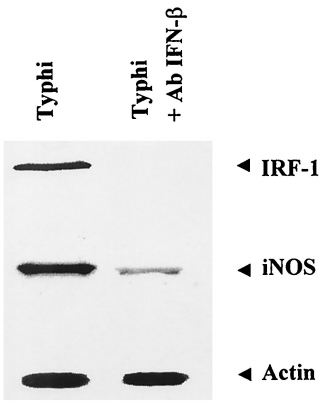

FIG. 3. IFN-β-neutralizing antibody attenuates the expression of iNOS and IRF-1 in macrophages infected with *Salmonella* serovar Typhi. Mouse macrophages were infected with *Salmonella* serovar Typhi at an MOI of 2:1 in the presence or absence of anti-IFN-β (10 μg/ml). At 1 h after infection, the cells were washed with PBS before being cultured in medium containing 250 μg of kanamycin/ml. The antibody (Ab) was kept in the culture medium throughout the experiment. At 8 h after infection, iNOS, IRF-1, and actin levels produced by the infected cells were determined by immunoblotting.

Salmonella serovar Typhi at an MOI of 2:1 for 1 h in the presence or absence of neutralizing MAb against IFN-β (10 μg/ml). In this experiment, only *Salmonella* serovar Typhi was used because under the experimental conditions, no iNOS was detected in *B. pseudomallei*-infected macrophages. The cells were washed with PBS before being cultured in medium containing 250 μg of kanamycin/ml and MAb against IFN-β. At 6 h after infection, the cells were lysed with lysis buffer and analyzed for iNOS and IRF-1 production by immunoblotting. As shown in Fig. 3, the MAb against IFN-β totally abolished IRF-1 expression from macrophages infected with *Salmonella* serovar Typhi, indicating that the expression of IRF-1 was mediated by IFN-β. However, under these conditions, a very low level of iNOS could still be observed. These results implied that IFN-β endogenously produced by the bacterium-infected macrophages could enhance iNOS production in an autocrine and/or paracrine fashion.

Exogenous IFN-β enhances the iNOS expression of macrophages infected with *B. pseudomallei*. In this experiment, mouse macrophages were infected with *B. pseudomallei* at an MOI of 2:1 in the presence or absence of exogenous IFN-β (100 U/ml). At different times, iNOS and IRF-1 levels were determined by immunoblotting. IFN-β alone did not stimulate iNOS production at concentrations that could be detected by immunoblotting (Fig. 4). However, macrophages activated with IFN-β expressed IRF-1 after only 1 h of exposure, and expression peaked at 4 h before being significantly degraded. Recall that macrophages infected with *B. pseudomallei* expressed neither iNOS nor IRF-1 even after 8 h of infection (Fig. 2). However, in this experiment, when macrophages were simultaneously exposed to IFN-β and *B. pseudomallei*, a high level of iNOS could be detected within 4 h of stimulation (Fig. 4). After 8 h of infection, the level of NO produced by macrophages infected with *B. pseudomallei* was under the limit for analysis by the Griess reaction (7). These results indicated that exogenous IFN-β could enhance the expression of iNOS in *B. pseudomallei*-infected macrophages.

Exogenous IFN-β decreases the intracellular survival and multiplication of *B. pseudomallei* in macrophages. Having established that IFN-β could enhance iNOS expression in macrophages infected with *B. pseudomallei*, we next tested whether this phenomenon could control the intracellular growth and survival of *B. pseudomallei*. In this experiment, macrophages were simultaneously exposed to IFN-β and *B. pseudomallei* for 8 h, and the number of live *B. pseudomallei* organisms inside the macrophages was determined by a standard antibiotic protection assay as described in Materials and Methods. In the absence of exogenous IFN-β, a high number of intracellular bacteria was observed (Fig. 5). However, when the cells were simultaneously exposed to exogenous IFN-β and *B. pseudomallei*, the number of intracellular bacteria was significantly reduced. Consistent with this observation, enhancement of the antimicrobial activity of macrophages stimulated with IFN-β was reduced in the presence of L-NAME, indicating that iNOS

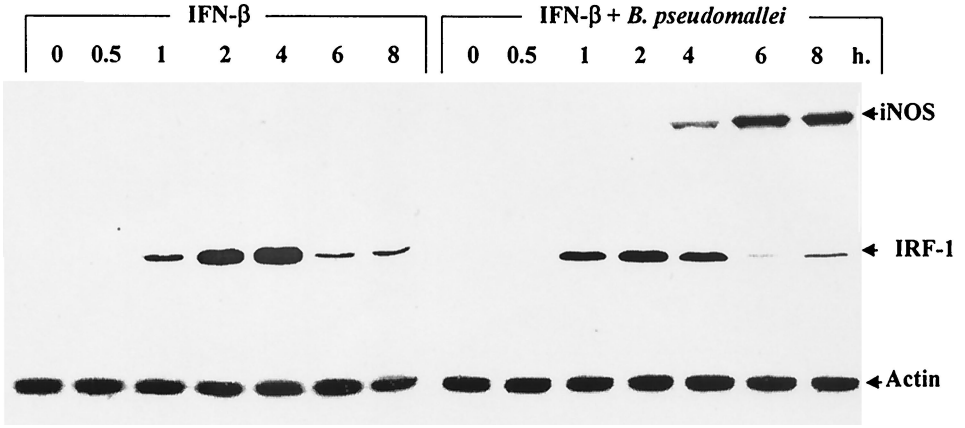

FIG. 4. Exogenous IFN-β enhances the expression of iNOS in *B. pseudomallei*-infected mouse macrophages. Macrophages were activated with IFN-β (100 U/ml) in the absence or presence of *B. pseudomallei* (MOI, 2:1) for 1 h, washed with PBS, and then cultured in medium containing 250 μg of kanamycin/ml. At different time intervals, the cells were lysed and subjected to immunoblotting with anti-iNOS, anti IRF-1, and antiactin.

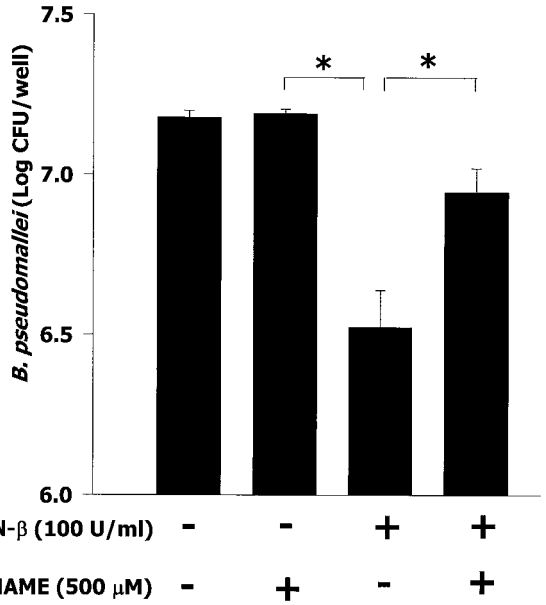

FIG. 5. Exogenous IFN-β suppresses the intracellular survival and multiplication of *B. pseudomallei* in mouse macrophages. Macrophages were simultaneously exposed to *B. pseudomallei* (MOI, 2:1) and IFN-β (100 U/ml) in the presence or absence of L-NAME (500 μM) for 1 h, washed with PBS, and cultured in medium containing 250 μg of kanamycin/ml. The iNOS inhibitor L-NAME was kept in the culture medium throughout the experiment. At 8 h after infection, the number of intracellular *B. pseudomallei* organisms was determined by a standard antibiotic assay as described in Materials and Methods. Error bars indicate standard deviations. Asterisks indicate significant differences.

is responsible for the intracellular killing of *B. pseudomallei* inside macrophages.

DISCUSSION

Macrophages are known to be one of the major cellular sources of IFN-β produced by the immune system. The production of IFN-β by macrophages is upregulated by microbial infection (e.g., *E. coli* or *L. major*) or by exposure to microbial products (e.g., LPS) (2, 3, 24). Among bacteria, only gram-negative ones can stimulate IFN-β production in mouse macrophages. *Listeria monocytogenes*, *Staphylococcus aureus*, or *Lactobacillus bulgaricus* and other gram-positive bacteria failed to activate IFN-β production (24). These results strongly suggested that LPS is the only bacterial component capable of inducing IFN-β production in mouse macrophages. Recent evidence indicated that LPS induced the expression of the gene encoding IFN-β through a Toll-like receptor 4 (TLR4) agonist but not a TLR2 agonist (25). However, LPS isolated from *Porphyromonas gingivalis*, which differs from enterobacterial LPS structurally and functionally, activated proinflammatory cytokines via TLR2 instead of TLR4 (10). *P. gingivalis* LPS did not stimulate IFN-β production by murine macrophages (25). In the present study, we demonstrated that *B. pseudomallei* also failed to stimulate IFN-β production in mouse macrophages (Fig. 1). The inability of this gram-negative bacterium to stimulate IFN-β may be due to the unique structure of *B. pseudomallei* LPS. It was reported that LPS isolated from this bacterium exhibited an unusual chemical structure in the acid-stable inner core region attached to the lipid A moiety which might lead to its weak macrophage activation activity (15, 26). Whether *B. pseudomallei* LPS is an agonist for TLR2 or TLR4 remains to be investigated.

The roles of IFN-β in innate immunity against microbial infections have been reported (2). Recombinant IFN-β was shown to protect mice against infection with *L. monocytogenes* (6). A similar protective effect of IFN-β was also observed in mice infected with *Trypanosoma cruzi* or *T. gondii* (17, 22). In mice infected with *Mycobacterium avium*, the continuous administration of IFN-β led to a reduction of the bacterial burden in the liver and spleen (5). The possible mechanism(s) by which IFN-β enhances the antimicrobial activity of macrophages may be related to the fact that it enhances iNOS and NO production by macrophages infected with the microorganism. In an in vitro study, costimulation of cells with IFN-β and *L. major* activated iNOS expression and NO production, leading to inhibition of the intracellular survival of parasites (20). In the present study, we demonstrated that a MAb against IFN-β significantly reduced iNOS expression by macrophages infected with *Salmonella* serovar Typhi (Fig. 3). This result implies that endogenous IFN-β, acting in an autocrine fashion, acted synergistically with the bacteria to increase the expression of iNOS. The enzyme iNOS is known to play an important role in the intracellular killing of macrophages (3). Recently, Utaisincharoen et al. reported that mouse macrophages infected with *B. pseudomallei* failed to trigger iNOS expression (27). However, preactivation with IFN-γ increased iNOS expression in *B. pseudomallei*-infected cells, resulting in a reduction in intracellular survival (27). The inability of *B. pseudomallei*-infected cells to produce iNOS may be due, at least in part, to the failure of *B. pseudomallei*-infected macrophages to produce IFN-β. However, in the presence of exogenous IFN-β, macrophages infected with *B. pseudomallei* were able to express iNOS at a high level (Fig. 4) and at the same time suppress the intracellular survival of *B. pseudomallei* (Fig. 5). However, in the presence of an iNOS inhibitor, L-NAME, the number of intracellular *B. pseudomallei* organisms in IFN-β-activated macrophages was significantly increased, indirectly indicating that the upregulation of iNOS in IFN-β-treated infected macrophages was associated with enhanced microbicidal activity leading to a reduction in the intracellular survival of *B. pseudomallei* (Fig. 5).

IRF-1 is a transcriptional activator which binds to sites within the promoters of a number of genes, including the iNOS gene (14, 19). Typically, IRF-1 is not expressed at a detectable level in unstimulated murine macrophages. However, IRF-1 expression can be induced by a variety of activators, including IFN-β (6). The expression of IRF-1 has been reported to be an important factor for the induction of the iNOS gene (14, 19). Moreover, IRF-$1^{-/-}$ mice were more susceptible to *M. bovis* than wild-type mice (14). This observation could be interpreted as indicating that the IRF-$1^{-/-}$ mice were unable to produce NO. In the present study, we demonstrated that a gram-negative bacterium like *Salmonella* serovar Typhi can stimulate IRF-1 expression and that this expression correlates with iNOS production (Fig. 2). However, when the MAb against IFN-β was added to *Salmonella* serovar Typhi-infected macrophages, IRF-1 was undetectable (Fig. 3). This result indicated that IFN-β acted in a paracrine fashion to autoregulate the expres-

sion of IRF-1 in macrophages infected with *Salmonella* serovar Typhi, leading to enhanced iNOS expression. In contrast, macrophages infected with *B. pseudomallei* did not produce IRF-1 at a level detectable by the immunoblotting procedure used in the present study (Fig. 2). The lack of IRF-1 production was most likely associated with the inability of *B. pseudomallei*-infected cells to produce IFN-β, because the addition of exogenous IFN-β to the system not only induced IRF-1 expression but also stimulated iNOS expression in these *B. pseudomallei*-infected macrophages (Fig. 4).

The pathogenesis of *B. pseudomallei* has been extensively investigated in a murine system, but very limited information on the mechanism(s) of intracellular bacterial survival is currently available. Previously, Utaisincharoen et al. demonstrated that *B. pseudomallei* could invade macrophages without significantly stimulating iNOS expression but that this expression could be enhanced by IFN-γ (27). In the present study, we showed that the inability of this bacterium to stimulate iNOS was related to the failure of *B. pseudomallei*-infected macrophages to produce IFN-β and IRF-1 (Fig. 1 and 2). However, exogenous IFN-β could reverse this defect and reinstall the antimicrobial activity of *B. pseudomallei*-infected macrophages (Fig. 5). It is possible that in an in vivo animal model, IFN-β in conjunction with IFN-γ produced from other sources can synergistically enhance resistance to and reduce the severity of *B. pseudomallei* infection. These points should be investigated to provide a clear understanding of the pathogenesis of melioidosis.

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Induction of iNOS expression and antimicrobial activity by interferon (IFN)-β is distinct from IFN-γ in *Burkholderia pseudomallei*-infected mouse macrophages

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SUMMARY

Burkholderia pseudomallei is a causative agent of melioidosis. This Gram-negative bacterium is able to survive and multiple inside both phagocytic and nonphagocytic cells. We previously reported that exogenous interferons (both type I and type II) enhanced antimicrobial activity of the macrophages infected with *B. pseudomallei* by up-regulating inducible nitric oxide synthase (iNOS). This enzyme thus plays an essential role in controlling intracellular growth of bacteria. In the present study we extended our investigation, analysing the mechanism(s) by which the two types of interferons (IFNs) regulate antimicrobial activity in the *B. pseudomallei*-infected macrophages. Mouse macrophage cell line (RAW 264·7) that was exposed simultaneously to *B. pseudomallei* and type I IFN (IFN-β) expressed high levels of iNOS, leading to enhanced intracellular killing of the bacteria. However, neither enhanced iNOS expression nor intracellular bacterial killing was observed when the macrophages were preactivated with IFN-β prior to being infected with *B. pseudomallei*. On the contrary, the timing of exposure was not critical for the type II IFN (IFN-γ) because when the cells were either prestimulated or co-stimulated with IFN-γ, both iNOS expression and intracellular killing capacity were enhanced. The differences by which these two IFNs regulate antimicrobial activity may be related to the fact that IFN-γ was able to induce more sustained interferon regulatory factor-1 (IRF-1) expression compared with the cells activated with IFN-β.

Keywords antimicrobial activity *Burkholderia pseudomallei* IFN-β IFN-γ iNOS IRF-1

INTRODUCTION

Burkholderia pseudomallei is a causative agent of melioidosis, an endemic disease in several tropical countries including south-east Asia and northern Australia [1–3]. This facultative intracellular Gram-negative bacterium is able to survive and multiply in both phagocytic and non-phagocytic cells [4]. After internalisation, *B. pseudomallei* can induce cell-to-cell fusion, resulting in multinucleated giant cell (MNGC) formation [5,6]. This phenomenon thus facilitates the spread of *B. pseudomallei* from one cell to another and has never been observed in any other bacteria. The mechanism(s) by which this microorganism can escape macrophage killing is not fully understood. However, we have reported that *B. pseudomallei* is able to invade mouse macrophages without activating inducible nitric oxide synthase (iNOS), an essential enzyme needed to generate reactive nitrogen intermediate (RNI) which regulates survival and multiplication of intracellular bacteria [7].

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Several cytokines including both type I [interferon (IFN)-β] and type II (IFN-γ) interferons have been reported to enhance iNOS expression [8,9]. Mouse macrophages prestimulated with IFN-γ could up-regulate iNOS expression in the cells infected with *B. pseudomallei* and this process, in turn, enhanced the intracellular killing of the bacterium [7]. The protective activity of IFN-γ has also been demonstrated in mice infected with *B. pseudomallei* [10]. Recently, we reported that exogenous IFN-β added simultaneously to the culture of mouse macrophages at the time of exposure to *B. pseudomallei* could also enhance the level of iNOS expression which increased the antimicrobial activity of *B. pseudomallei*-infected macrophages [11].

It is well documented that expression of interferon regulatory factor-1 (IRF-1) and the presence of IRF-1 binding site within the promoter region of the iNOS gene are necessary for the induction of iNOS expression in murine macrophages [12,13]. IRF-1 is not normally expressed in unstimulated murine macrophages. However, IRF-1 expression can be induced by a variety of stimuli including IFN-β and IFN-γ [13–16]. In this study, we demonstrated that the mechanism by which these two IFNs regulate the

antimicrobial activity of *B. pseudomallei*-infected macrophages is distinct from one another.

MATERIALS AND METHODS

Cell line and culture condition

Mouse macrophage cell line (RAW 264·7) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). If not indicated otherwise, the cells were cultured in Dulbeccco's modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA) at 37°C under a 5% CO_2 atmosphere.

Bacterial isolation

B. pseudomallei strain 844 (arabinose-negative biotype) used in this study was originally isolated from a patient admitted to Srinagarind hospital in the melioidosis endemic Khon Kaen province of Thailand. The bacterium was identified originally as *B. pseudomallei* based on its biochemical characteristics, colonial morphology on selective media, antibiotic sensitivity profiles and reactivity with polyclonal and monoclonal antibodies [17–19].

Protocol for activation of B. pseudomallei*-infected mouse macrophage cell line (RAW 264·7)*

In order to prestimulate mouse macrophages with IFNs, the cells (1×10^6 cells) were cultured in a six-well plate in the presence of IFN-β (100 U/ml) or IFN-γ (10 U/ml) for 18 h and then excess IFNs were removed by washing with phosphate buffered saline (PBS). For the co-stimulation experiments, the macrophages were exposed to either IFN-β or IFN-γ at the time of infection. Thereafter, the protocols for both prestimulation and co-stimulation experiments were the same. In brief, the IFN-treated macrophages were exposed to *B. pseudomallei* at a multiplicity of infection (MOI) of 2 : 1 for 1 h. Excess bacteria were removed by washing three times with 2 ml of PBS. Residual extracellular bacteria that might adhere to the cell surface were killed by incubating with DMEM containing 250 μg/ml of kanamycin (GIBCO Laboratories) for 2 h before switching to the medium containing 20 μg/ml of kanamycin.

Standard antibiotic protection assay

To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed as described previously [7]. The macrophages (1×10^6) were cultured overnight in a 24-well plate and then exposed to bacteria at MOI of 2 : 1. After 1 h of incubation, extracellular bacteria were removed and the cells were washed three times with 2 ml of PBS. Residual bacteria that adhered to the cell surface were killed by incubating in DMEM containing 250 μg/ml of kanamycin for 2 hrs before incubating further in DMEM containing 20 μg/ml of kanamycin. Eight hours after infection, the cells were washed three times with PBS and intracellular bacteria were liberated by lysing the macrophages with 0·1% Triton X-100 and plating the released bacteria in tryptic soy agar. The number of intracellular bacteria, expressed as colony forming units (CFU), was determined by bacterial colony counting.

Quantification of multinucleated giant cells (MNGCs) in B. pseudomallei*-infected macrophages*

In order to quantify the degree of MNGC formation, the macrophages (1×10^6) were first cultured overnight on a coverslip as described previously [6]. After 1 h of incubation with *B. pseudomallei* at MOI of 2 : 1, the macrophages were washed three times with PBS and then incubated in DMEM containing 250 μg/ml of kanamycin for 2 h to kill residual extracellular bacteria. The macrophages were incubated further in DMEM containing 20 μg/ml of kanamycin as indicated above. At 4, 6 and 8 h after infection the coverslips were washed with PBS, fixed for 15 min with 1% paraformaldehyde and then washed sequentially with 50% and 90% ethanol for 5 min each. The coverslips were air-dried before staining with Giemsa [6]. For enumeration of the MNGC formation, at least 1000 nuclei per coverslip were counted using light microscope at a magnification of 40× and the percentage of multinucleated cells was calculated [6]. The MNGC was defined as the cell possessing more than one nuclei within the same cell boundary.

Immunoblotting

Mouse macrophage preparations were lysed in buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates containing 30 µg of protein were electrophoresed on SDS-PAGE at 10% polyacrylamide and then electrotransferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with 5% milk for 1 h before incubating overnight with polyclonal rabbit antibody to mouse iNOS or IRF-1 (Santa Cruz, Santa Cruz, CA, USA). The blots were then allowed to react with horseradish peroxidase-conjugated swine antirabbit IgG (Dako, Glostrup, Denmark). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany).

RESULTS

One of the unique characteristics of *B. pseudomallei* is its ability to induce a cell-to-cell fusion leading to MNGC formation and the extensiveness of MNGC formation parallels with the severity of infection by *B. pseudomallei* [6]. In order to distinguish the regulatory mechanism of these two types of IFNs on bacterial induced pathology, mouse macrophage cell lines (RAW 264·7) were either preactivated with the IFN for 18 h (prestimulated) or simultaneously activated (co-stimulated) at the time of infection with *B. pseudomallei*. Eight hours after the infection, the infected cells were visualized under inverted microscope (40×). As shown in Fig. 1, the MNGC formation (indicated by arrow) could be observed readily in the controlled unactivated macrophages infected with *B. pseudomallei* (Fig. 1b). The MNGC formation was observed only sparingly in the experiment where the cells were co-stimulated with IFN-β (Fig. 1d) and the cell culture exhibited a morphology which was not noticeably different from the uninfected control (Fig. 1a). Unlike the results described from the co-stimulation experiment, a high number of MNGC formation could be observed in the cultures which were prestimulated with IFN-β prior to being infected with *B. pseudomallei* (Fig. 1c). In contrast to the results noted with the IFN-β, the degree of MNGC formation was reduced markedly in the cells that were either pre- or co-stimulated with IFN-γ, indicating that the IFN-γ was effective in activating the cells regardless of the time of exposure. To be quantitative, the number of MNGC was quantified after Giemsa staining (Fig. 2). At 8 h, the number of MNGC was as high as 30% in unactivated *B. pseudomallei*-infected macrophages and this number was reduced significantly to less than 10% with the IFN-β co-stimulation ($P \leq 0{\cdot}05$) (Fig. 2a). However,

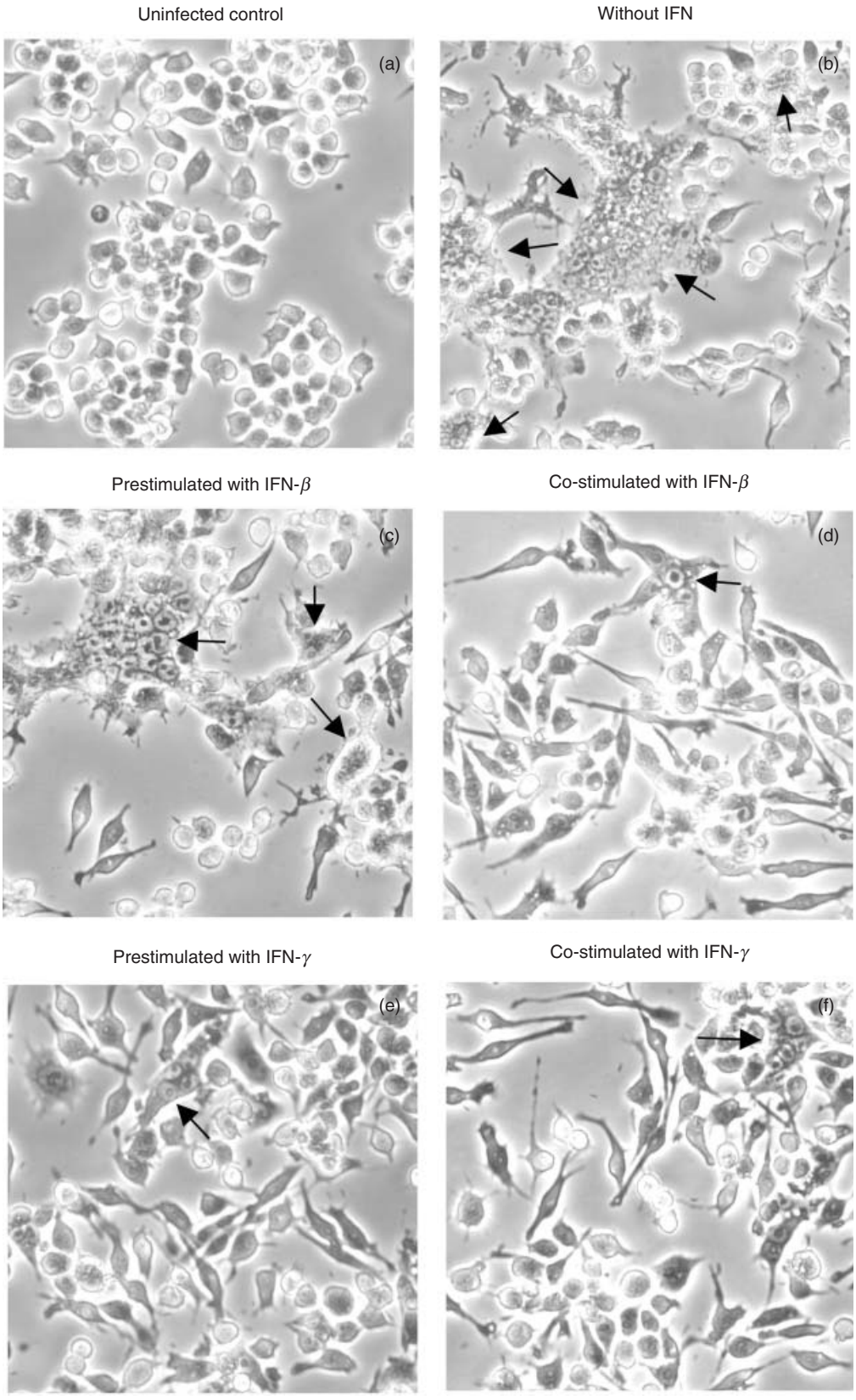

Fig. 1. Effect of IFN-β and IFN-γ on MNGC formation induced by *B. pseudomallei*. Mouse macrophage cell line (RAW 264·7) (1×10^6 cells/well) was either prestimulated with IFN (100 U/ml of IFN-β or 10 U/ml of IFN-γ) for 18 h (c) and (e) or simultaneously co-stimulated (d) and (f) with *B. pseudomallei* (MOI 2 : 1). After 8 h, the cells were visualized under inverted microscope (20×). Uninfected cells (a) and *B. pseudomallei*-infected cells alone without IFN (b) were included for comparison. MNGC formation is indicated by arrow.

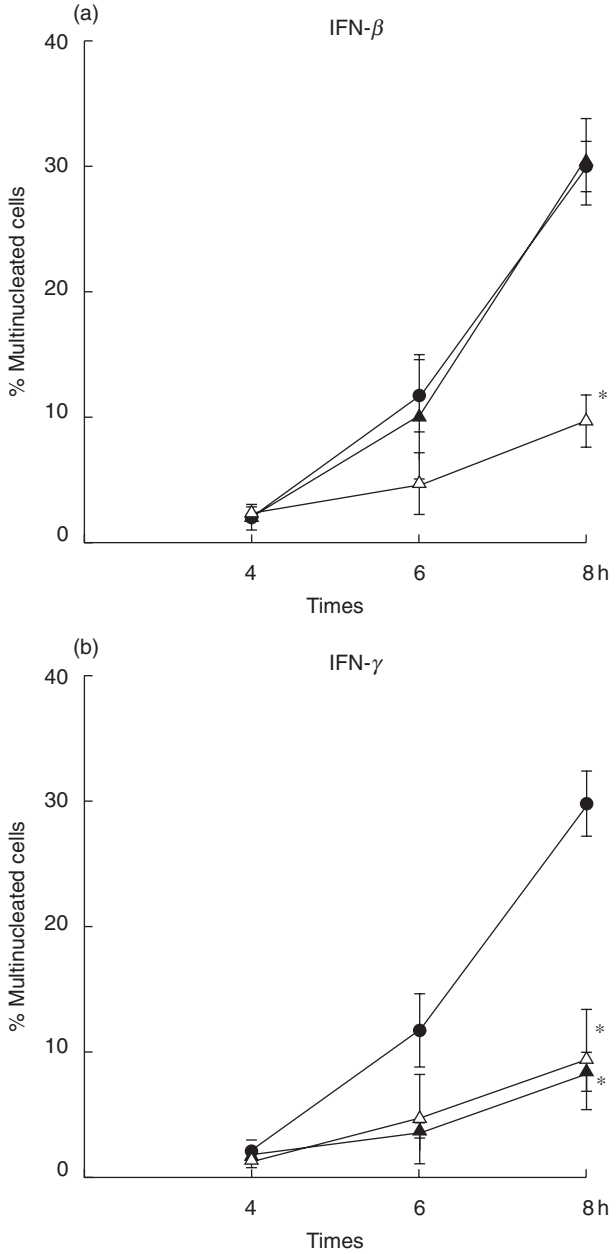

Fig. 2. Quantification of MNGC formation induced by *B. pseudomallei*. The macrophages were either prestimulated (▲) with IFN (100 U/ml of IFN-β (a) or 10 U/ml of IFN-γ (b)) for 18 h or simultaneously (△) co-stimulated with *B. pseudomallei* (MOI 2 : 1). The *B. pseudomallei*-infected macrophages without IFN stimulation served as control (●). At 4, 6 and 8 h post-infection, the cells were fixed, stained with Giemsa, and the number of MNGC was determined by microscopic examination (40×). Data represent mean and s.d. of three separate experiments, each carried out in duplicate. *$P \leq 0{\cdot}05$ by Student's *t*-test.

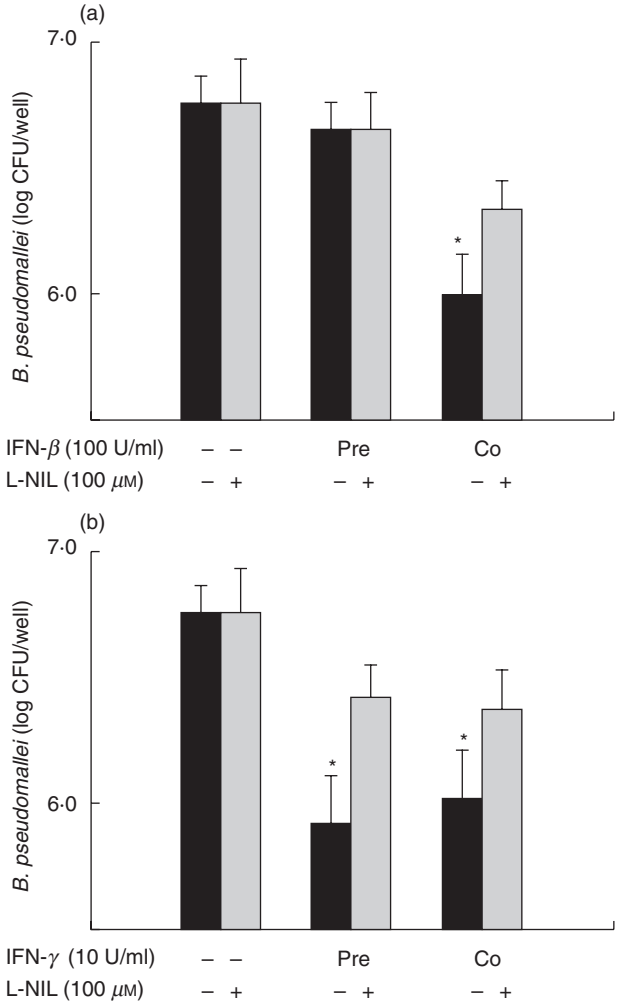

Fig. 3. IFNs inhibit intracellular survival of *B. pseudomallei* in mouse macrophages. Macrophages were prestimulated with either 100 U/ml of IFN-β (a) or 10 U/ml of IFN-γ (b) for 18 h or co-stimulated simultaneously with *B. pseudomallei* (MOI 2 : 1). The experiment was carried out either without or with L-NIL (100 μM) which was added to the cells 2 h prior to the bacterial infection and kept in the culture medium until the experiment was terminated. At 8 h post-infection, the number of intracellular *B. pseudomallei* was determined by standard antibiotic protection assay as described in Materials and methods. Data represent mean and s.d. of three separate experiments, each carried out in duplicate. *$P < 0{\cdot}05$ by Student's *t*-test.

preactivating the macrophages with IFN-β prior to the time of bacterial exposure did not reduce the number of MNGC. In contrast to IFN-β, the cells that were either prestimulated or co-stimulated with IFN-γ showed significantly lower numbers of MNGC formation compared with the unactivated *B. pseudomallei*-infected macrophages ($P \leq 0{\cdot}05$) (Fig. 2b).

The degree of MNGC formation induced by *B. pseudomallei* was reported to correlate directly with the number of intracellular survival of this bacterium [11]. In order to compare the ability of IFN-β and IFN-γ to activate macrophages, judging by their ability to kill intracellular *B. pseudomallei*, standard antibiotic protection assay was used. Consistent with the results based on reduction of the MNGC formation, the IFN-β was able to enhance antimicrobial activity of the macrophages infected with *B. pseudomallei* only under the co-stimulation condition (Fig. 3a). Again, prestimulating the cells with IFN-β did not increase its intracellular killing capacity. On the contrary, the cells that were either preactivated or co-activated with IFN-γ exhibited a significantly lower number of viable intracellular bacteria (Fig. 3b). The enhanced antimicrobial activity against *B. pseudomallei* by either IFN-β or IFN-γ was abrogated when a specific iNOS inhibitor, L-NIL, was added to the cell culture (Fig. 3).

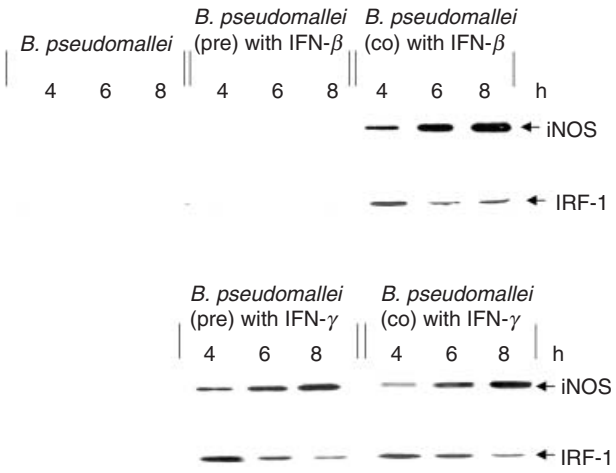

Fig. 4. IFNs induce expression of iNOS and IRF-1 in *B. pseudomallei* infected macrophages. Mouse macrophages were prestimulated with either 100 U/ml of IFN-β or 10 U/ml of IFN-γ for 18 h before or co-stimulated at the time of exposure to *B. pseudomallei* (MOI 2 : 1). At 4, 6 and 8 h post-infection, the cells were lysed with lysis buffer and cell lysate was subjected to immunoblotting using anti-iNOS and anti-IRF-1.

The ability of L-NIL to suppress antimicrobial activity in IFN-activated macrophages prompts us to investigate the regulation of iNOS and IRF-1 in *B. pseudomallei* infection. The expression of iNOS and IRF-1 was determined from the lysate of the infected cells by immunoblotting using antibody against iNOS and IRF-1, respectively. As shown in Fig. 4, exposure to *B. pseudomallei* by itself was not sufficient to stimulate the expression of either iNOS or IRF-1. However, the expression of both iNOS and IRF-1 were observed when the cells were co-stimulated with IFN-β. Consistent with the results of MNGC formation and intracellular killing presented above, the cells which were preactivated with IFN-β prior to being infected with *B. pseudomallei* expressed neither iNOS nor IRF-1. In contrast, the macrophages which were either prestimulated or co-stimulated with IFN-γ were able to produce both iNOS and IRF-1. Altogether, the data presented showed that the expression of iNOS paralleled the activation of the transcription factor IRF-1 in the *B. pseudomallei*-infected macrophages. It should be mentioned that the macrophages activated with the IFN alone were not able to up-regulate iNOS (data not shown).

It is well documented that both IFN-β and IFN-γ, alone, can stimulate a significant degree of IRF-1 expression [15]. The presence of this transcription factor is required for iNOS gene transcription. We therefore analyse the way by which these two IFNs differentially regulate the IRF-1 expression by immunoblot. In this experiment, the IFN was added to uninfected macrophages and the levels of IRF-1 expression in the lysate were followed by immunoblotting as described. The results presented in Fig. 5 demonstrated that the expression of IRF-1 in the macrophages stimulated with IFN-β gradually increased and reached a maximum after 2–4 h before being degraded and finally disappeared altogether by 18 h. Unlike the IFN-β, IFN-γ was not only able to stimulate IRF-1 but in addition it was also able to sustain the activation state of IRF-1 for a period as long as 24 h. In fact, a trace of IRF-1 expression could be detected at 36 h, when the experiment was terminated. It should be noted that in the unstimulated cells, neither IRF-1 nor iNOS were observed by the immunoblotting (data not shown).

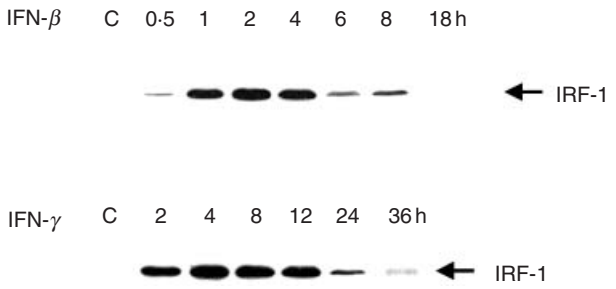

Fig. 5. Kinetics of IRF-1 expression from the macrophages activated with IFNs. Mouse macrophages were stimulated with either IFN-β (100 U/ml) or IFN-γ (10 U/ml). At different time intervals, the cells were lysed with lysis buffer and cell lysate was subjected to immunoblotting using anti-IRF-1.

DISCUSSION

Inducible nitric oxide synthase is known to play a significant role in host defence against a number of microbial infections. Intracellular microbial killing is often associated with the expression of iNOS and NO [20]. When *Leishmania major* promastigotes were injected into the peritoneal cavity of wild-type ($iNOS^{+/+}$) and iNOS-knock-out ($iNOS^{-/-}$) mice, 40-fold more parasites was recovered from the knock-out mice 24 h after the parasite injection [21]. In other models, the survival rate of $iNOS^{-/-}$ mice infected with *Listeria monocytogenes* and *Mycobacterium tuberculosis* was also reduced compared with the wild-type counterpart, indicating that iNOS is an essential enzyme in protective immunity against these bacterial infections [22,23]. The expression of iNOS could be differentially regulated by different microbial products. In addition to live bacteria, bacterial components such as endotoxin, lipoproteins or exotoxins could also effectively stimulate macrophages to express iNOS [9]. The iNOS expression from the macrophages infected with microbes or exposed to microbial products could be further enhanced by cytokines such as IFN-β and IFN-γ. In the mouse macrophages activated with ManLam (the cell wall lipoglycan of *M. tuberculosis*) or lipopolysaccharide (LPS) from Gram-negative bacteria, the expression of iNOS and NO production was greatly increased in the presence of IFN-γ or IFN-β [11,24–26]. In contrast to IFN-γ, a specific sequence of stimulation by IFN-β is required for up-regulating iNOS expression. Only simultaneous exposure of the macrophages to IFN-α/β and *L. major* promastigotes also enhanced the induction of iNOS, thus resulting in increased anti-leishmanial activity of these macrophages [27].

Unlike most other bacteria that have been examined, *B. pseudomallei* by itself fails to stimulate iNOS expression in macrophages [7]. The failure to induce the expression of this enzyme may be related to the fact that *B. pseudomallei* possess LPS which is different from that of other Gram-negative bacteria and it is also a poor macrophage activator [28]. The inability of *B. pseudomallei*-infected macrophages to produce iNOS would allow this bacterium to survive and multiply intracellularly. However, in the presence of IFN-γ, iNOS expression could be up-regulated, thus enhancing the intracellular killing of *B. pseudomallei* [7,29]. The protective roles of IFN-γ in mice infected with *B. pseudomallei* have also been demonstrated [10]. Recently, we reported that IFN-β could also enhance the macrophage ability to kill intracellular *B. pseudomallei* by up-regulating iNOS expression [11]. Although both IFN-γ and IFN-β could activate

microbicidal activity of macrophages, in the present study we demonstrated the evidence showing that the two IFNs possess different regulatory mechanisms on iNOS expression. With regard to the IFN-β, the up-regulation of iNOS in the macrophages infected with *B. pseudomallei* was observed only in the co-stimulating situation which paralleled the increase of the intracellular killing (Fig. 3), simultaneously decreasing MNGC formation (Figs 1 and 2).

The inability of IFN-β to enhance iNOS expression from the macrophages infected with *B. pseudomallei* when the cells were prestimulated with this cytokine may be related to the fact that IFN-β was unable to sustain the activation of IRF-1 expression, like the cells prestimulated with IFN-γ. IRF-1 is a crucial transcriptional activator which binds to the sites within the promoter region of the iNOS gene [12,13]. In IRF-1 knock-out ($IRF^{-/-}$) mice, the animals were more susceptible to bacterial infection than the wild-type and this is probably related to the fact that the $IRF^{-/-}$ mice were unable to produce NO even in the presence of IFN-γ [12]. The role of IRF-1 in regulating iNOS expression has also been demonstrated *in vitro*. In the presence of antibody against IFN-β, the mouse macrophages infected with *Salmonella typhi* failed to produce IRF-1, resulting in a significant decrease of iNOS expression [11]. When the cells that were prestimulated with IFN-β prior to the *B. pseudomallei* infection, these cells were not able to express either iNOS or IRF-1 protein. On the other hand, when the cells were prestimulated with IFN-γ and followed by exposure to living *B. pseudomallei*, high levels of both iNOS and IRF-1 were observed. In this communication, we extended our finding to demonstrate that the kinetics of IRF-1 expression induced by IFN-β is distinct from that of the IFN-γ (Fig. 5). IFN-β can stimulate IRF-1 expression and sustains it for only a short period. In contrast, the macrophages stimulated with IFN-γ were able to maintain the IRF-1 level for more than 36 h after activation. The difference in IRF-1 expression may be due to the fact that IFN-β and IFN-γ use different molecules to transduce the signal [30,31]. The difference in signalling pathways between IFN-β and IFN-γ may result in different kinetics of IRF-1 expression. The results presented reveal new information on innate immune mechanism and the way in which we may immunomodulate the host to respond favourably to severe infection and sepsis such as that caused by *B. pseudomallei*.

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