# Decrease of spleen invariant natural killer T and natural killer cell numbers in *Burkholderia thailandensis*-infected C57BL/6 mice

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## Abstract

**Background:** *Burkholderia thailandensis* is a non-pathogenic bacterium that is closely related to *B. pseudomallei*. Invariant natural killer T (iNKT) cells are innate lymphoid cells that play a role in bacterial infections; however the iNKT cells in *B. thailandensis* infections are still uncharacterized.

**Objectives:** To study the cytokine production in α-galactosylceramide (α-GalCer)-stimulated lymphocytes from mouse organs. The numbers of spleen iNKT cells, natural killer (NK) cells, dendritic cells and macrophages in *B. thailandensis*-infected C57BL/6 (B6) mice were investigated.

**Methods:** Lymphocytes, obtained from mouse lungs, livers, and spleens, were cultured for 48 hours with α-GalCer, and their cytokine levels were determined. iNKT, dendritic, macrophage and NK cells in the spleen of *B. thailandensis*-infected B6 mice or iNKT knock out (KO) mice, stimulated with either phosphate-buffered saline (PBS) or α-GalCer, were analyzed by flow cytometry. This was also done in adoptive cell transfer experiments.

**Results:** Interferon gamma (IFN-γ) was predominantly produced in α-GalCer-stimulated mouse spleen and liver lymphocytes, while interleukin (IL)-13 was the main cytokine found in the lungs. *B. thailandensis*-infected mice had a significantly lower number of splenic iNKT, NK and dendritic cells, but not macrophages, compared to the control. Interestingly, the number of NK cells was significantly decreased in iNKT wild type and iNKT KO mice after *B. thailandensis* infection. The number of NK cells recovered by activation with α-GalCer or after adoptive transfer of iNKT cells into KO mice. The iNKT cell-mediated reduction of dendritic and NK cells might be related to infection by *B. thailandensis*.

**Conclusion:** *B. thailandensis* decreased the number of iNKT and NK cells in the spleen of infected mice.

**Keywords:** iNKT cells, *B. thailandensis*, α-GalCer, iNKT knockout B6 mice, Melioidosis

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## Introduction

Invariant Natural Killer T (iNKT) cells are a subset of T cells that have functions in both innate and adaptive immunity. They express both the T cell receptor (TCR) and some surface receptors of natural killer (NK) cells. These cells recognize glycolipid antigens, presented by the monomorphic CD1d molecule, such as α-galactosylceramide (α-GalCer), a glycolipid originally isolated from a marine sponge. α-GalCer, a specific antigen that activates iNKT cells, has been used broadly both for *in vitro* and *in vivo* experiments involving this cell type from mice and humans.[1-3] Activated iNKT cells can rapidly secrete a large amount of Th1 and Th2 cytokines and chemokines.[4] They can also activate dendritic cells (DCs), NK cells, macrophages, and T and B cells, leading to activation of both innate and adaptive immunity.[5,6] Based on innate immune responses, iNKT cells can rapidly eradicate a number of infectious organisms, as well as stimulating cytokine production.[6-8] In addition, interferon gamma (IFN-γ) produced from iNKT cells has been demonstrated to play a protective role against bacterial infections, such as those caused by *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Borrelia burgdorferi*.[9,10]

Melioidosis is a fatal disease caused by the Gram-negative bacterium *Burkholderia pseudomallei*. It is endemic in Southeast Asia, Northern Australia, and Thailand. It has been estimated that at least 3,000–5,000 patients contact this disease each year, mostly in northeastern Thailand.[11] *B. pseudomallei* is an intracellular pathogen, with both innate and adaptive immune host responses playing a role against this bacterial infection. In particular, innate responses are important in protecting the host against infection,[12] with IFN-γ and neutrophils being involved.[13]

iNKT cells participate in IFN-γ production, though their role in melioidosis is still unknown. As a result, iNKT knockout (KO) mice are used in such studies; however, as they are not available in Thailand, the experiments have to be performed in Japan. In this current study, *B. thailandensis*, a non-pathogenic member of the same genus was used as a model system of *B. pseudomallei* infection, as it did not require biosafety level 3 (BSL-3) facilities, and had a similar pathogenesis mechanism in inbred mice. To explore the role of iNKT cells in melioidosis, a pilot experiment was done at BSL-1 to determine the levels of splenic iNKT, NK, dendritic and macrophages cells in *B. thailandensis*-infected C57BL/6 mice, and the cytokines produced in α-GalCer-stimulated lymphocytes from mouse organs. The results led to further investigation of iNKT cells in melioidosis.

## Methods

### Bacterial strains and culture conditions

*B. thailandensis* E264 (ATCC 700388) was obtained from Professor Yasuhiko Horiguchi, Research Institute for Microbial Diseases, Osaka University, Japan. It was an environmental isolate from northeast Thailand.[14,15] The median lethal dose (LD50) of this bacterium was $1.8 \times 10^6$.[14] Bacteria from a glycerol stock were streaked on Ashdown's agar and incubated at 37°C for 48 hours. To prepare a mid-log phase bacterial culture, a single colony was inoculated into 3 ml Luria-Bertani (LB) broth and incubated overnight at 200 rpm on a rotary shaker at 37°C. The bacterial suspension was then transferred into 50 ml LB as a 1% inoculum ($OD_{550}$ = 0.1). At mid-logarithmic phase ($OD_{550}$ = 0.3–0.5), cultures were harvested by centrifugation, and washed twice in sterile phosphate-buffered saline (PBS) pH 7.2. Bacterial cells were resuspended in PBS to the desire concentration compared with the growth curve. The actual number of viable bacteria in the suspension was confirmed by plating on Ashdown's agar and incubation at 37°C for 48 hours. Based on these results, bacterial suspensions were adjusted to approximately $10^8$ colony forming units (CFU)/ml for infection.

### C57BL/6, BALB/c, and iNKT KO mice

Male 6–8 week-old wild type (WT),[16] *Traj18-/-* KO (lacking iNKT cells) C57BL/6 (B6)[17] and BALB/c mice were kept under specific pathogen-free conditions, following protocols approved by the Animal Care and Use Committee, The University of Tokyo (Ref:65/2016). For the adoptive transfer experiment, six WT B6 mice and six iNKT KO B6 mice were separated into six groups (**Figure 1**). Via a tail vein, each group was intravenously injected with PBS or α-GalCer (1μg in 200μl PBS), or iNKT cells (750,000 cells/mouse) and 1 μg α-GalCer. After 12 hours, mice were infected with *B. thailandensis* ($10^7$ cells/mouse) or PBS via an intraperitoneal (IP) route. All mice were sacrificed 24 hours after infection, and their spleens were collected for measuring iNKT cells, macrophages, dendritic cells and NK cells by flow cytometry.

### Cytokine measurements

Total lymphocytes ($1 \times 10^6$ for spleens, livers, and lungs) were isolated from BALB/c mice stimulated with 0, 1 or 10 ng/ml α-GalCer. Supernatants were collected after 48 hours incubation. Concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, interleukin (IL)-4, IL-10, IL-13, and IL-17A were measured with a Cytometric Bead Array (CBA; BD Biosciences), according to the manufacturer's instructions. Data were analyzed by FCAP software (BD Biosciences).
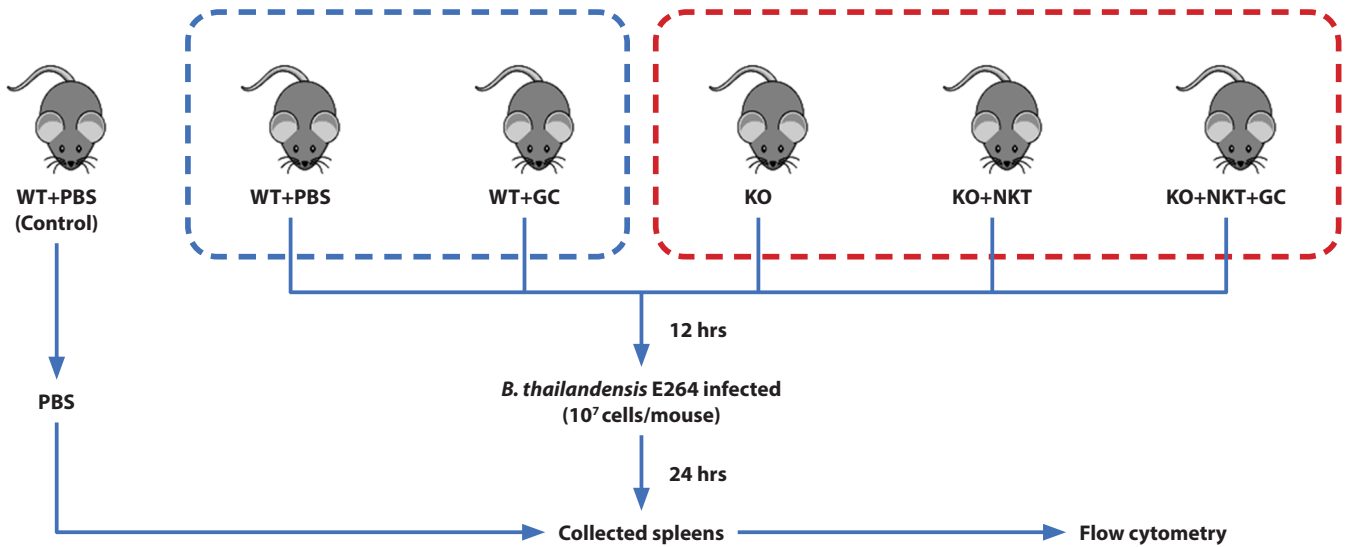
NKT cells = 750,000 cells/mouse
GalCer = 1 ug/mouse

**Figure 1. The experimental design of all mice in *B. thailandensis* infected experiment.**
Six wild type B6 mice were separated into 3 groups including uninfected WT mice (injected with PBS used as a control), WT mice injected with either PBS or with α-GalCer for 12 hours before infected with *B. thailandensis*. Six iNKT knockout B6 (KO) mice were separated into 3 groups including KO mice injected with PBS 12 hours before infected with *B. thailandensis*, KO mice injected with PBS and adoptive transferred of iNKT cells 12 hours before infected with *B. thailandensis* and KO mice injected with α-GalCer and adoptive transferred of iNKT cells 12 hours before infected with *B. thailandensis*. All mice were sacrificed at 24 hours after infection and their spleens were collected for measuring of iNKT cells, macrophages, dendritic cells and NK cells by flow cytometer.

### Adoptive transfer of iNKT cells

Ten B6 mice were sacrificed and their spleens were harvested for lymphocyte isolation. iNKT cells were enriched by magnetic-activated cell sorting (MACS; Miltenyi Biotec, Germany), before being separated on a FACSAriaIII sorter (BD Biosciences, NJ). In brief, pellets of mononuclear cells (MNCs) were added to allophycocyanin (APC)-labeled α-Gal-Cer/mouse CD1d dimers (2 μl/$10^6$ cells) and incubated for 30 minutes at 4°C. The cell pellet was washed twice with FACS staining buffer, with centrifugation at 800 × g for 5 min at 4°C, and resuspended with MACS enrichment buffer. Ten μl of anti-APC MicroBeads per $1 \times 10^7$ cells was added, followed by incubation for 10 minutes at 4°C in the dark. Purification followed the manufacturer's instructions. The cells were then passed over a LS column, and the enriched iNKT cells were recovered by positive selection. Enriched iNKT cells were then further purified by a FACSAriaIII cell sorter. Purified iNKT cells were resuspended in PBS (750,000 cells/mouse) and adoptively transferred by intravenous injection into a tail vein.

### Flow cytometry

Antibodies (BD Biosciences, eBioscience or BioLegend) used for flow cytometry detection were: fluorescein isothiocyanate (FITC) anti-TCRβ (H57-597), phycoerythrin-cyanine7 (PE-Cy7) anti-NK1.1 (PK136), APC-Cy7 anti-CD11b (M1/70), PE-Cy7 anti-CD11c (N418), peridinin chlorophyll protein (PerCP)-Cy5.5 anti-B220 (RA3-6B2), PE anti-IFN-γ (XMG1.2). α-GalCer-loaded CD1d (α-GalCer/CD1d) dimer

for iNKT cell enrichment and detection were prepared as described previously. Cells were analyzed and sorted with a FACSAriaIII system (BD Biosciences), and data were analyzed by FlowJo software.

### Statistical analysis

One-way ANOVA was used to analyze differences between multiple groups, and Student's t-test was used to compare data between the groups of mice in each parameter. The results were presented as mean ± the standard deviation of the mean. The level of significance for all statistical analyses was set at *p* < 0.05.

## Results

### Cytokine production in lymphocytes from different BALB/c mouse organs stimulated with α-GalCer

As *B. thailandensis* and *B pseudomallei* were found mostly in the spleen, liver and lungs of infected organisms, the cytokine levels from lymphocytes, obtained from different mouse organs activated *in vitro* with α-GalCer, were evaluated. Using α-GalCer in the cell culture, most activated lymphocytes would be iNKT cells, suggesting the level of cytokines from these cultures mostly came from iNKT cells. α-GalCer at 1 and 10 ng/ml could activate splenocytes to produce high levels of IFN-γ, IL-13, IL-4, IL-17A, IL-10 and GM-CSF (**Figure 2**). IL-13 levels were shown to be highest in activated lung lymphocytes, while levels of IFN-γ were highest in liver and spleen. The result indicated that α-GalCer-stimulated different cytokine levels in different organs.
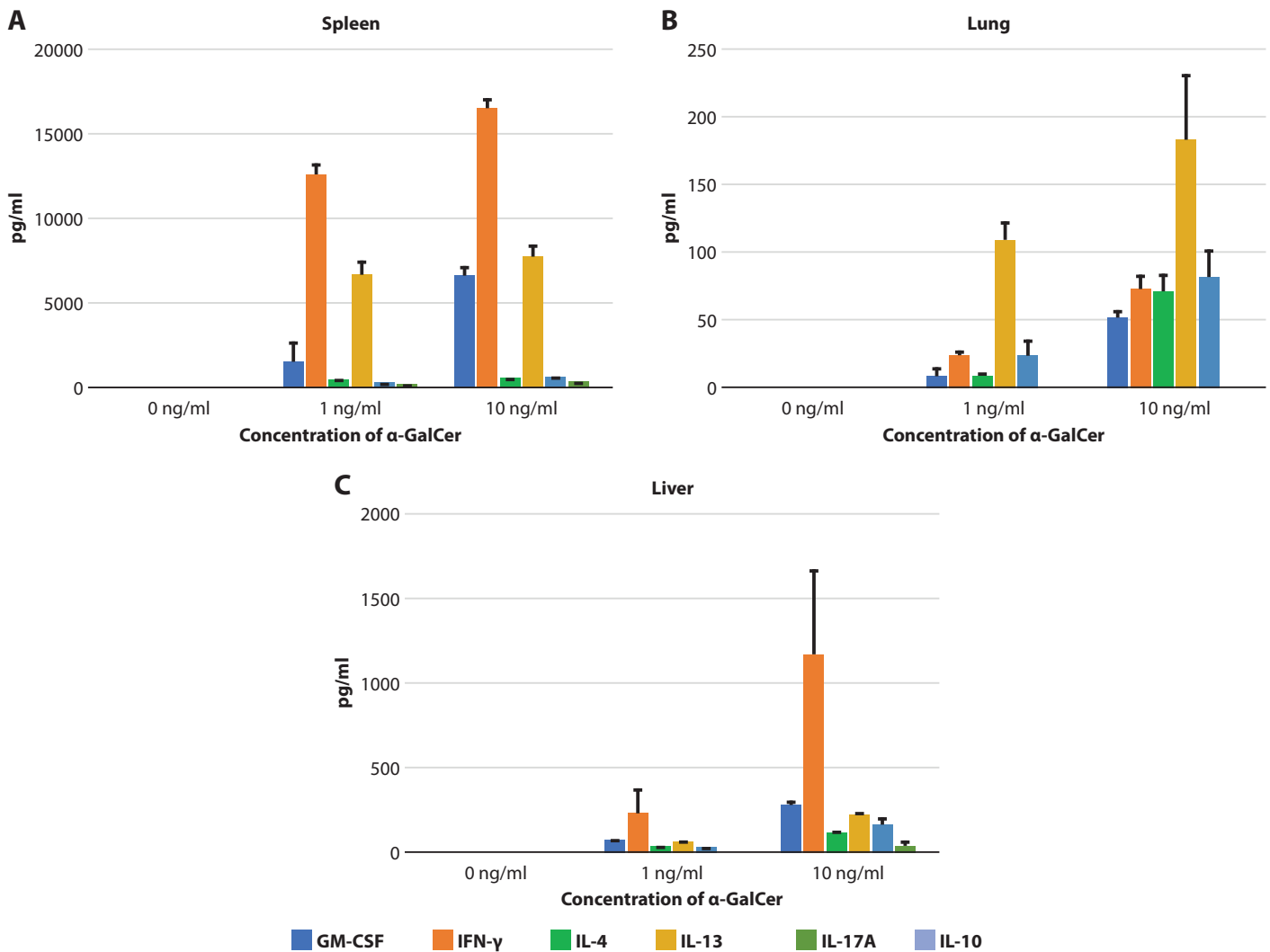
**Figure 2. Cytokines profiles in α-GalCer stimulation *in vitro*.**
Three BALB/c mice were sacrificed and their spleens (A), lungs (B) and livers (C) were harvested. Lymphocytes were separated from each organ and cultured with containing 0, 1 and 10 ng/ml α-GalCer in complete RPMI1640 media for 48 hours. The supernatants were then used for cytokine detections using BD Cytometric Bead Array. Data are mean ± SD of cytokine levels in organs.

### *Role of iNKT cells in B. thailandensis-infected mice*

To monitor the role of splenic iNKT cells during *B. thailandensis* infection, we investigated the responses of iNKT cells in WT, iNKT KO mice and iNKT KO mice adoptively transferred with iNKT cells. The experiments were done with and without activation with α-GalCer to monitor the role of iNKT cells. The numbers of spleen iNKT cells were significantly decreased in *B. thailandensis*-infected WT (WT+*Bt*) and α-GalCer-stimulated infected WT (WT+GC+*Bt*) mice (**Figure 3**). As expected, iNKT cells could not be detected in all iNKT KO mice groups. The decreased iNKT cell number could not be restored in infected iNKT KO mice adoptively transferred with sorted iNKT cells (**Figure 3**).

The number of macrophages from WT+*Bt* and infected iNKT KO (KO+*Bt*) mice were lower, though not significantly, while dendritic cell numbers were significantly reduced in α-GalCer-stimulated infected WT (WT+GC+*Bt*), iNKT KO (KO+NKT+GC+*Bt*) and iNKT KO (KO+*Bt*) mice compared to the control group. This result indicated that *B. thailandensis* infection of WT and iNKT KO mice did not affect the number of macrophages. Splenic NK cells in all infected groups were significantly lower than in the WT control group. Interestingly, for the iNKT KO mice group, the number of NK cells was significant lower, but increased when iNKT KO mice underwent adoptive cell transfer with iNKT cells.
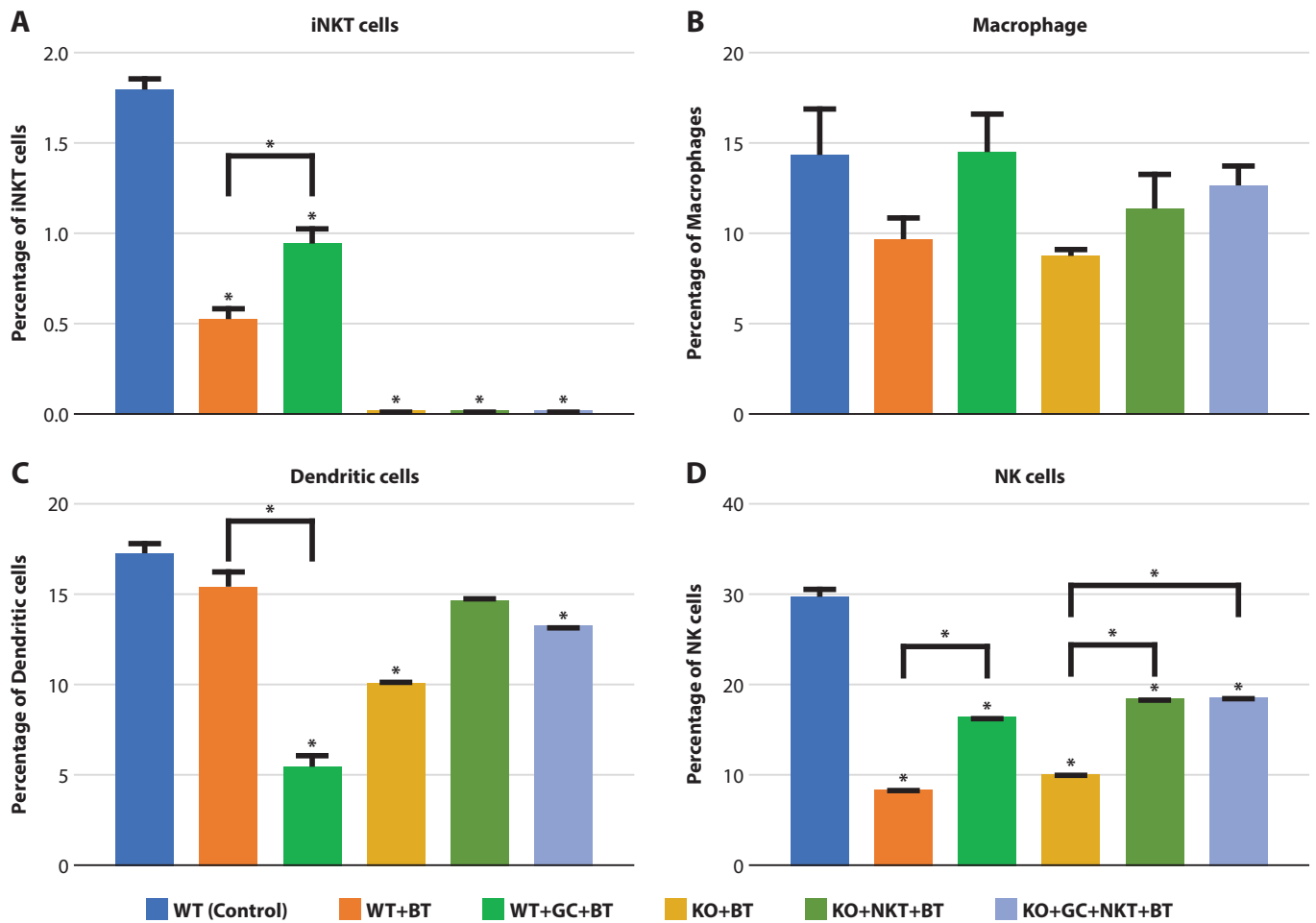
**Figure 3. Number of iNKT cells, macrophages, dendritic cells and NK cells in *B. thailandensis* infected mice.**
The iNKT cells (A), macrophages (B), dendritic cells (C) and NK cells (D) were obtained from spleens of *B. thailandensis* infected wild type (WT+BT), α-GalCer stimulated infected wild type (WT+GC+BT), infected iNKT cell knock out (KO+BT), infected iNKT knock out mice and adoptive transfer with iNKT cells (KO+iNKT+BT) and α-GalCer stimulated infected iNKT knock out mice and adoptive transfer with iNKT cells (KO+GC+iNKT+BT) compared to uninfected wild type group (control group). Lymphocyte cell suspensions were isolated from harvested spleens and $2 \times 10^6$/ml cells were stained with anti-mouse TCRβ, anti-mouse B220, CD1d-dimer, anti-mouse NK1.1, anti-mouse CD11b and anti-mouse CD11c then investigated the percent of cells by flow cytometer. Data are shown as means ± standard deviations. Statistically significant differences were evaluated using Student t-test. The asterisks (*) indicate statistical significance ($p < 0.05$) when compared with controls.

## Discussion

iNKT cells are a subset of T lymphocytes, which can be activated by lipid or glycolipid antigens presented by CD1d molecules on antigen-presenting cells. After activation, iNKT cells secrete substantial levels of pro-inflammatory and regulatory cytokines that regulate other immune cells through Th1, Th2 and Th17 cytokines, including dendritic cell maturation to prime CD4+ and CD8+ T cell responses. In addition, cytokine production from iNKT cells can rapidly activate NK cells to be effector cells and help B cells generate antibodies. Therefore, iNKT cells are not highly involved in optimal immunity; however, their restricted activation induces host resistance to disease.[18] α-GalCer is a glycolipid presented by CD1d that is specific for both human and mouse iNKT cell activation by strongly binding iNKT TCRs.[19,20]

In a previous experiment, the number of *B. pseudomallei* in blood, spleen, lungs and liver in infected BALB/c mice stimulated with α-GalCer for 1–2 days before infection, was significantly different from the infected control group. The results showed that the bacteremia, but not the bacterial load in the organs, in the α-GalCer-treated group could be cleared. Even so, the bacterial load in all infected organs, except the lungs, was significantly lowered (unpublished data). This indicated that α-GalCer might activate iNKT cells to play a role in the clearance of bacteria. In this current study, the stimulation of immune cells by α-GalCer was investigated *in vitro* in various mouse organs. α-GalCer was found to activate splenocytes to produce high levels of IFN-γ, IL-13 and GM-CSF.

The IL-13 levels were shown to be highest in activated lung lymphocytes, while IFN-γ was at its highest level in spleen and liver. These results indicated that iNKT cells might have different subtypes in different organs and tissues, resulting in different cytokine levels when stimulated with α-GalCer. IL-13 secretion in stimulated lung lymphocytes was similar to that previously obtained on intranasal administration of α-GalCer in BALB/c mice,[21] which resulted in neutrophils being recruited to the lung, allowing this bacterium to escaped from activated macrophages and spread to other organs.[22] This finding might explain why α-GalCer-stimulated mice infected with *B. pseudomallei* could clear or lower the bacterial load in blood and organs, except for the lungs. α-GalCer could stimulate high IFN-γ, a protective cytokine in melioidosis, in the spleen and liver, but not in the lungs (**Figure 2**). Many reports supported our findings and the hypothesis that α-GalCer-activated iNKT cells, resulting in cytokine production that eliminated the intracellular bacteria or extended the survival of infected mice.[23-26] Therefore, our results confirmed that α-GalCer-activated iNKT cells in spleen and liver gave high IFN-γ levels, similar to those found in *Listeria monocytogenes*, *Cryptococcus neoformans* and *Plasmodium chabaudi* infections.[27-29] This phenomenon might indicate a protective role of iNKT cells against such infections.[10]

To confirm the role of iNKT cells in *B.thailandensis* infections, iNKT KO mice were used. These KO mice were constructed using CRISPR/Cas9 technology and they have been shown to have no iNKT cells.[30] They were produced at the University of Tokyo, which did not have a facility to handle class III bacteria, such as *B. pseudomallei*. As a consequence, *B. thailandensis*, a related non-pathogenic bacterium was used. In addition to iNKT KO mice, adoptive cell transfer was also performed. The mouse spleen was used, as splenic abscesses are the most common finding in *B. thailandensis* and *B. pseudomallei* infections. Splenic iNKT cells were significantly decreased in *B. thailandensis*-infected WT mice and α-GalCer-dosed WT mice. This indicated that this bacterium might lower the number of iNKT cells in both normal and α-GalCer-activated mice, but activation resulted in significantly higher numbers of iNKT cells than in non-activated mice. This implied α-GalCer-activated iNKT cells, as observed with *B. pseudomallei*. The decrease in splenic iNKT cell numbers might be due to cell death or cell migration to other parts of the body. However, the down-regulation of TCRs, which are CD1d-dimer binding sites, might lead to decrease numbers of iNKT cells in WT mice, and undetectable iNKT cells in KO mice, on flow cytometry.[17,29] Previous studies suggested that glycolipid antigen-stimulation made iNKT cells undetectable because of down-regulation of TCRs on their cell surface within an hour of injection, and ongoing IFN-γ and IL-4 production.[27]

The number of macrophages in infected WT (WT+*Bt*) and infected iNKT KO (KO+*Bt*) mice was lower, but not significantly. This indirectly implied a non-related role of iNKT cells and macrophages in this infection. For NK cells, we showed that NK cells in all infected groups were significantly reduced (**Figure 3**), but their number was significantly increased in activated iNKT cells. This suggested that NK cells might play a role in this infection, which might be related to iNKT cell functions. Bearss *et al.* showed that the number of NK cells and macrophages was reduced at day 2 post-infection in *B. pseudomallei*-infected BALB/c and C57BL/c mice.[31] Dendritic cells showed a significant reduction in number in α-GalCer-activated WT and KO mice when infected with *B. thailandensis*, compared to control groups. This finding might be explained by some role of iNKT cells indirectly effecting dendritic cells, which have been shown to facilitate the spread of *B. pseudomallei* instead of protecting the host, though they have been shown to kill *B. pseudomallei in vitro*.[32] Interestingly, NK cells in all infected groups were significantly lower than in the WT group. Furthermore, the number of NK cells increased when iNKT cells were activated. This result was also confirmed by the adoptive cell transfer experiment using iNKT KO mice. We hypothesized that iNKT cells might have some influence on NK cells against this infection. Hayakawa *et al* reported that α-GalCer activation of iNKT cells could produce IFN-γ to rapidly activate NK cells, resulting in an increased number of NK cells and cluster of differentiation 69 (CD69) expression that was persistent for a week.[33] However, the lower number of NK cells found in the current study might be due to their migration out of the spleen or their apoptosis, as found in *Plasmodium berghei* infections when compared to uninfected mice.[28] Our findings indicated that iNKT cells might interact with other immune cells during *B. thailandensis* infection, suggesting an adjunctive role of iNKT cells in this infection.

In conclusion, we found that the activation of iNKT cells using α-GalCer produced different cytokines in diverse organs. The different cytokines produced might be related to differences in clearance of *B. thailandensis*, and by extrapolation *B.pseudomallei*. It also indirectly indicated that there was a variant or subpopulation of iNKT cells in each organ. Although *B. thailandensis* is a non-pathogenic bacterium, which cannot multiply inside cells, it could decrease the normal and activated iNKT cells. The lower number of these cells might lead to the significantly lower number of dendritic and NK cells. Such correlations in *B. thailandensis* infections might promote similar investigations of the related pathogen, *B. pseudomallei*.

## Acknowledgments

## Conflict of Interest

None

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