

## Invariant Natural Killer T (iNKT) cells response in human melioidosis

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### Abstract

**Background:** Melioidosis is an infectious disease caused by *Burkholderia pseudomallei*. In infected mice, IFN- $\gamma$  can provide protection against *B. pseudomallei* infection. Invariant Natural Killer T (iNKT) cells are a subpopulation of T lymphocytes, activated by recognition of glycolipid ligands such as  $\alpha$ -Galactosylceramide presented by CD1d, produce and secrete several cytokines, including IFN- $\gamma$  and IL-4. The response of iNKT cells in human melioidosis was then investigated.

**Objectives:** To determine the iNKT cells response in human melioidosis.

**Methods:** The number of human iNKT cells and its activation states were investigated in sepsis melioidosis patients compared with healthy controls using flow cytometry. The iNKT cells activation was confirmed *in vitro* using heat-killed *B. pseudomallei* with normal peripheral blood mononuclear cells. The components induced iNKT cell were also determined using different concentration of *B. pseudomallei* lipopolysaccharide (LPS), heat-killed *B. pseudomallei* treated with or without DNase, RNase, or proteinase.

**Results:** The number of human iNKT cells was significantly lower while the percentage of activated iNKT cells was higher in sepsis melioidosis when compared to control. In addition, *B. pseudomallei* can stimulate human iNKT cells *in vitro*. Heat-killed *B. pseudomallei* could activate iNKT cells but not relate to nucleic acid, proteins, or LPS.

**Conclusions:** We found for the first time that the iNKT cells were activated during *B. pseudomallei* infection in human. However, the roles and the mechanism of iNKT cells during early state of infection needed to be further investigated.

**Key words:** *Burkholderia pseudomallei*, melioidosis, iNKT cells, activation, human infection

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### Introduction

Melioidosis is an infectious disease caused by a Gram-negative bacterium, *Burkholderia pseudomallei*. Disease manifestations range from acute fulminant sepsis to chronic infection.<sup>1</sup> Most melioidosis patients present with sepsis with or without pneumonia.<sup>2</sup> In northeast Thailand, melioidosis shows a 40% mortality rate in septicemia, even after treatment.<sup>3</sup> Melioidosis patients have elevated several cytokines such as IFN- $\gamma$ , IL-8, IL-12, and IL-15 during sepsis melioidosis.<sup>4</sup> IFN- $\gamma$  that is produced from Natural killer (NK) cells and T cells have been implicated in survival from acute melioidosis.<sup>5</sup> When the PBMCs of those who recovered from melioidosis disease were stimulated with *B. pseudomallei* antigens, they generated an antigen-specific IFN- $\gamma$  immune response to *B. pseudomallei*. This indicated the role of IFN- $\gamma$  in protection against infection.<sup>6</sup>

Invariant natural killer T (iNKT) cell is a distinct population of T lymphocytes that express natural killer (NK) receptor (NK 1.1 in the mouse and NKR-P1A in humans) and a semi-invariant T cell receptor (TCR), V $\alpha$ 14Ja18 paired with V $\beta$ 8.2, V $\beta$ 7, or V $\beta$ 2 in mice and V $\alpha$ 24Ja18/V $\beta$ 11 in human.<sup>7,8</sup> The iNKT cells recognize lipid or glycolipid antigens presented by MHC class I-like antigen-presenting molecule, CD1d on antigen-presenting cells (APCs). During infection, iNKT cells produce cytokines such as IFN- $\gamma$  and IL-4 that recruit and stimulate other cell types, including macrophages, neutrophils, dendritic cells (DCs), NK cells, T, and B cells.<sup>9</sup> iNKT cells can be activated by direct recognition of microbial lipid antigens or modified endogenous lipid presented on CD1d via a T cell receptor (TCR)-dependent manner leading to rapid production of a large number of cytokines.<sup>10,11</sup> Further, iNKT cells can also be activated indirectly through cytokine-mediated activation or combination of both.<sup>12,13</sup> These properties enable iNKT cells to expand the protective response of the innate immune cells such as macrophages, and neutrophils that important in controlling *B. pseudomallei* infection.<sup>14,15</sup> Although there was reported a slightly lower number of NKT-like cells in dead melioidosis cases<sup>5</sup> however little is known about the role of iNKT cells during this disease. This study therefore intends to explore their role during *B. pseudomallei* infection.

In this study, we assessed the response of the iNKT cell in melioidosis by evaluating iNKT cells number and activation level in melioidosis patients as well as the disease outcome. This study demonstrated that *B. pseudomallei* could induce human iNKT cells activation both *in vivo* and *in vitro*. Moreover, the LPS, nucleic acids and proteins fractions from heat-killed *B. pseudomallei* did not involve in the activation of iNKT cells.

## Methods

### Sample collections

Human blood samples (10 ml) were collected from 13 patients within 7 days of admission to Srinagarind or Khon Kaen Provincial hospitals with acute melioidosis and culture-proven for *B. pseudomallei*. Normal blood samples (n = 10; male = 4, female = 6, age ranges 25–32 years-old) were also obtained from healthy adults as a control. The study was approved by the human ethics committee of Khon Kaen

University (*in-vivo* HE591293 and *in-vitro* HE621362) and the human ethics committee of Khon Kaen hospital (KE61048). Written inform consent was obtained from all participating patients. The inclusion and exclusion criteria for recruitment of patients with sepsis melioidosis<sup>16</sup> are shown in **table 1**.

### Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from whole blood samples by density gradient using the Ficoll-hypaque (GE Healthcare, Uppsala, Sweden). The cells were washed twice with phosphate buffer saline (PBS), pH 7.4 and re-suspended in an R10<sup>+</sup> medium comprising RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 U ml<sup>-1</sup> penicillin-streptomycin, 10 mM HEPES buffer solution and 5.5  $\mu$ M 2-Mercaptoethanol (2-ME).<sup>17</sup> Ten microliters of the cell were used to measure their viability using a trypan blue exclusion assay (Gibco, Thermo Fisher Scientific, Waltham, MA) and the rest were kept at 4°C. The final cell number was 0.5-1.0  $\times 10^7$  cells/ml with total of 1 ml for *in vitro* experiment and flow cytometry.

### Bacteria

A single colony of *B. pseudomallei* strain 1026b grown on selective Ashdown's agar was inoculated into 3 ml Luria-Bertani (LB) broth (Titan Biotech LTD., Rajasthan, India) and cultured overnight at 37°C with occasional shaking at 200 rpm. Subsequently, 2% of the culture was inoculated into 50 ml LB broth and cultured for 3 hours with shaking at 200 rpm in a 37°C shaker incubator (J.P. SELECTA, Barcelona, Spain) to reach the log phase. The bacterial cell pellets were centrifuged at 10,000 g for 10 minutes and washed with PBS, pH 7.4 before re-suspended in 1 ml PBS and kept at 4°C until use. To prepare heat-killed *B. pseudomallei*, 1  $\times 10^8$  CFU/ml of the bacterium were heated for 1 hour at 80°C and then checked for their viability.<sup>18</sup>

### Heat-Killed bacteria treated with enzymes

Five hundred microliters of 1  $\times 10^7$  CFU/ml of heat-killed bacterium were treated with 1 unit of DNase (Promega, Madison, WI) or 100  $\mu$ g of RNase (Thermo scientific, Carlsbad, CA) or 100  $\mu$ g of proteinase (Promega, Madison, WI) or PBS as a control for overnight at 37°C. After treatment,

**Table 1. Inclusion and Exclusion criteria for blood sample collection**

Group of samples	N	Inclusion criteria	Exclusion criteria
Healthy donors	10	Age more than 18 years-old	-
Melioidosis donors	13	<ol style="list-style-type: none"> <li>Age more than 18 years-old</li> <li>Culture-proven <i>B. pseudomallei</i> infection</li> <li>Sepsis caused by melioidosis was defined by the presence of <math>\geq 2</math> of the following characteristics               <ol style="list-style-type: none"> <li>Temperature &gt; 38°C or &lt; 36°C</li> <li>Pulse rate &gt; 90 beats/min</li> <li>Respiratory rate &gt; 20 breaths/min or partial pressure of CO<sub>2</sub> arterial &lt; 32 mmHg</li> <li>White blood cell (WBC) count &gt; 12,000 <math>\times 10^6</math> cells/l or band forms &gt; 10%</li> </ol> </li> </ol>	<ol style="list-style-type: none"> <li>Patients active with cancer</li> <li>Patients with other infection</li> <li>Patients received these anti-inflammatory drugs indicated as follows <math>\geq 2</math> weeks before recruit to this study               <ol style="list-style-type: none"> <li>Prednisolone &gt; 20 mg/day</li> <li>Hydrocortisone &gt; 80 mg/day</li> <li>Dexamethasone &gt; 3 mg/day</li> </ol> </li> </ol>

the enzymes were heated inactivated at 95°C for 15 minutes. The heat-killed bacteria were checked for bacterial lysis and the enzymes digestions were completed.

#### ***In vitro* iNKT cell activation with heat-killed *B. pseudomallei***

The MOI ratio used in this experiment was chosen from several ratios between heat-killed *B. pseudomallei*: PBMC, including 10 ( $1 \times 10^6$  CFU):1, 50 ( $5 \times 10^6$  CFU):1, and 100 ( $1 \times 10^7$  CFU):1. (**Supplement Figure 1**). The MOI 10 was selected to use in the experiment because it was sufficient to stimulate iNKT cells. The  $1 \times 10^5$  human PBMCs in 50  $\mu$ l of R10<sup>+</sup> medium was incubated with 50  $\mu$ l of  $1 \times 10^6$  CFU heat-killed *B. pseudomallei* or enzymes treated heat-killed *B. pseudomallei* (MOI = 10) in R10<sup>+</sup> media in a 96-well plate, U-Bottom (BD, Falcon, USA) at 37°C for 24 hours. PBMCs with R10<sup>+</sup> alone served as an unstimulated control. After incubation, cells were collected and their viability were checked using a trypan blue staining method as recommended by the manufacturer (Gibco, Thermo Fisher Scientific, Waltham, MA). The activation state of iNKT cells were then assessed by CD69 expression in comparison to isotype-matched controls mAb (mouse IgG1) using flow cytometry.<sup>19</sup>

#### ***In vitro* PBMC activation with *B. pseudomallei* lipopolysaccharide (LPS)**

The  $1 \times 10^5$  human PBMCs were cultured in 100  $\mu$ l of R10<sup>+</sup> medium in the presence of 1, 10, 100, and 1,000 ng/ml of *B. pseudomallei* LPS (kindly provided by Associate Prof. Narisara Chantratita, Mahidol University, Thailand)<sup>20</sup> for 24 hours. Then, the activated iNKT cells in the PBMCs were assessed by flow cytometry.

#### ***Flow cytometry analysis of human iNKT cells***

PBMCs ( $1 \times 10^5$  -  $1 \times 10^6$  cells) were suspended 50  $\mu$ l of staining buffer (2% (vol/vol) FBS, and 0.09% (wt/vol) sodium azide in PBS) and incubated with 2.5  $\mu$ g of Fc block (BD Biosciences, San Diego, CA) at room temperature for 10 minutes followed by staining with optimum concentration of antibodies recommended by the manufacturers; APC-labeled  $\alpha$ -GalCer/human CD1d dimer (BD Biosciences, San Diego, CA), and followed by FITC-conjugated anti-human  $\alpha\beta$  TCR (Thermo, Waltham, MA) mAb, peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-human CD19 mAb (Thermo, Waltham, MA), and brilliant violet (BV421)-conjugated anti-human CD69 mAb (BioLegend, San Diego, CA) for 15-30 minutes at 4°C in the dark.<sup>17</sup> Unloaded CD1d dimer or isotype matched control antibodies were used for detecting non-specific binding and set up cut-off value of negative and positive staining cells. The cells were then washed and re-suspended with staining buffer. Finally, samples were acquired by flow cytometer (Beckton Dickinson/FACSCanto II) and data were analyzed by FlowJo software (Tree star).

#### ***Statistical analysis***

Statistical analyses were performed by using SPSS version 20 and GraphPad Prism 5.0. Mann-Whitney U-test was used to compare unpaired data. The two-way analysis of variance (ANOVA) was used to analyze differences in multiple groups. A *p*-value of < 0.05 was considered as statistically significant.

## **Results**

### ***iNKT cells in melioidosis patients***

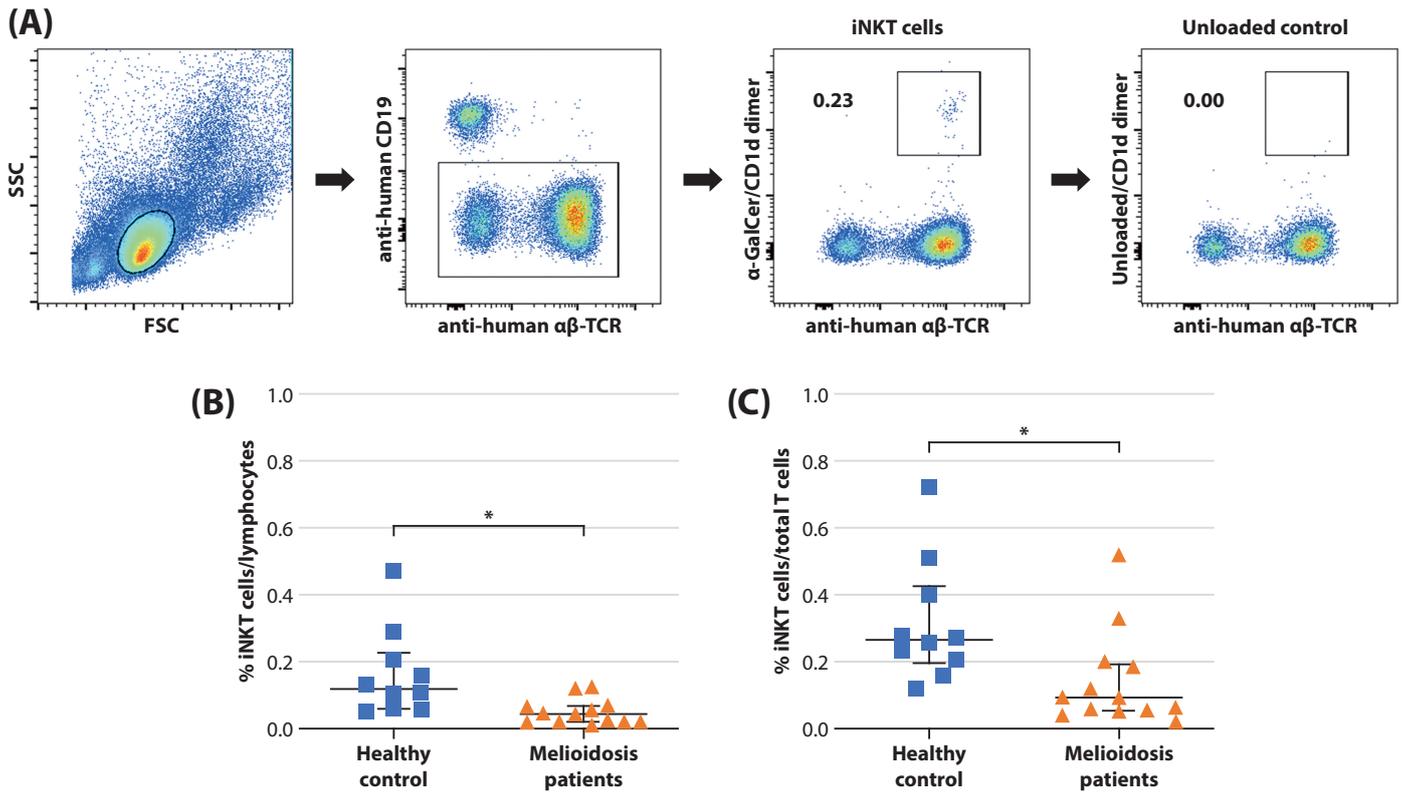
The responses of human iNKT cells to *B. pseudomallei* was investigated in melioidosis patients compared with healthy controls. Their demographic data for healthy controls (**Table 2**) and melioidosis patients were shown (**Table 3**). Most patients were males and diabetes were a major underlying disease. The percentage of circulating iNKT cells in total lymphocytes was significantly lower (*p* = 0.0019) in melioidosis patients (median 0.042%; interquartile range 0.02-0.07%) than in the healthy controls (median 0.117%; interquartile range 0.06-0.22%) (**Figure 1B**). The same result was also found when iNKT cells were calculated within absolute T cells (median 0.091%; interquartile range 0.05-0.19% and median 0.265%; interquartile range 0.19-0.43% for melioidosis patients and control respectively) (*p* = 0.0041) (**Figure 1C**).

**Table 2. Demographic data of healthy controls**

Demographic data	Number of donors
Ages (years, mean $\pm$ SD)	27 $\pm$ 2
Gender	
Male	4
Female	6

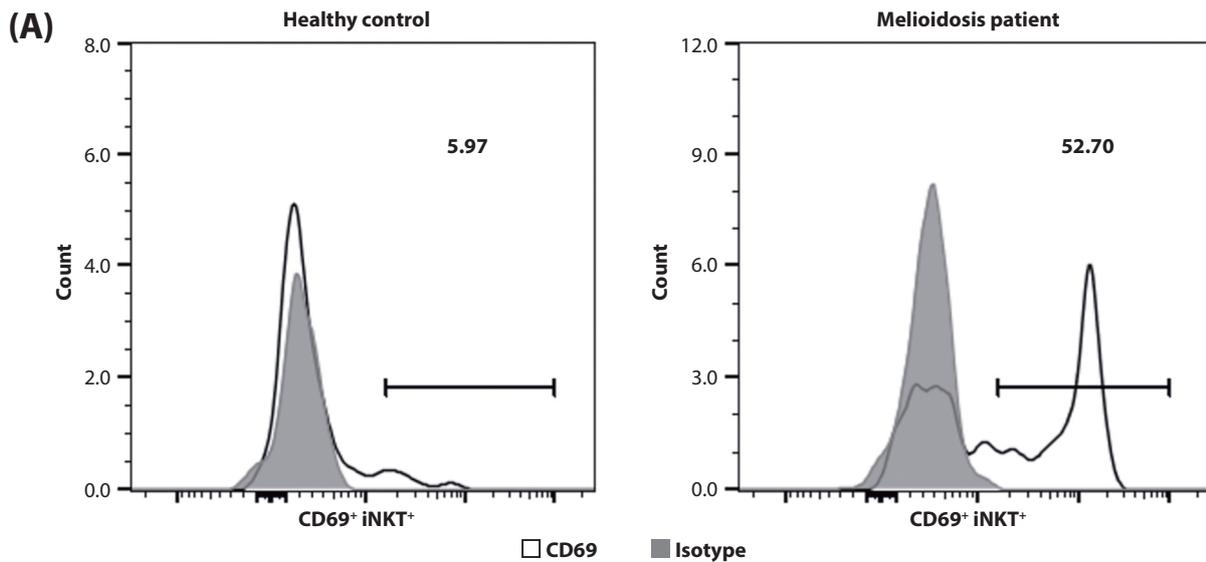
**Table 3. Demographic data of melioidosis patients**

Demographic data	Patients who survived	Patients who died
Number of patients, n	10	3
Ages (years, mean $\pm$ SD)	57 $\pm$ 8	64 $\pm$ 7
Gender		
Male	7	3
Female	3	0
Underlying disease		
None	2	0
Diabetes (type 2)	7	2
Chronic obstructive pulmonary disease	1	1
Type of specimen for bacterial culture		
Blood	7	3
Pus	3	0



**Figure 1. The frequency of circulating iNKT cells in melioidosis patients compared with healthy controls.**

PBMCs were incubated with monoclonal antibodies specific to iNKT cells and analyzed by flow cytometer. (A) Flow cytometry gating strategies for human iNKT cells. From left to right, after selection for lymphocytes by FSC-SSC, B cells were excluded by anti-human CD19. Finally, iNKT cells were identified by using (mAbs) anti-human  $\alpha\beta$  TCR and  $\alpha$ -GalCer/human CD1d dimer (in comparison with Unloaded/human CD1d dimer as control). (B) The percentage of circulating iNKT cells within total lymphocytes and (C) within total T cells obtained from melioidosis patients (N = 13; orange triangles) compared to healthy controls (N = 10; blue squares). Data represented as the median with interquartile range (\* $P < 0.05$  compared between two groups of samples).



**Figure 2. The activation of iNKT cells in melioidosis**

(A) Representative histograms showing the CD69 expression on iNKT cells (black line) as an activation marker compared with isotype control (gray shade) of a healthy control and a melioidosis patient. (B) Expression of CD69 in circulating iNKT cells obtained from melioidosis patients (n = 13; orange triangles) was compared to healthy controls (n = 10; blue squares). Data represented as the median with interquartile range (\* $P < 0.05$  compared between two groups of samples).

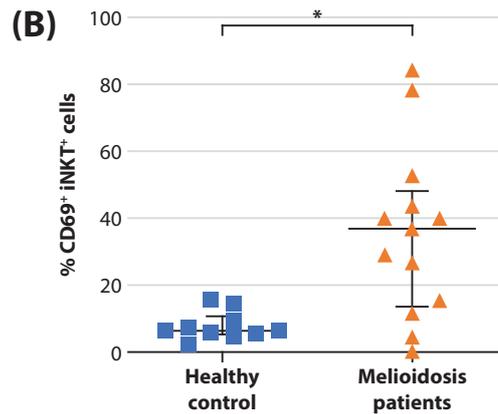


Figure 2. (Continued)

In contrast, the activated iNKT cells of melioidosis patients were significantly higher than in the controls (median 36.84%; interquartile range 13.48-48.09% and median 6.25%; interquartile range 5.16-10.58%, respectively) ( $p = 0.0062$ ) (Figure 2B).

#### *B. pseudomallei* stimulated iNKT cells in vitro

When human PBMCs were cultured with heat-killed *B. pseudomallei* in vitro, the number of iNKT cells was similar to unstimulated controls (median 0.161%; interquartile range 0.10-0.28% and median 0.161%; interquartile range 0.08-0.27%, respectively) (Figure 3A). Similar to what observed in patients, the number of activated iNKT cells in the human PBMCs stimulated with the heat-killed *B. pseudomallei* was also significantly higher than the unstimulated control (median 28.60%; interquartile range 20.25-58.47% and median 8.63%; interquartile range 3.78-13.00%, respectively) ( $p = 0.0005$ ) (Figure 3B).

Stimulation of PBMCs with heat-killed *B. pseudomallei* showed significantly higher of %CD69<sup>+</sup> iNKT cells (mean  $\pm$  SE =  $58.60 \pm 18.12$ ) when compared with the non-stimulated controls ( $1.20 \pm 0.22$ ) ( $p = 0.0309$ ) (Figure 3C). Furthermore, the activation was found to be dose-dependent (Supplement Figure 1). Additional treatment of heat-killed *B. pseudomallei* with DNase, RNase, or proteinase similarly showed higher iNKT cell activation ( $57.33 \pm 20.22$ ,  $57.17 \pm 19.60$ ,  $57.33 \pm 21.74$ , respectively) when compared to the control conditions ( $0.88 \pm 0.06$ ,  $0.82 \pm 0.01$  and  $0.80 \pm 0.40$ ,  $p = 0.0346$ ,  $0.0350$  and  $0.0342$ , respectively), however, no changes were found when compared to untreated heat-killed *B. pseudomallei* (Figure 3C). Taken together, heat-killed *B. pseudomallei* could activate iNKT cells in vitro and DNase, RNase and proteinase-treated heat-killed *B. pseudomallei* did not alter iNKT cells activation. This suggesting that nucleic acid and protein components are not responsible for iNKT cells activation. When *B. pseudomallei* LPS was used to activate the iNKT cells in human PBMC, it also did not response (Figure 3D).

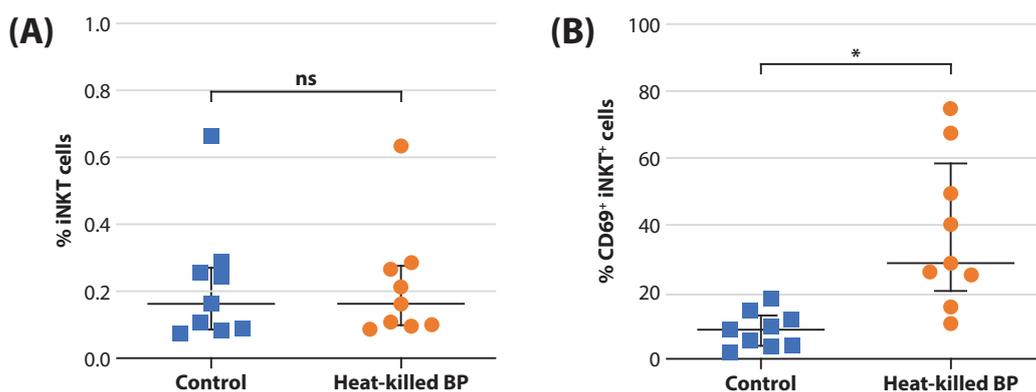


Figure 3. The percentage of iNKT cells and activated iNKT cells in human PBMCs when stimulated with heat-killed *B. pseudomallei* or LPS in vitro.

(A) The percentage of iNKT cells and (B) the number of CD69<sup>+</sup> iNKT cells were activated with heat-killed *B. pseudomallei* in vitro. The PBMCs obtained from healthy controls were treated with heat-killed *B. pseudomallei* (10:1 ratio; heat-killed *B. pseudomallei*: PBMCs) for 24 hours. (C) The iNKT cells activation from human PBMCs were cultured with heat-killed *B. pseudomallei* treated DNase, RNase, proteinase or PBS and heat-killed *B. pseudomallei* (HKBP) without treatment was used as positive control. (D) The percentage of iNKT cells was activated with different concentration of LPS from *B. pseudomallei* (LPS 1-1000 ng/ml) for 24 hours. The medium R10<sup>+</sup> and whole heat-killed *B. pseudomallei* were used as negative and positive control, respectively. After incubated, PBMCs were harvested and stained with antibodies to measure %CD69<sup>+</sup> iNKT cells by flow cytometer as in Methods. Data represented as the mean  $\pm$  SE (\* $P < 0.05$  compared between control and activation sample).

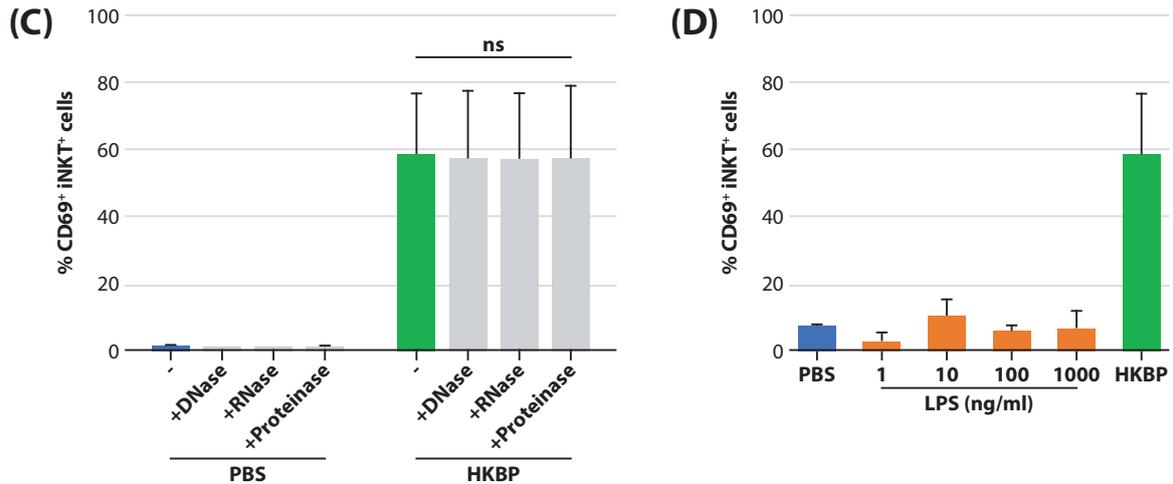


Figure 3. (Continued)

## Discussion

The iNKT cells play an important role against several infectious diseases.<sup>21,22</sup> We showed here for the first time that human iNKT cells are involved during melioidosis infection in human. The level of circulating iNKT cells in melioidosis patients was significantly lower than the healthy control. This finding is consistent with other reports such as autoimmune diseases, bacterial and viral infections.<sup>23,24</sup> In case of patients with tuberculosis, iNKT cells were decreased but had a stimulated phenotype.<sup>25,26</sup> On the other hand, circulating iNKT cells have been found to be increased in primary Sjogren syndromes while iNKT cells number were not different in comparison between patients with scrub typhus and healthy controls. The decrease in circulating iNKT cells might be the consequence from the *in vivo* activation of iNKT cells via T cell receptor (TCR) recognition, of which leading to the TCR internalization that cannot be detected by tetramer/dimer staining methods.<sup>27,28</sup> Another possibility is that *B. pseudomallei* might stimulate iNKT cells and induce apoptosis similar to what was found in *Mycobacterium tuberculosis* infection.<sup>28-30</sup> The apoptosis of iNKT cells in patients with pulmonary tuberculosis were from the chronic activation of glycolipid MTB antigen.<sup>28</sup> The decrease in iNKT cells may also come from *B. pseudomallei* stimulation that down-regulate the iNKT TCR rendering the lower number of iNKT cells as observed by flow cytometry during early stages of infection. Another possibility is the migration of iNKT cells out of the circulation to accumulate at the infection sites.<sup>28</sup> As the number of circulating iNKT cells in the patients prior to *B. pseudomallei* infection was not known, it is also possible that the individuals might have low levels of iNKT cells before infection that render them to be more susceptible to the disease.

In this study, the iNKT cells were found to be activated in blood samples of patients with sepsis melioidosis as well as when cultured *in vitro* with heat-killed *B. pseudomallei*, suggesting that *B. pseudomallei* could stimulate the iNKT cells. Unfortunately, there was no significant correlation between patients who survived and activation of iNKT cells. This might be due to the limited number of patients investigated. A follow up study to measure iNKT cells in those patients who survived after onset of melioidosis would be useful

for determining whether iNKT cells play any important role during infection. In other infections, it was reported that iNKT cells were activated in response to diverse range of infectious agents, including gram-positive and gram-negative bacteria, and mycobacteria.<sup>12</sup> The possible mechanisms by which iNKT cells were activated during infection can be separated into 2 major pathways including direct activation (microbial-antigen mediated) and indirect activation (endogenous-antigen mediated, cytokine mediated, or a combination).<sup>12</sup> The activation of iNKT cells may influence other adaptive immune cells such as T and B cell responses.<sup>9</sup>

Several bacterial components could stimulate iNKT cells such as  $\alpha$ -glucosyl diacylglycerol from *Streptococcus pneumoniae*,  $\alpha$ -galactosyl diacylglycerol from *Borrelia burgdorferi*, and  $\alpha$ -glucuronosyl from *Sphingomonas*.<sup>31</sup> We also attempted to determine the *B. pseudomallei* crude components, which induces iNKT cell activation. Our data showed that DNA, RNA, and protein of *B. pseudomallei* were not associated with iNKT cell stimulation (Figure 3C). There have been reports demonstrated that lipopolysaccharide from gram-negative bacteria such as *Salmonella* Typhimurium and *Escherichia coli* can stimulate iNKT cells during bacterial infections.<sup>32</sup> LPS from *Salmonella* Typhimurium can activate iNKT cells via interleukin 12 (IL-12) that produced from dendritic cells and CD1d recognition.<sup>33</sup> In the case of *Escherichia coli*, LPS-induced IL-12 and IL-18 from dendritic cells were sufficient to stimulate iNKT cells.<sup>34</sup> Accordingly, we hypothesized that LPS from *B. pseudomallei* might be able to activate iNKT cells. Surprisingly iNKT cells could not be activated after stimulated with *B. pseudomallei* LPS (Figure 3D). This finding led us to hypothesize that other molecules such as lipid, or glycolipid from this bacterium might be the major activators for iNKT cells. In mouse model, *B. pseudomallei* could produce a bioactive sphingolipid metabolite during intracellular infection and it was found to associate with bacterial virulence.<sup>35</sup> This lipid might be a possible molecule that could stimulate the iNKT cells. Alternatively, iNKT cells might be indirectly activated by cytokines mediated or endogenous lipid pathways. Further study is needed to identify the components of *B. pseudomallei* that induced iNKT cell stimulation.

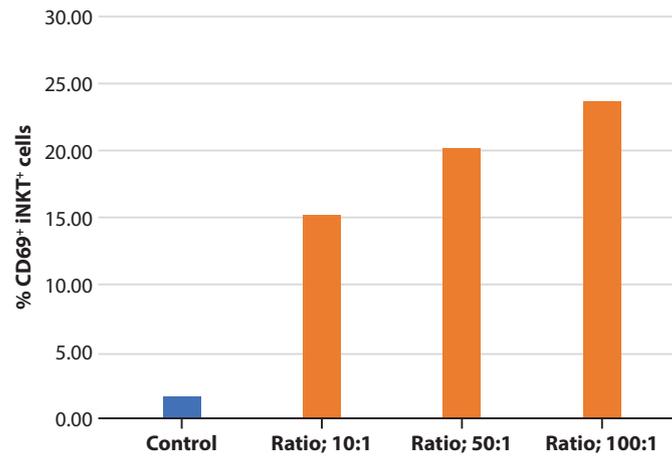
In summary, *B. pseudomallei* could stimulate iNKT cells and the molecules that involve in activation were not DNA, RNA, protein or LPS. These findings provide the first information about the role of human iNKT cells against *B. pseudomallei* infection. The lower number of iNKT cells in septic melioidosis patients obtained will be a beginning of iNKT cells study in melioidosis and lead to better understanding of its role in this bacterial infection.

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**Supplement Figure 1. The activation of human iNKT cells by heat-killed *B. pseudomallei*.** PBMC from healthy persons were incubated with heat-killed *B. pseudomallei* at various ratios of heat-killed bacteria: PBMC 10:1, 50:1, and 100:1). After incubation, cells were stained with antibodies and analyzed by flow cytometry for determine iNKT cells. Each bar represented percentage of CD69 of iNKT cells after stimulated with heat-killed *B. pseudomallei* (orange bars) and an unstimulated control (blue bar).