DR. GANJANA LERTMEMONGKOLCHAI (Orcid ID : 0000-0001-6972-585X)

Received Date : 13-Nov-2016

Revised Date : 30-Dec-2016

Accepted Date : 04-Jan-2017

Article type : Original Article

Boosting of post-exposure human T and B cell recall responses *in vivo* by *Burkholderia pseudomallei* related proteins

Arnone Nithichanon[1], Louise J. Gourlay[2], Gregory J. Bancroft[3], Manabu Ato[4], Yoshimasa Takahashi[4], Ganjana Lertmemongkolchai[1*]

[1] The Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand.

[2] Department of Biosciences, University of Milan, Milan 20133, Italy.

[3] London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.

[4] Department of Immunology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Short title: Boosting of human T and B cell recall responses *in vivo*

Keywords: B cell; Memory; T cell; Antibodies; Neutrophil

* Correspondence to ganja_le@kku.ac.th; g.lert@yahoo.co.uk

Summary

Burkholderia pseudomallei is the causative agent of melioidosis, an infectious disease with high incidence and mortality in South East Asia and Northern Australia. To date there is no protective vaccine and antibiotic treatment is prolonged and not always effective. Most people living in endemic areas have been exposed to the bacteria and have developed some immunity, which may have helped prevent disease. Here, we used a humanized mouse model (hu-PBL-SCID), reconstituted with human peripheral blood mononuclear cells (PBMCs) from seropositive donors, to illustrate the potential of three known antigens (FliC, OmpA and N-PilO2) for boosting both T- and B-cell immune responses. All three antigens boosted the production of specific antibodies in vivo, and increased the number of antibody and interferon gamma (IFN-γ) secreting cells, and induced antibody affinity maturation. Moreover, antigen-specific antibodies isolated from either seropositive individuals or boosted mice, were found to enhance phagocytosis and oxidative burst activities from human polymorphonuclear cells. Our study demonstrates that FliC, OmpA and N-PilO2 can stimulate human memory T and B cells and highlight the potential of the hu-PBL-SCID system for screening and evaluation of novel protein antigens for inclusion in future vaccine trials against melioidosis.

Introduction

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei*, Gram negative bacilli bacteria, commonly found in wet soil and water in South East Asia and Northern Australia [1]. According to previous reports, people living in endemic areas have an increased chance of exposure to the bacterium; some acquire the infection and progress to disease, whereas others do not. The clinical manifestations of melioidosis are vast, ranging from acute to chronic infection phases [2]. Despite the high incidence of melioidosis in endemic areas, with high mortality rates [3], to date, no licensed vaccine for melioidosis prevention exists [4, 5].

Melioidosis shares several clinical and immunological characteristics with tuberculosis including induction of granulomatous pathology, a requirement for Interferon gamma (IFN-γ) activated macrophages for bacterial killing, the presence of extended periods of clinical latency and the requirement for prolonged antibiotic treatment [6, 7]. In the case of tuberculosis, mathematical modelling indicates the most effective strategies for the elimination of tuberculosis will require both pre-exposure and post-exposure vaccines, in developing countries with high incidence rate [8]. Applying this concept to melioidosis, Northeast Thailand is a highly endemic region. Populations in this area are frequently exposed to *B. pseudomallei*, and some individuals generate immunological memory against *B. pseudomallei*, exhibiting high titers of *B. pseudomallei*-specific antibodies and possessing memory T cells [9]. Although, immunological memory is not sufficient for complete protection, boosting protective immunity in seropositive people in endemic areas may be considered [5, 8, 10]. To date, several vaccine antigen candidates have been identified and tested for *in vivo* protection in murine models of pre-exposure vaccination [11, 12]. However,

validation of abilities of vaccine antigen candidates for boosting human immune responses for post-exposure vaccination *in vivo* is lacking.

In a previous protein microarray study, we identified a number of *B. pseudomallei* proteins as potential antigen candidates, based on their recognition by antibodies from healthy seropositive individuals and those recovered from melioidosis [13, 14]. Furthermore, some of these proteins have been shown to induce the production of IFN-γ, a key cytokine with an established role in protection against melioidosis [14]. Some antigens have been further studied *in vivo*. In particular, the peptidoglycan-associated lipoprotein (OmpA; BPSL2765) has been shown to be immunogenic in both mice and melioidosis patients [15]. Recently, a multi-antigen formulation containing BPSL2765, in combination with three other chronic phase associated antigens, was found to offer enhanced protection against mice challenged with *B. pseudomallei* [16]. Another seroreactive antigen candidate that has been tested *in vivo* is flagellin (FliC; BPSL3319), which has been shown to trigger IFN-γ responses from human T cells, and antibodies raised against FliC have been shown to protect mice in passive immunization trials [14, 17-20]. A third candidate that is recognized by antibodies from melioidosis recovery individuals is BPSS1599 or type IV pilus assembly protein 2 (PilO2) [14]. Based on such findings, we selected OmpA, FliC and PilO2 for further study.

Effective antigen candidates are those that are recognized by human immune responses and that can boost pre-existing immune responses in seropositive individuals [21]. To address the ability of these antigens for boosting of human immune responses *in vivo*, we made us of the ability to measure human lymphocyte frequency and function following transplantation into severely immunocompromised mice [22-25]. The humanized non-obese diabetic / severe combined immunodeficiency

(NOD/SCID) mouse model has been a useful tool to study human immune responses studies against a variety of pathogens including Epstein Barr virus (EBV) [26], Hepatitis C virus (HCV) [27], Human Immunodeficiency virus (HIV) type 1 [28-31], influenza virus [32], and *Salmonella typhi* [33, 34]. The NOD/SCID/JAK3^{null} mouse is a powerful model of *in vivo* human immunity studies, due to the complete lack of murine T, B, NK and NKT cell function. In addition, into this type of mouse, various type of human cells can be transplanted without graft rejection [22, 24, 35]. Humanized mice, reconstituted with human PBMCs (hu-PBL-SCID mice), represent a suitable preclinical *in vivo* model to address human immunity boosting and to test for vaccine candidates [24, 36, 37].

In this report, we tested the potential of recombinant OmpA, FliC and the N-terminal domain (residues 1-192) of PilO2 (N-PilO2) to boost human seropositive immune responses in hu-PBL-SCID mice *in vivo*. Our findings show that all three antigens boosted antibody production and affinity maturation from human B cells. The cognate antibodies stimulated bacterial uptake by host phagocytes. Moreover, boosting of hu-PBL-SCID mice also enhanced IFN-γ production from human T cells, a mechanism that may also enhance protection against *B. pseudomallei*. This study provides the first report on the potential of hu-PBL-SCID mice as a tool to identify protein antigens from *B. pseudomallei* that can boost both T and B cell immune responses from seropositive individuals *in vivo*, in order to facilitate the development of a vaccine against *B. pseudomallei*.

Materials and Methods

Human samples

Heparinized blood samples from seronegative and seropositive (Indirect heamagglutination assay; IHA titer ≤ 40 and > 40 respectively) healthy individuals [38, 39] were obtained from the Blood Bank Center, Khon Kaen Hospital, Khon Kaen, Thailand. All donors were adults and had received research information before signing the consent form. The project was approved by the Khon Kaen University (KKU) and National Institute of Infectious Diseases in Japan (NIID) Ethics Committee for Human Research no. HE470506, HE561234, and 471. The study was carried out in accordance with the approved guidelines and informed consent was obtained from all subjects. Plasma and peripheral blood mononuclear cells (PBMCs) were collected and frozen at -80^{o}C until use. Demographic distribution and sample quality after thawing are shown in Table 1.

Mouse strain

NOD/SCID/JAK3^{null} mice (aged between 6-8 weeks) were kindly provided by S. Okada (Kumamoto University) and maintained under specific pathogen free condition. All animal procedures were approved by the Animal Ethics Committee of the NIID (114022-2) and carried out in accordance with the NIID guidelines.

Recombinant antigen production

FliC, OmpA and N-PilO2 were generated as recombinant proteins, as previously described and exchanged into 1X PBS or sterile water (N-PilO2) [17, 40, 41]. LPS removal was carried out by incubating each purified protein sample overnight at

4°C, in a Pierce High Capacity Endotoxin Removal Spin Column (Pierce), according to the manufacturer's instructions. LPS removal was assayed using the Pierce LAL Chromogenic Endotoxin Quantitation Kit (Pierce).

Transplantation of human peripheral blood mononuclear cells (PBMCs) and boosting

The transfer of human PBMCs into recipient NOD/SCID/Jak^{null} mice was performed as previously described [42]. Briefly, 3-5 x 10^7 human PBMCs were transferred to mice by intravenous (i.v.) injection via the lateral tail vain. After 24 h, mice were boosted with 80 μg of FliC, OmpA or N-PilO2 by i.v. administration or not boosted (given PBS only). Mice were maintained for 14 days prior to sacrifice by cervical dislocation. Blood was taken by heart puncture, and the spleens were collected for further analyses.

Human cell surface marker staining and analysis by flow cytometry

Erythrocyte depleted splenocytes from hu-PBL-SCID mice were stained for live/dead marker (AmCyan), and then stained for human leukocyte surface markers with fluorescent anti-human CD4 (Alexa Fluor 700), anti-human CD3 (Pacific Blue), anti-human CD45 (phycoerythrin) and anti-human CD19 (fluorescein isothiocyanate) monoclonal antibodies. After washing twice with 1 ml of FACS buffer, cells were fixed with 100 μl of 2% paraformaldehyde in PBS and stored on ice in the dark. Then, cell populations from 2 x 10^5 acquired cells were analyzed by FACS Canto II. Cells within the viable lymphocyte gate were further analyzed for human T cell ($CD45^+$, $CD3^+$) and B cell ($CD45^+$, $CD19^+$) populations. Human helper T cells

($CD45^+$, $CD3^+$, and $CD4^+$) were revealed on $CD3^+$ gated panel. Gating strategies are shown in Fig. S1.

Detection of human antibodies in hu-PBL-SCID mouse sera by indirect enzyme linked immunosorbent assay (ELISA)

Purified *B. pseudomallei* derived protein antigens were coated onto 96 well polystyrene plates at 10 µg/ml overnight. After blocking with 1% BSA in PBS, mouse sera were added in 4-fold serial dilutions in duplicate, and incubated at RT for 2 h. Plates were washed 5 times with 0.1% Tween20 in PBS (PBST), and in the case of measuring antibody affinity, 7 M urea (treated) or PBS (untreated) was added and incubated for 15 min [43]. Then horse radish peroxidase (HRP) conjugated anti-human IgM (Southern Biotech No. 2020-05) or anti-human IgG (Southern Biotech No. 2040-05) detection antibodies were added and incubated at RT for 1h. Plates were washed 5 times with PBST, and then 100 ⌈l of O-Phenylenediamine Dihydrochloride (OPD) substrate was added. The reaction was stopped with 2 N H_2SO_4 and the optical density (O.D.) was measured at 490/595 nm.

Concentration of detected antibodies were analyzed by dose-response curves [44] in comparison with reference serum, (defined as 1000 U/ml serum), and expressed as U/ml of antibody activity. Antibody affinity was represented as % Urea Resistance, calculated from dose-response curves and compared between with or without urea treatment.

Detection of human antibody secreting cell (ASC) detection by Enzyme Linked Immunospot (ELISpot) assay

Nitrocellulose membranes were pre-coated with 20 µg/ml *B. pseudomallei* protein antigens overnight. On the day of assay, antigen coated membranes were blocked with 1% BSA in PBS for 2 h at RT. Erythrocyte depleted spleen cells in 200 µl of DMEM media were added onto the membrane at 1 x 10^5 cells per well in duplicate, incubated at 37^{o}C with 5% CO_2 for 4 h. The membrane was then washed with 10 mM EDTA in PBS until all attached cells were completely removed. Next, the membrane was soaked in HRP conjugated anti-human IgM or IgG detection antibodies solution, incubated at RT for 2 h. After 5 washes, the membrane was soaked in HRP substrate solution for 5-10 min and the reaction stopped with tap water. The number of spots on the membrane was counted and presented as antibody-secreting cell (ASC) per 2 x 10^5 spleen cells.

Restimulation of hu-PBL-SCID mouse spleen cells for secretion of human interferon gamma (IFN-γ)

Polyvinylidene difluoride (PVDF) membrane ELISpot plates were pre-coated with IFN-γ capture antibody (Mabtech) overnight. Prior to the assay, plates were washed with sterile PBS and blocked with 10% BSA in DMEM medium at RT for 2 h. Erythrocyte-depleted splenocytes at 5 x 10^5 cells per well were restimulated with their boosting antigen at 20 µg/ml in duplicate, and incubated at 37^{o}C for 48 h. Then, after splenocytes were detached from the PVDF membrane plate by washing with distilled water and 0.1% Tween20 in PBS, each well was probed with a biotinylated IFN-γ detection antibody (Mabtech), incubated at RT for 2 h, the membrane was washed 5 times with 0.1% Tween20 in PBS, HRP-streptavidin added, and incubated

at RT for an hour, the plates were washed again, the AEC (3-amino-9-ethylcarbazole) substrate was added, and spot forming units (SFU) were counted. Data are presented as IFN-γ SFU/10^6 spleen cells.

Purification of *B. pseudomallei* specific antibodies from human plasma by ammonium sulfate precipitation and affinity chromatography

B. pseudomallei-specific antibodies from human plasma were purified by ammonium sulfate precipitation with gel filtration affinity chromatography [45]. Briefly, human plasma from seropositive donors were pooled, and depleted of unwanted macromolecule proteins by adding an equal volume of saturated ammonium sulfate ($(NH_4)_2SO_4$), stirred 1 h at 4°C, then centrifuged for 10 min at 4°C. The supernatant was removed and the protein pellet was resuspended in PBS and dialyzed with stirring at 4°C against 2 L of PBS.

Meanwhile, Sepharose 4B gels (GE healthcare) were coupled to FliC, OmpA or N-PilO2 proteins, following the manufacture's guidelines. Protein-coupled gels were packed into the chromatography column case (equilibrated with 20 mM phosphate buffer containing 0.3 M NaCl until use). Next, the dialyzed plasma protein was introduced into the column at a flow rate at 0.5 ml/min. This step was repeated 3 times, prior to washing with 20 mM phosphate buffer containing 0.3 M NaCl. Finally, antibody specifically bound to the protein-coupled gel matrix were eluted in 0.17 M glycine/HCl (pH 2.7), and neutralized by addition of 1M Tris-HCl (pH 9.0). Eluted antibody was concentrated using with a centrifugal concentrator with a MW cut-off of 100 kDa (Vivaspin100, Sartorius, Germany), and the protein concentration was determined by measuring the absorbance at 280 nm.

Phagocytosis and oxidative burst activity of human PMNs

The detailed protocol adopted for the phagocytosis and oxidative burst analyses are previously described [41, 46]. Briefly, *B. pseudomallei* were grown in Luria-Bertani broth, and killed by using 1% paraformaldehyde (PFA) in PBS. Then, 10^8 CFU/ml PFA fixed *B. pseudomallei* were labeled with 1 µg/ml fluorescein isothiocyanate (FITC) (Sigma, United States) in the dark at RT for 1 h. Unbound FITC was removed by washing in PBS twice. FITC intensity was measured by flow cytometry prior to opsonization tests, using 20 µg/ml of each purified human antibody against the cognate protein antigen.

Whole blood from *B. pseudomallei* seropositive individuals were assayed by complete blood count, and the number of PMNs in whole blood was diluted to achieve 2 x 10^6 PMNs/ml. Diluted whole blood samples were incubated with previously-opsonized FITC-labeled dead *B. pseudomallei* at a ratio of 10:1 (bacteria: PMNs) at 37^{o}C for 30 min. Phorbol 12-myristate 13-acetate (PMA; 800 ng/ml) (Sigma) was used as a positive control for oxidative burst activities. Hydroethidine (HE; 2,800 ng/ml) (Sigma, United States) was added and incubated for 5 min at RT. During this step, respiratory oxidative activities were measured by following the oxidation of HE into ethidium bromide (EB), which can be directly detected by flow cytometry at excitation and emission wavelengths of 473 nm and 593 nm, respectively. Red cells were lysed with BD FACS Lysing Solution (BD Biosciences, United States), and the remaining leukocytes were washed twice, and fixed with 2% paraformaldehyde.

Phagocytosis of FITC labeled bacteria and oxidative burst activities were analyzed by flow cytometry (FACSCalibur, BD Biosciences). Results are represented as % Total Phagocytosis and % Oxidative Burst in phagocytosed cells.

% Total Phagocytosis = (%FITC$^+$, %EB$^+$) + (%FITC$^+$, %EB$^-$)

% Oxidative Burst in phagocytosed cell = (%FITC$^+$, %EB$^+$) / ((%FITC$^+$, %EB$^+$) + (%FITC$^+$, %EB$^-$)) x 100.

Statistical analysis

Analysis of statistical significance was performed using Prism version 5 (Graphpad). Multiple comparisons were carried out, using one-way ANOVA, and post-test, using Bonferroni's Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant. The correlation was done by linear regression. A P value < 0.05 was considered statistically significant.

Results

Recombinant FliC, OmpA and N-PilO2 induce specific human B cell clonal expansion and antibody (IgM and IgG) production *in vivo*.

We measured IFN-γ production by human seronegative and seropositive PBMCs in response to dead, whole *B. pseudomallei* bacteria and the three recombinant proteins. Results show that PBMCs from seronegative individuals induced low levels of IFN-γ in response against whole bacteria of *B. pseudomallei* and the three proteins, while PBMCs from seropositive individuals strongly produced IFN-γ upon stimulation (Figure 1). This data suggests that PBMCs from seronegative individuals have no pre-existing immunity in response to *B. pseudomallei* and FliC, OmpA and N-PilO2 proteins. On the contrary, seropositive PBMCs recognize *B. pseudomallei* and FliC, OmpA and N-PilO2 proteins, resulting in strong cell activation and production of IFN-γ upon stimulation. This implies that seropositive individuals have

developed immune memory against *B. pseudomallei* and FliC, OmpA and N-PilO2 proteins.

To study *in vivo* human immune responses from seropositive individuals against vaccine candidate antigens, we reconstituted NOD/SCID/JAK3^{null} mice [35] with human PBMCs from 5 healthy seropositive individuals from the melioidosis endemic region of Northeast Thailand. To characterize the immune status of these donors, baseline antibodies (IgM and IgG) for all human plasma samples were determined (Table 1), and the cellularity of frozen isolated human PBMC samples was determined before transplantation into the mice (Table 1). After thawing, all samples contained 96-99% viable cells as counted by trypan blue exclusion, and 86-93% based on AmCyan negative cells analyzed by FACS analysis. The compositions of T and B lymphocytes were 82-92% and 7-22% respectively (Table 1). These results revealed that the samples used for this study were mostly viable, and have normal composition of T and B lymphocytes [47]. These hu-PBL-SCID mice were then boosted by injection of OmpA, FliC, or N-PilO2, or phosphate buffer saline (PBS) as a negative control. At 14 days after boosting, hu-PBL-SCID mice were sacrificed and spleen cells were collected, to address the changes in human T and B cell populations between antigen-boosted and control mice. Results show that the number of human B cells (hCD45^{+} and hCD19^{+}) was significantly increased in mice boosted with recombinant FliC ($P < 0.05$), OmpA ($P < 0.05$) and N-PilO2 ($P < 0.01$), in comparison to the non-boosted control (Figure 2A and 2B). However, for the human T cell (human CD45+ and human CD3+) and helper T cell (human CD45+, human CD3+ and human CD4+) populations, no increase in cell number was observed (Figure 2C and 2D).

Subsequently, sera were collected from hu-PBL-SCID mice to determine whether FliC, OmpA and N-PilO2 protein antigens stimulate human B cells in hu-PBL-SCID mice to elicit the production of IgM and IgG antibodies. Specific IgM and IgG serum levels were measured by indirect enzyme linked immunosorbent assay (ELISA). All three proteins were found to significantly boost IgM and IgG antibody levels, when compared to control mice (Figure 3A; $P < 0.05$). Such findings were further supported by the results of enzyme-linked immunospot (ELISpot) assay for antibody secreting cells (ASCs) from spleen cells, showing that antigen-boosted mice exhibit more antigen-specific ASCs relative to the controls (Figure 3B, $P < 0.05$). Our findings imply that antibody production from human B cells in seropositive individuals increased upon boosting with FliC, OmpA or N-PilO2.

Recombinant FliC, OmpA and N-PilO2 increase the number of IFN-γ producing cells from boosted hu-PBL-SCID mice after antigen restimulation.

To examine the frequency and function of human *B. pseudomallei* specific T cells, spleen cells from boosted hu-PBL-SCID mice were restimulated *in vitro* with their respective protein antigen, and the number of IFN-γ producing cells was quantified by ELISpot. The results showed that spleen cells from boosted hu-PBL-SCID mice had significantly more IFN-γ producing cells, in comparison with control hu-PBL-SCID mice, after restimulation with the same protein antigen (Figure 4). In details, the number of IFN-γ producing cells was significantly increased in hu-PBL-SCID mice boosted with FliC (1.85 fold, ranged between 1.30 - 2.67, $P < 0.05$), OmpA (2.1 fold, ranged between 1.38 – 2.48, $P < 0.05$) and N-PilO2 (2.33 fold, ranged between 1.29 – 3.33, $P < 0.01$) when compared with mice, which had not been boosted.

Affinity maturation of anti-*B. pseudomallei* IgG affinity occurs after boosting hu-PBL-SCID mice with FliC, OmpA or N-PilO2 proteins *in vivo*.

The affinity of human IgM and IgG antibodies against *B. pseudomallei* antigens obtained from hu-PBL-SCID mice was estimated by the detachment of low-affinity antibodies through urea wash as previously described [43]. Results revealed that IgG antibodies from antigen boosted mice sera are more resistant to urea compared to antibodies from the same donor sera used to boost mice; $P < 0.01$. On the contrary, the affinity of IgM antibodies from both boosted mice and donor sera were the same (Figure 5). Overall, we propose that human B cells in hu-PBL-SCID mice are activated and develop into antigen-specific ASCs, in response to boosting with FliC, OmpA or N-PilO2. Moreover, FliC, OmpA or N-PilO2 protein antigens also induce T cell responses that help B cells in the process of affinity maturation.

Antisera against *B. pseudomallei* FliC, OmpA or N-PilO2 from boosted hu-PBL-SCID mice enhance bacterial phagocytosis and oxidative burst activities of human polymorphonuclear cells (PMNs).

We next investigated the ability of specific antibodies against FliC, OmpA and N-PilO2, present in plasma from seropositive donors to induce phagocytosis of *B. pseudomallei* and the oxidative burst response by PMN. Human anti-FliC, anti-OmpA and anti-N-PilO2 antibodies were purified from plasma pooled from five seropositive donors living in endemic areas. Antigen-specific recognition against each purified human antibody was assessed; no cross-reaction was observed between protein antigens (Figure S2). Purified antibodies were then incubated with FITC-labeled intact dead *B. pseudomallei*, and cultured with human whole blood. Oxidative burst

was detected by the addition of hydroethidine (HE), which is converted into ethidium bromide (EB) in the presence of oxidative radical species, and counting the number of FITC and/or EB positive cells in the PMN gate by flow cytometry. Purified antibodies from human plasma were found to enhance both PMN phagocytosis ($P < 0.05$) and oxidative burst activities ($P < 0.05$) (Figure 6A), suggesting that these antibodies may play a role in host defense against *B. pseudomallei* infection. We then tested for the enhancement of human PMN phagocytosis and oxidative burst activities, in the presence of humanized antibodies from antigen boosted hu-PBL-SCID mice. Sera from hu-PBL-SCID mice (antigen boosted and non-boosted controls) were incubated with FITC-labeled intact dead *B. pseudomallei*, and cultured with human whole blood. Sera from all antigen boosted hu-PBL-SCID mice were found to significantly enhance both phagocytosis ($P < 0.05$) and oxidative burst activities ($P < 0.01$) in human PMNs, in whole blood compared to sera from non-boosted mice (Figure 6B).

We then analyzed the correlation of the effects of PMN phagocytosis and oxidative burst enhancement and the concentration or affinity of IgG antibodies from FliC, OmpA and N-PilO2 boosted hu-PBL-SCID mice. We found a positive correlation of PMN phagocytosis and oxidative burst enhancement with the level and affinity of IgG in boosted mice sera (Figure 6C and 6D). For FliC or OmpA boosted mice, a significant positive correlation between % total phagocytosis and the level of IgG (FliC $P < 0.05$; OmpA $P < 0.01$) was observed, however the IgG affinity was not altered (FliC $P = 0.1698$; OmpA $P = 0.1743$). Accordingly, the % oxidative burst in phagocytosed cells was also significantly positively correlated to the level of IgG (FliC $P < 0.01$; OmpA $P < 0.05$) but not to IgG affinity (FliC $P = 0.2544$; OmpA $P = 0.0516$). With regards to N-PilO2, we did not observe any significant changes. Our

data suggest that enhancement of PMN phagocytosis and oxidative burst activities by antibodies from boosted hu-PBL-SCID mice may depend on the level of antibodies and/or affinity.

The summary of this humanized mouse model (hu-PBL-SCID) reconstituted with human peripheral blood mononuclear cells (PBMCs) from *B. pseudomallei* seropositive donors, to illustrate the potential of three known antigens (FliC, OmpA and N-PilO2) for boosting both T- and B-cell immune responses is shown in Figure 7.

Discussion

In this study, we used the humanized NOD/SCID/Jak3^{null} mouse model to study *in vivo* boosting of seropositive PBMCs by three protein antigen candidates known to induce human antibody responses in seropositive and/or melioidosis recovery individuals [14]. The humanized NOD/SCID/Jak3^{null} mouse model can receive PBMCs from seropositive donors as it completely lacks T-, B-, NK- and NKT cell functions [35, 48]. All three target antigens pertain to protein families that are known immunogens across diverse bacterial species, namely flagellar proteins (FliC), outer membrane proteins (OmpA) and pilus subunits (N-PilO2) [13-16, 49, 50].

Here, we show that the IFN-γ response from PBMCs from seropositive individuals living in a melioidosis endemic area, upon stimulation with *B. pseudomallei* antigens, is significantly greater than from seronegative individuals. This correlates with our previous studies showing that human antibodies and PBMC can recognize FliC and OmpA proteins, and magnitude of the response from human seropositive individuals is higher than seronegative individuals [14, 17, 41]. Additional

studies on larger sample sizes from endemic area of Thailand also confirm that the T cells from seropositive healthy or melioidosis recovery individuals against *B. pseudomallei* antigens is significantly greater than seronegative healthy individuals [9, 51, 52]. During IFN-γ production upon stimulation of human seropositive PBMC with *B. pseudomallei*, NK cells are transient and are prominent in the first 24 h of stimulation whereas CD4 and CD8 T cells have more contribution in the later phase of stimulation by primarily response through terminally differentiated effector memory T cell (T_{EMRA}) [9]. Our use of purified proteins here rather than intact bacteria, and that we boosted mice for 14 days prior to restimulation ex vivo makes it less likely for us to adequately probe the human NK cell response under these conditions, and is consistent with our results that the majority of IFN-γ production from humanized spleen cells after stimulation for 48 h was T cell mediated. In other studies with such donors there is no difference in IFN-γ production upon stimulation of human seronegative versus seropositive PBMC with cytomegalovirus, Epstein Barr virus and influenza virus (CEF) pooled peptides [9, 52]. Thus, the difference of IFN-γ production in response to *B. pseudomallei* between seropositive and seronegative individuals seen here in hu-PBL-SCID mice most likely reflects differences in human T cell memory against the bacteria.

Our analyses of the human lymphocyte population in spleen samples collected from hu-PBL-SCID mice boosted with each of the three target antigens, showed an increase in the number of B cells ($CD45^+$, $CD19^+$), but not T cells. In previous examples of human PBMC transplantation, the number of T and B cells substantially decreased due to cell death, however, from day 3 to day 7, the number of B cells sharply increased, and then slowly declined. On the other hand, T cell numbers slowly increased from day 3 onwards [53]. Interestingly, despite a gradual

increase in T cell numbers, they were converted into a reversible anergy state [54, 55]. When human T cells were reconstituted in the SCID mouse, the human T cell phenotypes were $CD45RO^+$ and $HLA\text{-}DR^+$, suggesting that they were mature memory T cells [55]. Moreover, those T cells also expressed CD25, CD69 and CD71, suggesting that human memory T cells were continuously stimulated by xenoantigens, and lead to anergy and loss of function in the hu-SCID mouse [55, 56]. This anergy was found to be reversible upon T cell re-activation by a TCR activator and IL-2 *in vitro* [54, 55]. These humanized models may also underestimate the magnitude of the human CD8+ T cell response [57], but CD4 T cells proliferation and responses can clearly be found [58]. Here we did not observe T-cell proliferation *in vivo* by flow cytometry, however, the number of IFN-γ secreting cells increased when antigen boosted hu-PBL-SCID mice spleen cells were restimulated *in vitro* with the relative antigen. We propose that when human T cells from healthy seropositive donors were reconstituted into NOD/SCID mice, these T cells (especially naïve T cells) which have never been exposed to *B. pseudomallei* die upon transplantation, but some memory T cells survive and respond to the cognate antigen by producing IFN-γ.

Next, we examined the production of specific human IgM and IgG antibodies in response to boosting antigens. We found that both antigen-specific human IgM and IgG antibodies, in mouse sera and ASCs, were increased in boosted mice in comparison with the controls. Furthermore, we also found that after boosting with specific antigen, human IgG antibody was affinity-matured, implying that antigen-specific memory B cells with higher affinity were preferentially restimulated by booster antigens. Our findings support the report that human cells, immunized with tetanus toxoid and transplanted into SCID mice, upon a second boosting with

tetanus toxoid, resulted in an increase in antigen-specific IgG titers in a T-cell dependent manner, and also induced affinity maturation [59].

Finally, we have shown that human antibodies against FliC, OmpA and N-PilO2, from both seropositive donor plasma and antigen boosted hu-PBL-SCID mice are able to enhance phagocytosis and oxidative burst activities of human PMN against intact *B. pseudomallei*. This is consistent with our previous studies that antibodies raised against FliC [17] and OmpA [41] enhance bacterial uptake and oxidative burst by neutrophils. *B. pseudomallei* binding antibody enhances complement deposition which subsequently enhances bacterial uptake and killing by neutrophils [60, 61], even though, *B. pseudomallei* can evade and survive inside macrophage-like cells upon infection [62, 63]. Our previous study on primary human neutrophils infected with *B. pseudomallei* has demonstrated that neutrophils could kill intracellular *B. pseudomallei* through autophagy [64]. Moreover, induction of autophagy in neutrophils leads to formation of neutrophil extracellular traps (NETs) [65, 66], and both autophagy and NETs are important antibacterial mechanisms against *B. pseudomallei* [67, 68]. Thus, the enhancement of bacterial uptake and oxidative burst of neutrophils by antibodies against FliC, OmpA and N-PilO2 would be an important mechanism in host defense against *B. pseudomallei* infection.

In conclusion, we have shown that FliC, OmpA and N-PilO2 can boost memory B and T cell responses *in vivo* in hu-PBL-SCID mice reconstituted with PBMC from *B. pseudomallei*-exposed seropositive individuals. Such boosting effects resulted in the enhancement of host immune function likely to be important in defense against *B. pseudomallei* infection. Our data indicate that hu-PBL-SCID mice provide a useful tool to identify and evaluate bacterial proteins which can boost

human immune responses to *B. pseudomallei* and support the possibility of using FliC, OmpA and N-PilO2 as vaccine candidates in the future.

Acknowledgements

Financial support from the Thailand Research Fund and Khon Kaen University through the Royal Golden Jubilee Ph. D. Program (Grant No. PHD/0167/2553 to AN and GL) is acknowledged. Recombinant antigen production was supported by Fondazione CARIPLO (Progetto Vaccini, contract number 2009-3577). The authors acknowledge Ms. Jeerawan Dhanasen for sample collection, and Dr. Darawan Rinchai for her scientific comments and help on sample shipment.

Author contribution

AN & LG performed the experiments; AN, GB, MA, YT, GL designed the study; AN, LG, GB & GL wrote the paper.

Conflict of Interest

The authors have declared that no competing interests exist.

References

1. Currie BJ, Dance DA, Cheng AC. The global distribution of *Burkholderia pseudomallei* and melioidosis: an update. Trans R Soc Trop Med Hyg 2008; 102 Suppl 1:S1-4.

2. Cheng AC, Currie BJ, Dance DA, Funnell SG, Limmathurotsakul D, Simpson AJ, et al. Clinical definitions of melioidosis. Am J Trop Med Hyg 2013; 88:411-3.

3. Limmathurotsakul D, Wongratanacheewin S, Teerawattanasook N, Wongsuvan G, Chaisuksant S, Chetchotisakd P, et al. Increasing incidence of human melioidosis in Northeast Thailand. Am J Trop Med Hyg 2010; 82:1113-7.

4. Cheng AC, Currie BJ. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev 2005; 18:383-416.

5. Choh LC, Ong GH, Vellasamy KM, Kalaiselvam K, Kang WT, Al-Maleki AR, et al. *Burkholderia* vaccines: are we moving forward? Front Cell Infect Microbiol 2013; 3:5.

6. Koh GC, Schreiber MF, Bautista R, Maude RR, Dunachie S, Limmathurotsakul D, et al. Host responses to melioidosis and tuberculosis are both dominated by interferon-mediated signaling. PLoS One 2013; 8:e54961.

7. Limmathurotsakul D, Peacock SJ. Melioidosis: a clinical overview. Br Med Bull 2011; 99:125-39.

8. Lietman T, Blower SM. Potential impact of tuberculosis vaccines as epidemic control agents. Clin Infect Dis 2000; 30 Suppl 3:S316-22.

9. Tippayawat P, Saenwongsa W, Mahawantung J, Suwannasaen D, Chetchotisakd P, Limmathurotsakul D, et al. Phenotypic and functional characterization of human memory T cell responses to Burkholderia pseudomallei. PLoS Negl Trop Dis 2009; 3:e407.

10. Bader MS, McKinsey DS. Postexposure prophylaxis for common infectious diseases. Am Fam Physician 2013; 88:25-32.

11. Patel N, Conejero L, De Reynal M, Easton A, Bancroft GJ, Titball RW. Development of vaccines against *Burkholderia pseudomallei*. Front Microbiol 2011; 2:198.

12. Muruato LA, Torres AG. Melioidosis: where do we stand in the development of an effective vaccine? Future Microbiol 2016; 11:477-80.

13. Felgner PL, Kayala MA, Vigil A, Burk C, Nakajima-Sasaki R, Pablo J, et al. A *Burkholderia pseudomallei* protein microarray reveals serodiagnostic and cross-reactive antigens. Proc Natl Acad Sci U S A 2009; 106:13499-504.

14. Suwannasaen D, Mahawantung J, Chaowagul W, Limmathurotsakul D, Felgner PL, Davies H, et al. Human immune responses to *Burkholderia pseudomallei* characterized by protein microarray analysis. J Infect Dis 2011; 203:1002-11.

15. Hara Y, Mohamed R, Nathan S. Immunogenic *Burkholderia pseudomallei* outer membrane proteins as potential candidate vaccine targets. PLoS One 2009; 4:e6496.

16. Champion OL, Gourlay LJ, Scott AE, Lassaux P, Conejero L, Perletti L, et al. Immunisation with proteins expressed during chronic murine melioidosis provides enhanced protection against disease. Vaccine 2016; 34:1665-71.

17. Nithichanon A, Rinchai D, Gori A, Lassaux P, Peri C, Conchillio-Sole O, et al. Sequence- and Structure-Based Immunoreactive Epitope Discovery for *Burkholderia pseudomallei* Flagellin. PLoS Negl Trop Dis 2015; 9:e0003917.

18. Musson JA, Reynolds CJ, Rinchai D, Nithichanon A, Khaenam P, Favry E, et al. CD4+ T cell epitopes of FliC conserved between strains of *Burkholderia*: implications for vaccines against melioidosis and cepacia complex in cystic fibrosis. J Immunol 2014; 193:6041-9.

19. Chen YS, Hsiao YS, Lin HH, Liu Y, Chen YL. CpG-modified plasmid DNA encoding flagellin improves immunogenicity and provides protection against *Burkholderia pseudomallei* infection in BALB/c mice. Infect Immun 2006; 74:1699-705.

20. Brett PJ, Woods DE. Structural and immunological characterization of *Burkholderia pseudomallei* O-polysaccharide-flagellin protein conjugates. Infect Immun 1996; 64:2824-8.

21. Chentoufi AA, Kritzer E, Yu DM, Nesburn AB, Benmohamed L. Towards a rational design of an asymptomatic clinical herpes vaccine: the old, the new, and the unknown. Clin Dev Immunol 2012; 2012:187585.

22. Ito R, Takahashi T, Katano I, Ito M. Current advances in humanized mouse models. Cell Mol Immunol 2012; 9:208-14.

23. Brehm MA, Shultz LD, Greiner DL. Humanized mouse models to study human diseases. Curr Opin Endocrinol Diabetes Obes 2010; 17:120-5.

24. Koo GC, Hasan A, O'Reilly RJ. Use of humanized severe combined immunodeficient mice for human vaccine development. Expert Rev Vaccines 2009; 8:113-20.

25. Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. Nat Rev Immunol 2012; 12:786-98.

26. Melkus MW, Estes JD, Padgett-Thomas A, Gatlin J, Denton PW, Othieno FA, et al. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat Med 2006; 12:1316-22.

27. Billerbeck E, Horwitz JA, Labitt RN, Donovan BM, Vega K, Budell WC, et al. Characterization of human antiviral adaptive immune responses during

hepatotropic virus infection in HLA-transgenic human immune system mice. J Immunol 2013; 191:1753-64.

28. Sun Z, Denton PW, Estes JD, Othieno FA, Wei BL, Wege AK, et al. Intrarectal transmission, systemic infection, and CD4+ T cell depletion in humanized mice infected with HIV-1. J Exp Med 2007; 204:705-14.

29. Denton PW, Othieno F, Martinez-Torres F, Zou W, Krisko JF, Fleming E, et al. One percent tenofovir applied topically to humanized BLT mice and used according to the CAPRISA 004 experimental design demonstrates partial protection from vaginal HIV infection, validating the BLT model for evaluation of new microbicide candidates. J Virol 2011; 85:7582-93.

30. Denton PW, Estes JD, Sun Z, Othieno FA, Wei BL, Wege AK, et al. Antiretroviral pre-exposure prophylaxis prevents vaginal transmission of HIV-1 in humanized BLT mice. PLoS Med 2008; 5:e16.

31. Stoddart CA, Maidji E, Galkina SA, Kosikova G, Rivera JM, Moreno ME, et al. Superior human leukocyte reconstitution and susceptibility to vaginal HIV transmission in humanized NOD-scid IL-2Rgamma(-/-) (NSG) BLT mice. Virology 2011; 417:154-60.

32. Yu CI, Gallegos M, Marches F, Zurawski G, Ramilo O, Garcia-Sastre A, et al. Broad influenza-specific CD8+ T-cell responses in humanized mice vaccinated with influenza virus vaccines. Blood 2008; 112:3671-8.

33. Libby SJ, Brehm MA, Greiner DL, Shultz LD, McClelland M, Smith KD, et al. Humanized nonobese diabetic-scid IL2rgammanull mice are susceptible to lethal *Salmonella typhi* infection. Proc Natl Acad Sci U S A 2010; 107:15589-94.

34. Firoz Mian M, Pek EA, Chenoweth MJ, Ashkar AA. Humanized mice are susceptible to *Salmonella typhi* infection. Cell Mol Immunol 2011; 8:83-7.

35. Okada S, Harada H, Ito T, Saito T, Suzu S. Early development of human hematopoietic and acquired immune systems in new born NOD/Scid/Jak3null mice intrahepatic engrafted with cord blood-derived CD34 + cells. Int J Hematol 2008; 88:476-82.

36. Spranger S, Frankenberger B, Schendel DJ. NOD/scid IL-2Rg(null) mice: a preclinical model system to evaluate human dendritic cell-based vaccine strategies in vivo. J Transl Med 2012; 10:30.

37. Ramer PC, Chijioke O, Meixlsperger S, Leung CS, Munz C. Mice with human immune system components as in vivo models for infections with human pathogens. Immunol Cell Biol 2011; 89:408-16.

38. Barnes JL, Warner J, Melrose W, Durrheim D, Speare R, Reeder JC, et al. Adaptive immunity in melioidosis: a possible role for T cells in determining outcome of infection with *Burkholderia pseudomallei*. Clin Immunol 2004; 113:22-8.

39. Cheng AC, O'Brien M, Freeman K, Lum G, Currie BJ. Indirect hemagglutination assay in patients with melioidosis in northern Australia. Am J Trop Med Hyg 2006; 74:330-4.

40. Lassaux P, Conchillo-Sole O, Manjasetty BA, Yero D, Perletti L, Belrhali H, et al. Redefining the PF06864 Pfam family based on *Burkholderia pseudomallei* PilO2(Bp) S-SAD crystal structure. PLoS One 2014; 9:e94981.

41. Gourlay LJ, Peri C, Ferrer-Navarro M, Conchillo-Sole O, Gori A, Rinchai D, et al. Exploiting the *Burkholderia pseudomallei* acute phase antigen BPSL2765

for structure-based epitope discovery/design in structural vaccinology. Chem Biol 2013; 20:1147-56.

42. Moens L, Wuyts M, Meyts I, De Boeck K, Bossuyt X. Human memory B lymphocyte subsets fulfill distinct roles in the anti-polysaccharide and anti-protein immune response. J Immunol 2008; 181:5306-12.

43. Khurana S, Verma N, Yewdell JW, Hilbert AK, Castellino F, Lattanzi M, et al. MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines. Sci Transl Med 2011; 3:85ra48.

44. Innis BL, Seriwatana J, Robinson RA, Shrestha MP, Yarbough PO, Longer CF, et al. Quantitation of immunoglobulin to hepatitis E virus by enzyme immunoassay. Clin Diagn Lab Immunol 2002; 9:639-48.

45. Grodzki AC, Berenstein E. Antibody purification: ammonium sulfate fractionation or gel filtration. Methods Mol Biol 2010; 588:15-26.

46. Chanchamroen S, Kewcharoenwong C, Susaengrat W, Ato M, Lertmemongkolchai G. Human polymorphonuclear neutrophil responses to *Burkholderia pseudomallei* in healthy and diabetic subjects. Infect Immun 2009; 77:456-63.

47. Autissier P, Soulas C, Burdo TH, Williams KC. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytometry A 2010; 77:410-9.

48. Park SY, Saijo K, Takahashi T, Osawa M, Arase H, Hirayama N, et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. Immunity 1995; 3:771-82.

49. Margarit I, Rinaudo CD, Galeotti CL, Maione D, Ghezzo C, Buttazzoni E, et al. Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. J Infect Dis 2009; 199:108-15.

50. Krebs SJ, Taylor RK. Protection and attachment of *Vibrio cholerae* mediated by the toxin-coregulated pilus in the infant mouse model. J Bacteriol 2011; 193:5260-70.

51. Tippayawat P, Pinsiri M, Rinchai D, Riyapa D, Romphruk A, Gan YH, et al. *Burkholderia pseudomallei* proteins presented by monocyte-derived dendritic cells stimulate human memory T cells in vitro. Infect Immun 2011; 79:305-13.

52. Jenjaroen K, Chumseng S, Sumonwiriya M, Ariyaprasert P, Chantratita N, Sunyakumthorn P, et al. T-Cell Responses Are Associated with Survival in Acute Melioidosis Patients. PLoS Negl Trop Dis 2015; 9:e0004152.

53. Depraetere S, Verhoye L, Leclercq G, Leroux-Roels G. Human B cell growth and differentiation in the spleen of immunodeficient mice. J Immunol 2001; 166:2929-36.

54. Wagar EJ, Cromwell MA, Shultz LD, Woda BA, Sullivan JL, Hesselton RM, et al. Regulation of human cell engraftment and development of EBV-related lymphoproliferative disorders in Hu-PBL-scid mice. J Immunol 2000; 165:518-27.

55. Tary-Lehmann M, Saxon A. Human mature T cells that are anergic in vivo prevail in SCID mice reconstituted with human peripheral blood. J Exp Med 1992; 175:503-16.

56. Cao T, Leroux-Roels G. Antigen-specific T cell responses in human peripheral blood leucocyte (hu-PBL)-mouse chimera conditioned with radiation and an

antibody directed against the mouse IL-2 receptor beta-chain. Clin Exp Immunol 2000; 122:117-23.

57. Sato Y, Takata H, Kobayashi N, Nagata S, Nakagata N, Ueno T, et al. Failure of effector function of human CD8+ T Cells in NOD/SCID/JAK3(-)/(-) immunodeficient mice transplanted with human CD34+ hematopoietic stem cells. PLoS One 2010; 5.

58. Terahara K, Ishige M, Ikeno S, Mitsuki YY, Okada S, Kobayashi K, et al. Expansion of activated memory CD4+ T cells affects infectivity of CCR5-tropic HIV-1 in humanized NOD/SCID/JAK3null mice. PLoS One 2013; 8:e53495.

59. Brams P, Nguyen ML, Chamat S, Royston I, Morrow PR. Antigen-specific IgG responses from naive human splenocytes: in vitro priming followed by antigen boost in the SCID mouse. J Immunol 1998; 160:2051-8.

60. Mulye M, Bechill MP, Grose W, Ferreira VP, Lafontaine ER, Wooten RM. Delineating the importance of serum opsonins and the bacterial capsule in affecting the uptake and killing of *Burkholderia pseudomallei* by murine neutrophils and macrophages. PLoS Negl Trop Dis 2014; 8:e2988.

61. Woodman ME, Worth RG, Wooten RM. Capsule influences the deposition of critical complement C3 levels required for the killing of *Burkholderia pseudomallei* via NADPH-oxidase induction by human neutrophils. PLoS One 2012; 7:e52276.

62. Jones AL, Beveridge TJ, Woods DE. Intracellular survival of Burkholderia pseudomallei. Infect Immun 1996; 64:782-90.

63. Gong L, Cullinane M, Treerat P, Ramm G, Prescott M, Adler B, et al. The *Burkholderia pseudomallei* type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. PLoS One 2011; 6:e17852.

64. Rinchai D, Riyapa D, Buddhisa S, Utispan K, Titball RW, Stevens MP, et al. Macroautophagy is essential for killing of intracellular *Burkholderia pseudomallei* in human neutrophils. Autophagy 2015; 11:748-55.

65. Remijsen Q, Vanden Berghe T, Wirawan E, Asselbergh B, Parthoens E, De Rycke R, et al. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. Cell Res 2011; 21:290-304.

66. Tang S, Zhang Y, Yin SW, Gao XJ, Shi WW, Wang Y, et al. Neutrophil extracellular trap formation is associated with autophagy-related signalling in ANCA-associated vasculitis. Clin Exp Immunol 2015; 180:408-18.

67. Riyapa D, Buddhisa S, Korbsrisate S, Cuccui J, Wren BW, Stevens MP, et al. Neutrophil extracellular traps exhibit antibacterial activity against *Burkholderia pseudomallei* and are influenced by bacterial and host factors. Infect Immun 2012; 80:3921-9.

68. de Jong HK, Koh GC, Achouiti A, van der Meer AJ, Bulder I, Stephan F, et al. Neutrophil extracellular traps in the host defense against sepsis induced by *Burkholderia pseudomallei* (melioidosis). Intensive Care Med Exp 2014; 2:21.

Table 1: Human plasma antibody baseline (IgM and IgG) against FliC, OmpA and N-PilO2 from *B. pseudomallei*, and cellular quality of PBMC samples from seropositive donors before transfer to NOD/SCID/Jak3^{null} mice.

Total number of samples = 5	
Protein specific antibody baseline	**U/ml (range)**
anti-FliC	
IgM	1801 (585-3500)
IgG	1192 (797-1755)
anti-OmpA	
IgM	1434 (581-2542)
IgG	1490 (1062-2450)
anti-N-PilO2	
IgM	1305 (435-2054)
IgG	1209 (510-2490)
Cellular properties	**% Average (range)**
Viability (tryphan blue exclusion)	97 (96-99)
Lymphocyte viability (FACS analysis)	89 (86-93)
CD3+	89 (82-92)
CD3+, CD4+	*49 (34-70)*
CD19+	14 (7-22)

Note: Human PBMCs were stained for human surface markers including CD19, CD3 and CD4. Dead cells were stained with AmCyan before analysis by flow cytometry.

Figure Legends

Figure 1: IFN-γ production upon stimulation of human PBMC from seronegative and seropositive individuals. Isolated human PBMCs (5 x 10^5) from seronegative (N = 3) and seropositive (N = 5) individuals were stimulated at 37°C for 48 hours induplicate with dead *B. pseudomallei* whole bacteria (Bp), 3 μg/ml phytohaemagglutinin (PHA) or 10 μg/ml *B. pseudomallei* proteins; FliC, OmpA or N-PilO2. IFN-γ production upon stimulation was determined by ELISA. Statistical significance was analyzed using one-way ANOVA, and post-test using Bonferroni's Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant.

Figure 2: Expansion of human B cell population induced by *B. pseudomallei* FliC, OmpA and N-PilO2 protein antigens *in vivo* in hu-PBL-SCID mice. NOD/SCID/Jak3^{null} (SCID) mice were reconstituted with PBMCs from seropositive donors and boosted with PBS (non-boosted controls) or *B. pseudomallei* FliC, OmpA or N-PilO2 proteins. After 14 days, spleen cells from hu-PBL-SCID mice were collected, processed and stained with the viability marker (AmCyan), and markers of human CD45, CD19, CD3 and CD4, before analysis by flow cytometry. Statistically significant differences between the results obtained from non-boosted mice versus antigen-boosted mice were analyzed, using one-way ANOVA, and post-test, using Bonferroni's Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant.

Figure 3: *In vivo* human IgM and IgG antibodies are induced by boosting hu-PBL-SCID mice with FliC, OmpA and N-PilO2. Mouse spleen and sera were collected at day 14 after boosting with PBS, FliC, OmpA or N-PilO2. Levels of

specific human antibodies, produced against boosting antigens in hu-PBL-SCID mice sera, were measured by indirect ELISA (panel A). The number of specific antibody secreting cells (ASC) in antigen boosted or control mice were enumerated by ELSpot assay (panel B). Statistical significant differences between results from control and antigen-boosted hu-PBL-SCID mice were analyzed, using one-way ANOVA, and post-test, using Bonferroni's Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant.

Figure 4: *B. pseudomallei* antigen specific IFN-γ secreting cells are present in hu-PBL-SCID mice and are increased by boosting *in vivo* with FliC, OmpA and N-PilO2. After boosting for 14 days, spleen cells were removed from hu-PBL-SCID mice, and were restimulated *in vitro* with cell culture medium, containing FliC, OmpA or N-PilO2 for 48 h, prior to IFN-γ detection, counting the number of IFN-γ spot forming units (SFU) by ELISpot assay. Statistically significant differences were analyzed by using one-way ANOVA, and post-test by using Bonferroni's Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant.

Figure 5: Affinity maturation of anti-*B. pseudomallei* IgG antibody after boosting hu-PBL-SCID mice with FliC, OmpA and N-PilO2. Antibody avidities of IgM and IgG were evaluated by using indirect ELISA following treatment with 7 M urea (N = 5). % Urea resistance of IgM and IgG antibodies from human sera (Human) and antigen boosted hu-PBL-SCID mice sera (Hu-mice) were compared, statistically significant differences were analyzed, using one-way ANOVA, and post-test, using Bonferroni's Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant.

Figure 6: *B. pseudomallei* specific antibody in hu-PBL-SCID mice promotes phagocytosis and oxidative burst activities of human PMNs, in a concentration and affinity dependent manner. Antigen-specific purified pooled human plasma antibodies by affinity chromatography (A) and sera from hu-PBL-SCID mice 14 days after boosting with either PBS (non-boosted), FliC, OmpA and N-PilO2 (B) were used for FITC-labeled opsonization of dead, intact *B. pseudomallei*. Elution buffer passed through a column with uncoated beads (No Ab) was used as a negative control for purified human antibody (A), while sera from non-boosted hu-PBL-SCID mice (Non-boosted) were base line control for hu-PBL-SCID sera (B). Whole blood from seropositive donors were cultured with pre-opsonized FITC *B. pseudomallei*, and oxidative burst activities from human PMNs were detected by flow cytometry. Statistical significance was analyzed using one-way ANOVA, and post-test using Bonferroni's Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant compared between results from conditions with and without antibody. The correlation between level (C) or avidity (D) of IgG antibody in each antigen boosted hu-PBL-SCID mice and % total phagocytosis (closed circles) and % Oxidative burst in phagocytosed cells (open circle) was analyzed by linear regression.

Figure 7: Summary of the humanized mouse model (hu-PBL-SCID) reconstituted with human peripheral blood mononuclear cells (PBMCs) from *B. pseudomallei* seropositive donors, to illustrate the potential of three known antigens (FliC, OmpA and N-PilO2) for boosting both T- and B-cell immune responses.

Supplementary Figure Legends

Figure S1: Gating strategies of human cell surface marker staining in hu-PBL-SCID mice spleen cells. Hu-PBL-SCID mice spleen cells were stained for human leukocyte surface markers including CD45, CD19, CD3 and CD4. Dead cells were excluded by AmCyan. Firstly, we gated on lymphocyte population and focused on viable cells (AmCyan negative). Then, we analyzed the proportion of human B cells (CD45+, CD19+), T cells (CD45+, CD3+) and helper T cells (CD45+, CD3+, CD4+).

Figure S2: Dose dependent antigen specific binding of purified human anti-FliC, anti-OmpA and anti-PilO antibodies. Heparinized human plasma samples were collected and pooled together (N = 5) for purification of human antibodies against FliC, OmpA and N-PilO2 by affinity chromatography using antigen coated sepharose gel. Specific binding of purified human antibodies, both IgM and IgG, were determined by indirect ELISA with serial 3 fold dilutions of each purified antibody.