Human Invariant Natural Killer T (iNKT) cells response to *Burkholderia thailandensis in vitro*

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ABSTRACT

Burkholderia thailandensis is a non-pathogenic bacterium closely related to *Burkholderia pseudomallei*, the causative agent of melioidosis. Invariant Natural Killer (iNKT) cells are subpopulation of T lymphocytes that express natural killer receptors and unique T cell receptors that recognized lipid antigen. Upon activation, iNKT cells rapidly produced and secreted a number of cytokines such as IL-4 and IFN-γ to modulate the other immune cells. The iNKT cells play a crucial role in several bacterial infections. However, the human iNKT cells response to *B. thailandensis* infection has never been investigated. Accordingly, this study purposed to investigate iNKT cells response to *B. thailandensis in vitro*. Heat-killed *B. thailandensis* (HKBT) was used to stimulate human peripheral blood mononuclear cells, the iNKT cells number and activated levels were evaluated by flow cytometer compared to heat-killed *B. pseudomallei* (HKBP) and unstimulated controls. Results showed that the percentage of number iNKT cells in total T cells were all the same (0.40%, 0.41% and 0.41%) in with HKBT and HKBP and unstimulated control. We found similar result when the percentage of number iNKT cells was calculated as absolute lymphocytes (0.18% for PBS, 0.15% for HKBT and 0.18% for HKBP). Interestingly, when the percentage of activated cells (%$CD69^+$ $iNKT^+$ cells) was analyzed, HKBT gave higher stimulation (48.7%) than those of HKBP (34.6%) and unstimulated control (0.64%). The components of *B. thailandensis* that associated with iNKT cells stimulation were found to relate with nucleic acid and proteins. Our finding demonstrated that HKBT could activate human iNKT cells stronger than HKBP. This dissimilar might describe the different in host immune stimulation leading to better bacteria clearance of *B. thailandensis* hence make it not cause disease.

Keywords: iNKT cells, *Burkholderia thailandensis, Burkholderia pseudomallei*

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Introduction

Burkholderia thailandensis is a bacterium closely related to *Burkholderia pseudomallei*, the causative agent of melioidosis [1]. The clearly different between these two species is the capability of *B. thailandensis* to assimilate L-arabinose (Ara^+), but *B. pseudomallei* does not have this ability (Ara^-) [2]. In addition, *B. thailandensis* has been revealed low virulence in animal models and rarely cause disease in human though it presents various phenotypic characteristics similar to *B. pseudomallei* [3-4]. Immune cross-reactivity between *B. pseudomallei* and *B. thailandensis* were found both in human and murine models [5-6]. Moreover, melioidosis patients reveal strong cellular and humoral cross-immunity between pathogenic *B. pseudomallei* and less pathogenic *B. thailandensis* [6]. Recently there are reports of heat-killed and live *B. thailandensis* are capable provide protection against *B. pseudomallei* infection [7].

Invariant natural killer T (iNKT) cells are an unconventional T lymphocytes population that express a semi-invariant $\alpha\beta$ T cell receptor (TCR), Vα14Jα18 paired with Vβ8.2, Vβ7, or Vβ2 in mice and Vα24Jα18/Vβ11 in human and natural killer (NK) receptor (NK 1.1 in the mouse and CD161 in humans) [8-9]. This unique TCR of iNKT cells can recognize lipid or glycolipid antigen such as α-galactosylceramide (α-GalCer) presented by MHC class I-like antigen-presenting molecule, CD1d on antigen-presenting cells (APCs) [10]. Upon TCR ligation, iNKT cells release several cytokines to contribute the innate and adaptive immune cells activation [11]. This mechanism has been demonstrated relate to defense against several microbes, including bacteria, virus, fungi, and parasites. [12]. Our previous study demonstrated that human iNKT cells could be stimulated by *B. pseudomallei* during infection rendering decreased circulating iNKT cells number [13]. Moreover, the number of mouse iNKT cells was also reduced when infected with *B. pseudomallei* (unpublished observation) and in *B. thailandensis* [14]. These finding make us to hypothesize that *B. thailandensis* may activate human iNKT cells like those found in *B. pseudomallei.*

In this study we aimed to determine iNKT cells response to *B. thailandensis in vitro* by co-culture PBMC with heat-killed *B. thailandensis* and evaluated the activated iNKT cells using CD69, known as activation marker, by flow cytometry. Subsequently, the components of *B. thailandensis* that induce iNKT cell stimulation were explored using heat-killed *B. thailandensis* treated with DNase, RNase, or proteinase before culture with healthy PBMC and iNKT cells activation state was then determined by flow cytometry.

Methodology

1. Peripheral blood mononuclear cells (PBMCs) isolation

All procedures were approved by The Human Ethic committee of Khon Kaen University (HE621362). PBMCs were isolated from healthy blood sample using Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. The mononuclear cells were washed twice with sterile 0.05 Molar phosphate-buffered saline (PBS) pH 7.4 and re-suspended with 1 ml of $R10^+$ (RPMI 1640

comprising 10% fetal bovine serum (FBS), 100 U ml-1 penicillin-streptomycin, 10 mM HEPES buffer solution and 5.5 µM 2-mercaptoethanol (2-ME); all reagents was purchased from Gibco (Gibco, Thermo Fisher Scientific, Waltham, MA) [15]. The number of cells viability was estimated by trypan blue exclusion assay (Gibco, Thermo Fisher Scientific, Waltham, MA). Finally, the cells number was adjusted to 1 x 10^7 cells/ml with total of 1 ml for the following experiments.

2. Heat-killed bacteria preparation

B. pseudomallei strain 1026b and *B. thailandensis* strain E264 were grown on Ashdown's agar and incubated at 37 °C to obtain a single colony. To prepare mid-log-phase bacteria, single colony was inoculated into 3 ml of Luria-Bertani (LB) broth (Titan Biotech LTD., Rajasthan, India), incubated overnight at 37°C with occasional shaking at 200 rpm. Subsequently, 2% of overnight culture were transferred into 50 ml LB broth and cultured for 3 hours with shaking at 200 rpm in a 37°C incubator shaker (J.P. SELECTA, Barcelona, Spain). The bacterial suspension was centrifuged at 10,000 g for 10 minutes and washed with sterile PBS, pH 7.4. After that, pellet was suspended in 1 ml PBS and bacterial number were determined by plating on LB agar. Bacteria (1 x 10^8 CFU/ml) were heated-inactivated for 1 hour at 80°C and then checked their viability by plating on LB agar [16].

3. Heat-killed *B. thailandensis* treated with enzymes

Five hundred microliters of 1 x 10^7 CFU/ml heat-killed *B. thailandensis* was treated overnight with 1 unit of DNase (Promega, Madison, WI) or 100 µg of RNase (Thermo scientific, Carlsbad, CA) or 100 µg of proteinase (Promega, Madison, WI) at 37°C. After that the activity of enzymes was inhibited by incubating at 95°C for 15 minutes. The heat-killed bacteria were checked for bacterial lysis and enzymes digestions were completed [13].

4. PBMC stimulation with heat-killed bacteria

PBMC 1x10^5 cells in 50 µl of $R10^+$ medium was incubated with 50 µl of 1x10^6 CFU heat-killed *B. thailandensis* or heat-killed *B. pseudomallei* (MOI = 10) or sterile PBS as negative control in U-Bottom of 96 wells plates (BD Biosciences, San Diego, CA) at 37°C for 24 hours. Then, cells were harvested and measured viability of cells using trypan blue staining method. The iNKT cells were then determined their numbers and activation levels by flow cytometry.

5. PBMC activation with heat-killed *B. thailandensis* treated with and without enzymes

The 1x10^5 human PBMCs in 50 µl of $R10^+$ medium was incubated with 50 µl of 1x10^6 CFU heat-killed *B. thailandensis* or enzymes treated heat-killed *B. thailandensis* (MOI = 10) in $R10^+$ media in 96-well plates (BD Biosciences, San Diego, CA) at 37°C for 24 hours. PBS alone or PBS treated with enzyme served as unstimulated controls. After incubation, PBMCs were collected and iNKT cells activation were measured by CD69 expression [17] in comparison to isotype-matched controls mAb (mouse IgG1) using flow cytometry.

6. Immunofluorescence staining for iNKT cells detection

PBMCs ($1x10^5$ cells) were suspended with 50 µl of staining buffer (2% (vol/vol) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA), and 0.09% (wt/vol) sodium azide in PBS) then incubated with 2.5 µg of Fc block (BD Biosciences, San Diego, CA) for 10 minutes at room temperature. The cells were stained with optimum concentration of monoclonal antibodies (mAbs) recommended by the manufacturers; APC-labeled α-GalCer/human CD1d dimer (BD Biosciences, San Diego, CA), and followed by peridinin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-conjugated anti-human CD19 mAb (Thermo, Waltham, MA), FITC-conjugated anti-human $\alpha\beta$ TCR (Thermo, Waltham, MA) mAb, and brilliant violet (BV421)-conjugated anti-human CD69 mAb (BioLegend, San Diego, CA) for 15-30 minutes at 4°C in the dark [15]. 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 version 10 (Tree Star).

7. Statistical analysis

Statistical analyses were performed by using GraphPad Prizm 5.0. The one-way analysis of variance (ANOVA) was used to analyze difference in multiple groups and unpaired t-test was used to compared data between the groups of samples in each parameter. A *p*-value of < 0.05 was considered as statistically significant.

Results

1. Heat-killed *B. thailandensis* could activate human iNKT cells

Human PBMCs were stimulated with heat-killed *B. thailandensis* and the iNKT cells were analyzed by flow cytometry using gating strategies as shown in Figure 1A. To evaluate iNKT cells, lymphocytes were gated on a forward scatter (FSC) – side scatter (SSC), then B cells were exclude using anti-human CD19. Finally, iNKT cells were identified as anti-human $\alpha\beta$ TCR and α-GalCer/human CD1d dimer double-positive cells in combination with monoclonal antibody (mAb) against CD69 expression as activation marker of iNKT cells (Figure 1A). The result showed that the percentage of iNKT cells in total T cells were 0.40%, 0.41% when activated with heat-killed *B. thailandensis* (HKBT) and heat-killed *B. pseudomallei* (HKBP) which did not different from unstimulated control (0.41%) (Figure 1B). The similar result was also found when iNKT cells when calculated as absolute lymphocytes (0.18% for PBS, 0.15% for HKBT and 0.18% for HKBP). In contrast, when activation marker (CD69) was used, iNKT cells activation levels (%$CD69^+$ $iNKT^+$ cells) from HKBT were significantly higher (48.7%) than unstimulated control (0.64%, $P < 0.0001$) and HKBP (34.6%, $P < 0.0001$) (Figure 1C). This result indicated that heat-killed *B. thailandensis* could activate human iNKT cells higher than HKBP.

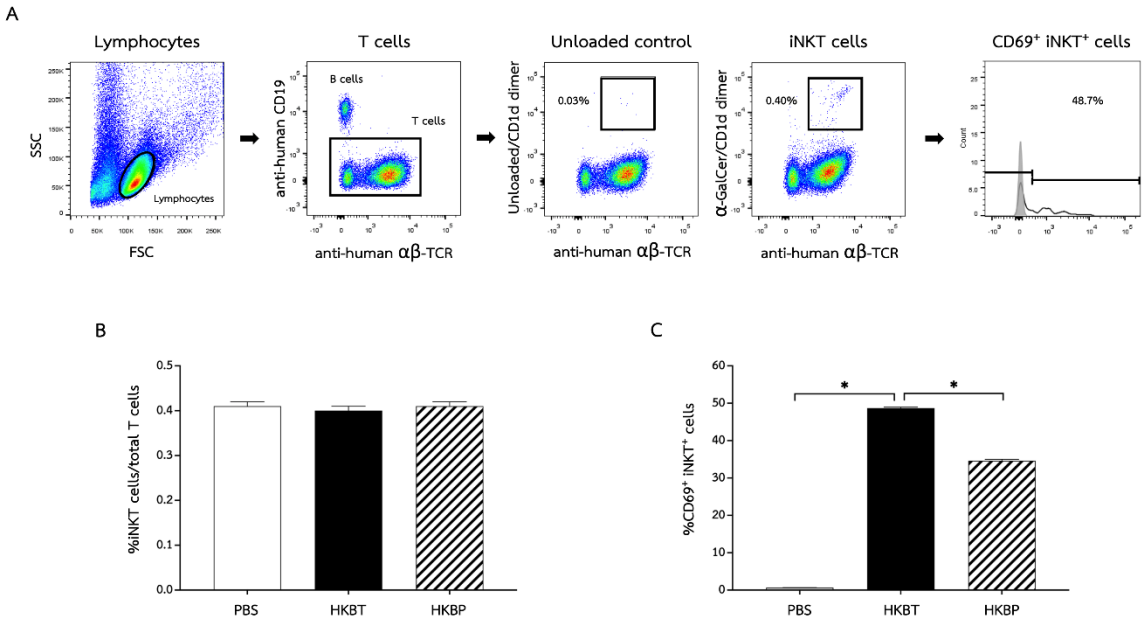

Figure 1 The percentage of iNKT cells and their activation levels when stimulated with heat-killed *B. thailandensis*.

The healthy human PBMCs (1 x 10^5 cells) were stimulated with heat-killed *B. thailandensis* (HKBT) at the ratio 10:1 (HKBT: PBMC) for 24 hours. PBS and heat-killed *B. pseudomallei* (HKBP) was used as unstimulated and positive controls. After incubation, cells were harvested and stained with monoclonal antibodies which specific to iNKT cells and analyzed by flow cytometry. **(A)** Flow cytometry gating strategies for identify iNKT cells. From the left to right, total lymphocytes were selected by FSC-SSC, B cells were excluded by anti-human CD19. The iNKT cells were identified using anti-human αβ TCR and α-GalCer/human CD1d dimer (in comparison with Unloaded/human CD1d dimer as control). Representative histogram showing the CD69 expression on iNKT cells as an activation marker (black line) compared with isotype control (gray shade). **(B)** The percentage of iNKT cells and **(C)** iNKT cells activation level when cultured with PBS as unstimulated control (white bar), heat-killed *B. thailandensis* (black bar), and heat-killed *B. pseudomallei* (pattern bar). The data presents as mean ± standard deviation of mean (SD). Statistically significant differences were evaluating using the unpaired-t test. The asterisks (*) indicate statistical significance ($P < 0.05$).

2. Nucleic acid and proteins of *B. thailandensis* might relate to iNKT cells activation

In order to investigate the components of *B. thailandensis* that could stimulate iNKT cells, we utilized heat-killed *B. thailandensis* treated with enzymes including DNase, RNase, and proteinase to remove either DNA, RNA, or proteins before incubating with healthy PBMC. The levels of iNKT cells activation (%$CD69^+$ $iNKT^+$ cells) of HKBT treated with DNase, RNase, or proteinase were 18.4%, 18.2% and 21.2%, respectively which were significantly higher than PBS control conditions (0.6%, 1.4%, 1.7%

and 0.6%, P < 0.0001) (Figure 2) whereas the intact heat-killed *B. thailandensis* could stimulate significantly high level of iNKT cells (48.7%, P < 0.0001). This result suggested that either DNA, RNA, or protein of *B. thailandensis* might be necessary for activation of iNKT cells.

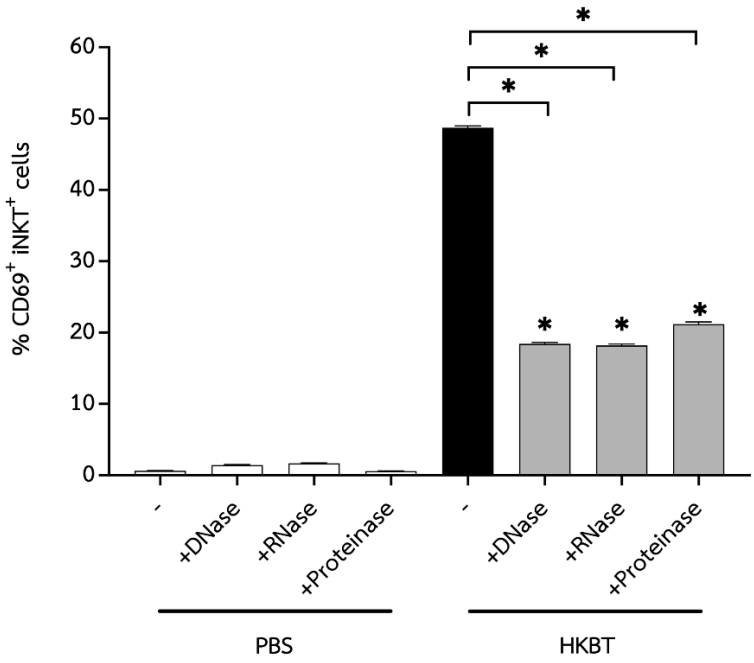

Figure 2 Nucleic acid or proteins of *B. thailandensis* were needed for human iNKT cells activation.

The healthy human PBMCs were cultured with heat-killed *B. thailandensis* (HKBT) treated enzymes (gray bars) DNase, RNase, proteinase at MOI = 10 for 24 hours while PBS (white bars) and whole heat-killed *B. thailandensis* (black bar) were used as negative and positive controls. After incubation, cells were harvested and stained with monoclonal antibodies to determine %$CD69^+$ $iNKT^+$ cells by flow cytometer as described in methods. Data are shown as mean ± SD. Statistically significant differences were evaluating using the ANOVA. The asterisks (*) indicate statistical significance (P < 0.05).

Discussions

B. thailandensis is a bacterium that closely related to *B. pseudomallei*, the causative agent of melioidosis. This bacterium was used as a model of *B. pseudomallei* infection, because it has similar pathogenesis in mice but not cause diseases thus make it did not require biosafety level 3 (BSL-3) facilities for study [18]. *B. pseudomallei* has been found to stimulate human iNKT cells [13]. Moreover, we recently found that it also stimulated mice iNKT cells (unpublished observation). For *B. thailandensis*, our colleague revealed that it could stimulate mice iNKT cells similar to *B. pseudomallei* [14]. Accordingly, we showed here for the first time that *B. thailandensis* could stimulate iNKT cells *in vitro*. Although they could be stimulated, the percentage of iNKT cell number was not different from

unstimulated control (Figure 1B) suggested that HKBT did not effect on iNKT cells numbers similar to those found in HKBP [13]. However, *in vivo* study demonstrated the number of iNKT cells was decreased after *B. thailandensis* infection in C57BL/6 mice [14]. Such finding might be due to the live *B. thailandensis* that induced host cell death or cell migration to other parts of body. This finding was also found in *Mycobacterium tuberculosis* infection [19]. Another possible explanation is the down-regulation of TCRs which are CD1d-dimer binding sites rendering undetectable by flow cytometry led to decreased number of iNKT cells after infection [20].

The activation level of iNKT cells when co-cultured with heat-killed *B. thailandensis* was higher than unstimulated control (Figure 1C), suggesting that *B. thailandensis* could activate human iNKT cells. Consistent with other studies, several bacteria including Gram-negative and Gram-positive were also stimulate iNKT cells [21]. Our result demonstrated that HKBT could activated iNKT cells better than HKBP. This phenomenon leads to a new hypothesis whether HKBT activates more iNKT cells giving higher primed innate immune cells with significant IFN-γ levels making the efficient bacterial clarence by the host. However, the limitation of this study is the dead bacteria which may give different result when live *B. thailandensis* is evaluated both *in vivo* and *in vitro*. The mechanism of iNKT cells activation by microbial and their products could divide into 2 major pathways including CD1d-dependent pathway that TCRs on iNKT cells directly interacts with microbial antigens presented by CD1d molecule. Another pathway is CD1d-independent pathway that induce iNKT cells activation by cytokines produced from antigen presenting cells such as dendritic cells (DCs) after Toll-like receptor (TLR)-mediated activation by microbial compound [22-24].

Subsequently, we explored the crude components of *B. thailandensis* that stimulate iNKT cells. Our data showed that bacterial DNA, RNA, and proteins might be related to induce iNKT cells activation (Figure 2) which was different from those uncovered with heat-killed *B. pseudomallei* when their DNA, RNA, and proteins did not associate iNKT cells stimulation [13]. This dissimilarity might be reflecting the difference of host clearance to these bacteria. Interestingly, the residual of iNKT cells activation state in each group of heat-killed *B. thailandensis* treated with enzymes (Figure 2) made us hypothesize that carbohydrate or other lipid components which could not be destroyed by enzymes may induce iNKT cells stimulation. Several bacterial components were reported to activate iNKT cells including α-glucuronosyl from *Sphingomonas*, α-glucosyl diacylglycerol from *Streptococcus pneumoniae*, and α-galactosyl diacylglycerol from *Borrelia burgdorferi* [25]. Additionally, lipopolysaccharides (LPS) from *Escherichia coli* and *Salmonella* Typhimurium can stimulate iNKT cells during infection [24]. Previous study has shown that LPS structure of *B. thailandensis* was different from *B. pseudomallei* [26]. Novem and colleagues demonstrated that LPS synthesized from *B. pseudomallei* was induced weak immunological activities rendering evade host defense while *B. thailandensis* LPS can activate the innate immune systems more strongly, resulting in efficient bacterial clearance [27]. The difference immunological activities of *B. thailandensis* and *B. pseudomallei* may be led to different of iNKT cells

activation (Figure 1C). Therefore, further study is needed to investigate purified LPS and identify more components of *B. thailandensis* that associate to induce iNKT cells activation.

Our study showed that *B. thailandensis* could activate iNKT cell similar to *B. pseudomallei*. Other molecules beside DNA, RNA, and proteins of the bacteria may associate to iNKT cells stimulation. These findings provide information about iNKT cells in human immune responses to *B. thailandensis* that may be helpful for the treatment and developing vaccine adjuvants to promote protective immunity against pathogenic *B. pseudomallei* in the future.

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