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Burkholderia pseudomallei Proteins Presented by Monocyte-Derived Dendritic Cells Stimulate Human Memory T Cells *In Vitro*▽

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Melioidosis is a severe infectious disease caused by the saprophytic facultative intracellular pathogen *Burkholderia pseudomallei*. The disease is endemic in Southeast Asia and Northern Australia, and no effective vaccine exists. To describe human cell-mediated immune responses to *B. pseudomallei* and to identify candidate antigens for vaccine development, the ability of antigen-pulsed monocyte-derived dendritic cells (moDCs) to trigger autologous T-cell responses to *B. pseudomallei* and its products was tested. moDCs were prepared from healthy individuals exposed or not exposed to *B. pseudomallei*, based on serological evidence. These were pulsed with heat-killed *B. pseudomallei* or purified antigens, including ABC transporters (LolC, OppA, and PotF), Bsa type III secreted proteins (BipD and BopE), tandem repeat sequence-containing proteins (Rp1 and Rp2), flagellin, and heat shock proteins (Hsp60 and Hsp70), prior to being mixed with autologous T-cell populations. After pulsing of cells with either heat-killed *B. pseudomallei*, LolC, or Rp2, coculturing the antigen-pulsed moDCs with T cells elicited gamma interferon production from $CD4^+$ T cells from seropositive donors at levels greater than those for seronegative donors. These antigens also induced granzyme B (cytotoxic) responses from $CD8^+$ T cells. Activation of antigen-specific $CD4^+$ T cells required direct contact with moDCs and was therefore not dependent on soluble mediators. Rp peptide epitopes recognized by T cells in healthy individuals were identified. Our study provides valuable novel data on the induction of human cell-mediated immune responses to *B. pseudomallei* and its protein antigens that may be exploited in the rational development of vaccines to combat melioidosis.

Burkholderia pseudomallei is a Gram-negative facultative intracellular bacterium that causes melioidosis, a severe invasive disease that is endemic in Southeast Asia, Northern Australia, and several other tropical regions (7, 32). Acute septic melioidosis has a high mortality rate in northeast Thailand (approximately 50%), even in patients who receive appropriate antibiotics. Serological evidence based on indirect hemagglutination assay (IHA) indicates that 80% of people living in areas of endemicity have been exposed to *B. pseudomallei* with no clinical symptoms, and the prevalence of positive serology increases with age (4, 9). Recurrent melioidosis can also occur, either as a relapse after antibiotic treatment or as reinfection (21). *B. pseudomallei* is classified as an NIAID category B potential agent for biological terrorism (24). The mechanisms by which the organism evades the bactericidal effects of the host immune response are not fully understood, and there are no licensed vaccines at the time of this writing.

Recently, we used a *B. pseudomallei* protein microarray to identify proteins of the organism recognized by the human humoral immune system (13). However, development of effective melioidosis vaccines also requires consideration of the cell-mediated immune response owing to intracellular persistence of the bacterium. In animal models, the crucial mediator of resistance to acute *B. pseudomallei* infection is gamma interferon (IFN-γ) produced by multiple cell types, including NK cells and both CD4 and CD8 T cells (17, 20, 26). T-cell-mediated responses to some recombinant proteins of *B. pseudomallei* have been studied. Immunization of mice with a live-attenuated *ilvI* mutant of *B. pseudomallei* (2D2) induced antigen-specific T cells, including $CD4^+$ T cells that respond to proteins secreted by the Bsa type III secretion system (T3SS) (BopE and BipD) (16). *B. pseudomallei* 2D2-primed T cells also recognize bacterial proteins of the ATP-binding cassette (ABC) family, including LolC, PotF, and OppA, and respond by producing IFN-γ (18). In an assay to evaluate candidate vaccine antigens, immunization of mice with adjuvant-mixed Bip proteins did not elicit protection

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against *B. pseudomallei* infection (11, 27). However, antibodies to BipB, BipC, and BipD have been detected in sera of melioidosis patients (11, 28). Furthermore, a DNA vaccine encoding the flagellin protein and carrying CpG motifs enhanced resistance of immunized mice to *B. pseudomallei* (5, 6). Owing to the incomplete protection afforded by candidate antigens to date, it is necessary to search for additional proteins for vaccine development. Tandem repeat (TR) sequence-containing proteins of *B. pseudomallei* (such as Rp1 and Rp2) may be considered vaccine candidates, as proteins containing TRs are known targets of B-cell responses (15). Heat shock proteins (HSPs) have also been found to play a role in intracellular antigen processing, presentation, and cross-presentation (36). Indeed, a DNA vaccine encoding a *Mycobacterium bovis* BCG heat shock protein could enhance T-cell activation against mycobacterial infection (19). The HSPs of *B. pseudomallei* have not been studied for vaccine development to date.

Dendritic cells (DCs) are the control center of the immune system and are specialized antigen-presenting cells that regulate the initiation of T-cell responses to eradicate invading pathogens (31). In mice, immunization with DCs pulsed with heat-killed *B. pseudomallei* in the presence of CpG adjuvant led to the induction of both cell-mediated and humoral responses to the organism (12). However, little is known about the biology of DCs in relation to *B. pseudomallei* antigens and the generation of effective T-cell immunity in humans. In a previous study, we determined the ability of the ABC transporter proteins LolC, OppA, and PotF to restimulate T cells in whole blood or peripheral blood mononuclear cells (PBMC) from seropositive healthy humans and recovered melioidosis patients living in areas where melioidosis is endemic (29). Here we extended these observations by examining the ability of *B. pseudomallei* and a panel of its antigens to pulse human monocyte-derived dendritic cells (moDCs) to stimulate autologous T-cell populations *in vitro*.

Our data show that autologous human DCs efficiently processed and presented heat-killed *B. pseudomallei* and its purified antigens to CD4 T cells and, to a lesser extent, CD8 T cells, in a contact-dependent manner. Using this approach, tandem repeat sequence-containing proteins were found to be novel T-cell immunogens of *B. pseudomallei*. We provide novel data on the *B. pseudomallei* antigens presented by human DCs, some of which are candidates for inclusion in vaccines to elicit cell-mediated immunity against melioidosis.

(This work was presented in part at the European Melioidosis Network Conference, London, England, 11 September 2009.)

MATERIALS AND METHODS

Human subjects. The consent form for the study was approved by the Khon Kaen University Ethics Committee for Human Research (project number HE470506). All subjects signed the consent form, and the data collected were not disclosed. Leukocyte-rich samples from healthy blood donors were collected as leftover samples from the Blood Transfusion Center, Faculty of Medicine, Khon Kaen University. The healthy subjects were divided into either seronegative or seropositive groups, based on titers of antibody to *B. pseudomallei* quantified by IHA. Seronegative subjects were selected on the basis of IHA titers of <1:40, whereas the seropositive subjects presumed to have been exposed to *B. pseudomallei* were selected by titers of 1:40 or greater (8). Both the seronegative and seropositive donors were age matched and had no clinical history of melioidosis but lived in an area where melioidosis is endemic (predominantly the northeast region of Thailand).

moDCs. PBMC were isolated by Ficoll-Hypaque density gradient centrifugation of leukocyte-rich samples. Monocytes ($CD14^+$) and $CD4^+$ and $CD8^+$ T cells were then isolated by positive selection using immunomagnetic beads (Miltenyi Biotec, Germany), and their purity was determined by flow cytometry as described below. Immature DCs were generated from monocytes by culture in 300 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Preprotech) and 200 U/ml of interleukin-4 (IL-4) (Preprotech) for 6 days in 1 ml of complete RPMI 1640 (RPMI 1640 containing 10% [vol/vol] heat-inactivated fetal bovine serum [FBS], 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine [Sigma-Aldrich]). Every 2 days, 500 μl of culture supernatant was removed, and 500 μl of fresh culture medium containing cytokines at the same final concentrations was added.

Proteins and peptides. LolC, OppA, and PotF polypeptides lacking predicted membrane-spanning domains were expressed in *Escherichia coli* under kanamycin selection and purified as previously described (18). Expression and purification of BipD and BopE proteins in *E. coli* have been described elsewhere (16). The full-length *B. pseudomallei* Hsp60 (BpHsp60), Hsp70 (BpHsp70), and flagellin (Flg) genes were cloned and expressed in *E. coli* M15 cells and then purified as described previously (34). Rp proteins and peptides were synthesized by In Bios International, Inc. (Seattle, WA). The purity of affinity-tagged recombinant proteins was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and their concentration determined using bicinchoninic acid assays (Pierce Biotechnology). All recombinant *B. pseudomallei* proteins used in this study were negative when assayed for the presence of contaminating lipopolysaccharide by use of *Limulus* amebocyte lysate reagent (Pyrotell; Associates of Cape Cod), with a limit of detection of 0.03 endotoxin unit (EU)/ml.

Coculture of monocyte-derived dendritic cells with T cells. *B. pseudomallei* strain K96243 was cultured in Luria-Bertani medium and inactivated by heating at 100°C for 20 min. The number of viable bacteria prior to heat inactivation was determined by plating of serial dilutions to permit estimation of the multiplicity of infection (MOI). moDCs were pulsed with heat-killed *B. pseudomallei* (MOI of 10:1) or purified *B. pseudomallei* proteins (1 μg/ml) for 18 h. The pulsed moDCs were washed twice with phosphate-buffered saline (PBS), plated at 4×10^4 cells/well into 96-well plates, and cocultured with autologous $CD4^+$ and $CD8^+$ T cells at 4×10^5 cells/well (ratio of moDCs to T cells = 1:10). Culture supernatants were collected after 48 h of coculture, and IFN-γ levels were measured by enzyme-linked immunosorbent assay (ELISA) as described below. The polyclonal activator phytohemagglutinin (PHA; Biochrom AG, Germany) served as a positive control for T-cell activation. The IFN-γ-inducing cytokines interleukin-12 (IL-12) and IL-15 (BD Biosciences) were used as a positive control for cytokine activation of T cells via the T-cell receptor (TCR)-independent pathway (29) Cultures of moDCs or T cells alone were used as negative controls.

Transwell assay to demonstrate T-cell activation by pulsed DCs via TCR-dependent pathway. Transwell chambers consist of an upper and a lower chamber separated by a 0.4-μm-pore-size polycarbonate membrane (Corning) that allows diffusion of cytokines and chemokines through the membrane but avoids contact interaction of the cells in both chambers. moDCs were pulsed with heat-killed *B. pseudomallei* (MOI of 10:1) or purified *B. pseudomallei* protein (LolC or Rp2) (1 μg/ml) for 18 h as described above. The pulsed moDCs were washed twice with PBS and plated onto the upper chamber, and autologous purified $CD4^+$ T cells were placed in the lower chamber (DC/$CD4^+$ T-cell ratio = 1:10). The cultures were incubated at 37°C in 5% CO_2 for 48 h. The supernatant was collected and kept at −20°C for use in an ELISA to measure IFN-γ.

Quantification of IFN-γ in culture supernatants by ELISA. The supernatants of moDC–T-cell cocultures were assayed for IFN-γ production by use of an ELISA kit (BD Biosciences). Plastic 96-well microtitration plates were coated with a monoclonal antibody (MAb) to human IFN-γ in 0.1 M carbonate-bicarbonate buffer overnight at 4°C. The excess reagent was removed by washing the plate three times with 0.005% (vol/vol) Tween 20 in PBS, and the plate was then blocked with 10% (vol/vol) fetal calf serum (FCS) in PBS and incubated for 1 h. The plate was washed three times, and then test supernatants or a recombinant human IFN-γ standard was added and incubated for 2 h at room temperature (RT). After washing of the plate, biotinylated anti-human IFN-γ and avidin-horseradish peroxidase conjugate were added and incubated for 1 h at RT. The plate was washed seven times, and then a substrate solution was added and incubated for 30 min at RT in the dark. Reactions were stopped with 2 N H_2SO_4, and absorbance was read at 450 nm with an ELISA plate reader (Tecan, Austria).

Quantification of cells secreting IFN-γ and GrB. $CD4^+$ and $CD8^+$ T cells producing IFN-γ or granzyme B (GrB) were enumerated by enzyme-linked immunospot (ELISPOT) assay. In summary, precoated 96-well polyvinylidene difluoride (PVDF) plates (MSIP; Millipore) were incubated overnight with 15

μg/ml of IFN-γ- or GrB-specific antibody, and then 4×10^4 moDCs pulsed with heat-killed *B. pseudomallei* or purified *B. pseudomallei* proteins were added to the plate in duplicates. Pulsed moDCs were then cocultured with autologous $CD4^+$ or $CD8^+$ T cells for 48 h, and IFN-γ and GrB secretion was detected by addition of 1 μg/ml biotinylated anti-human IFN-γ and GrB at room temperature for 3 h. After washing of the plate, 1 μg/ml streptavidin-alkaline phosphatase was added for 1 h, and spots were revealed with a Bio-Rad alkaline phosphatase kit and enumerated under a stereomicroscope.

Flow cytometric analysis. Purities of $CD14^+$, $CD4^+$, and $CD8^+$ cells after separation by use of immunomagnetic beads were determined by flow cytometry after surface marker staining with fluorescein isothiocyanate (FITC)-conjugated marker-specific antibodies (Becton Dickinson) at a concentration of 0.06 to 1 μg/1×10^6 cells. Monocytes differentiated into DCs were enumerated by staining of DC-associated surface markers ($CD11c^+$ and HLA-DR) and decreased expression of CD14, using 0.06 to 1 μg/1×10^6 cells of FITC–anti-CD14, FITC–anti-CD11c, and phycoerythrin (PE)–anti-HLA-DR. Flow cytometry was used for the characterization of IFN-γ-producing memory $CD4^+$ T cells, using allophycocyanin (APC)–anti-IFN-γ, FITC–anti-CCR7, and PE–anti-CD45RA. Isotype-matched controls were included in each analysis. Staining of immune cell surface markers was performed for 30 min at 4°C. Data acquisition was performed with a Becton Dickinson FACSCalibur instrument and analyzed by CellQuest software (BD Biosciences). Forward scatter/side scatter was used as the first gate, with selection of lymphocytes in the first gate, followed by an IFN-γ dot plot for analysis of IFN-γ^+ cell frequency, with fast acquisition, at >1,500 total events/second. Following flow cytometry analysis, the purity of each cell type was over 96%. The memory T-cell subsets were identified by gating on IFN-γ^+ cells, and then combined analysis of CCR7 and CD45RA was used to characterize central memory T cells (T_{CM}; $CCR7^+$ $CD45RA^-$), effector memory T cells (T_{EM}; $CCR7^-$ $CD45RA^-$), effector memory RA T cells (T_{EMRA}; $CCR7^-$ $CD45RA^+$), and resting T cells ($CCR7^+$ $CD45RA^+$).

MHC class II epitope prediction. Epitope prediction programs, including the Immune Epitope Database (IEDB) (http://tools.immuneepitope.org/analyze/html/mhc_II_binding.html) and ProPred (http://www.imtech.res.in/raghava/propred/), were used to predict dominant human Th1-cell epitopes of the full-length Rp amino acid sequences in relation to major histocompatibility complex (MHC) class II restriction (10-mers to 15-mers, depending on the program used). Predicted epitopes that ranked highly across both programs were selected for further evaluation. Preference was given to epitopes that showed high hypothetical affinities for more than one HLA type.

***In vitro* stimulation of human PBMC with *B. pseudomallei* Rp peptides.** PBMC from healthy donors living in an area of melioidosis endemicity were isolated from buffy coat samples by density centrifugation on Histopaque 1077 (Sigma-Aldrich), and the number of cells was adjusted as required prior to stimulation. The isolated PBMC were stimulated with PHA as a mitogen, with 1 μg/ml LolC as a positive antigen control, with 3 μg/ml Rp proteins or peptides, or with medium alone as a negative control. Peptides 1 to 7 were test peptides derived from *B. pseudomallei* Rp proteins. The number of cells stimulated to secrete IFN-γ was evaluated by ELISPOT assay as described above.

HLA typing. HLA-DRB1 alleles in the samples used above were determined by PCR sequence-specific primer (PCR-SSP), using 20 pairs of primers (22, 23). The primer mixtures contained a pair of allele-specific primers and a pair of 256-bp internal control primers.

Statistical analysis. Statistical analysis (Mann-Whitney test) was performed using Graphpad Prism software, version 5 (GraphPad, San Diego, CA). A P value of <0.05 was considered statistically significant.

RESULTS

T-cell responses of seropositive versus seronegative healthy donors to moDCs pulsed with heat-killed *B. pseudomallei*. We demonstrated previously, using a whole-blood assay, that T cells from seropositive and recovered melioidosis subjects respond to heat-killed *B. pseudomallei* by producing IFN-γ (29). Here we first defined the contributions of IFN-γ production from $CD4^+$ and $CD8^+$ T cells cocultured with antigen-pulsed autologous moDCs. T-cell subsets were purified from 9 healthy donors who were seropositive for *B. pseudomallei* and from 8 seronegative donors. These were cocultured with heat-killed *B. pseudomallei*-pulsed moDCs for 48 h, and then the IFN-γ levels in cultured supernatants were assayed by ELISA. The ratio of moDCs to T cells was optimized by comparing responses to heat-killed *B. pseudomallei* at ratios of 1:5, 1:10, 1:50, and 1:100, with the highest IFN-γ level being observed at a moDC/T-cell ratio of 1:10. Moreover, the incubation period and the concentration of each protein were also optimized (data not shown). Cells were stimulated separately with PHA and medium alone, as positive and negative controls, respectively.

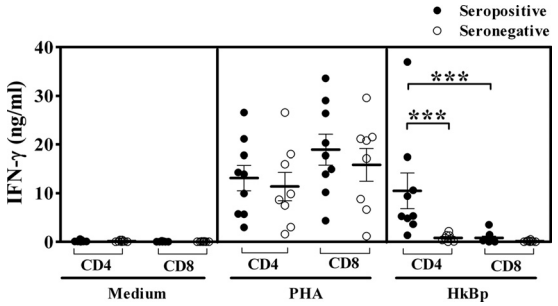

FIG. 1. IFN-γ production by T-cell subsets from seropositive compared to seronegative healthy donors in response to moDCs pulsed with heat-killed *B. pseudomallei* (HkBp). Monocyte-derived DCs were pulsed with heat-killed *B. pseudomallei* or medium for 18 h and then cocultured with autologous $CD4^+$ and $CD8^+$ T cells (ratio of moDCs to T cells of 1:10). PHA-stimulated T cells were used as a positive control. Culture supernatants were collected at 48 h, and IFN-γ levels were measured by ELISA. The controls included medium as a negative control and PHA as a positive control for mitogen stimulation. ***, $P < 0.001$ by Mann-Whitney test between IFN-γ production of autologous $CD4^+$ T cells in response to heat-killed *B. pseudomallei*-pulsed moDCs from seropositive ($n = 9$) and seronegative ($n = 8$) donors and between IFN-γ production of autologous $CD4^+$ T cells and $CD8^+$ T cells in response to heat-killed *B. pseudomallei*-pulsed moDCs from seropositive donors.

$CD4^+$ T cells of 6/8 seronegative donors showed statistically significant IFN-γ responses to heat-killed *B. pseudomallei* compared with the medium control, but the magnitude of IFN-γ secretion was low (Fig. 1). In contrast, 8 of 9 seropositive donors showed extensive IFN-γ production, with a mean value of 10.52 ng/ml, compared to 0.81 ng/ml for the seronegative group ($P < 0.001$). A response by $CD8^+$ T cells from 7/9 seropositive donors was also observed but was 13-fold lower than the $CD4^+$ T-cell response, showing that the latter were the dominant IFN-γ-producing T cells under these restimulation conditions ($P < 0.001$; Mann-Whitney test). These results are consistent with previous data from whole-blood assays (29).

Purified *B. pseudomallei* proteins are recognized by $CD4^+$ T cells of seropositive healthy donors. In an effort to identify the proteins of *B. pseudomallei* recognized by cell-mediated immunity in humans, the IFN-γ responses of $CD4^+$ T cells to 10 different purified Bps proteins presented by moDCs were evaluated. The proteins, namely, LolC, OppA, and PotF (ABC transporter proteins), BipD and BopE (Bsa T3SS proteins), Hsp60 and Hsp70 (heat shock proteins), Rp1 and Rp2 (tandem repeat-containing proteins), and flagellin, were expressed in *E. coli* and affinity purified as described in Materials and Methods. moDCs from 9 seropositive and 8 seronegative healthy donors were pulsed separately with each purified protein for 48 h. PHA and heat-killed *B. pseudomallei* served as positive controls owing to their ability to evoke strong responses of $CD4^+$ T cells from seropositive donors (Fig. 1), while negative controls were comprised of medium, moDCs

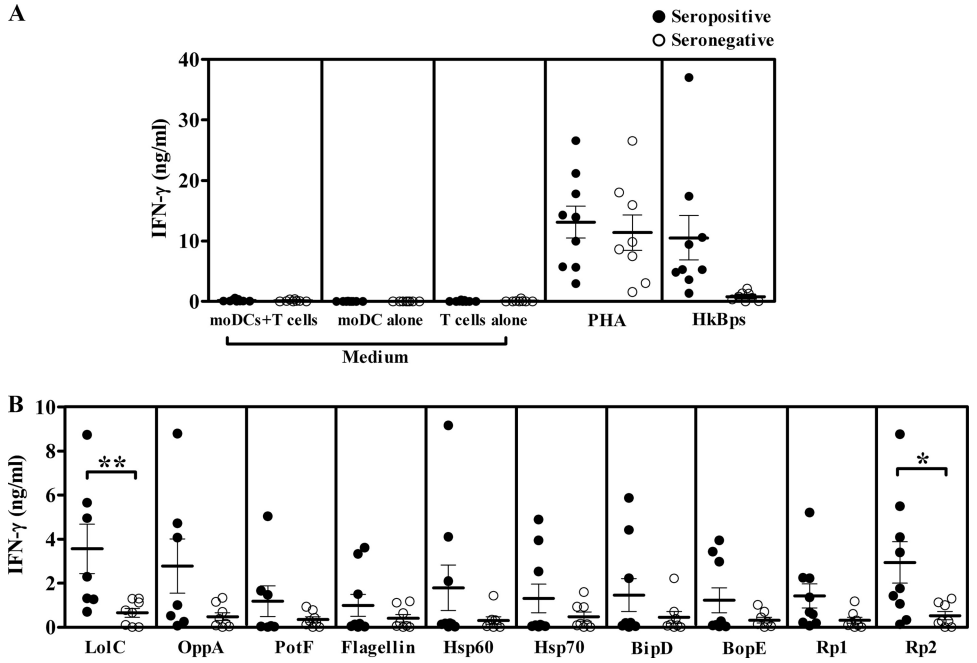

FIG. 2. IFN-γ production of $CD4^+$ T cells from seropositive versus seronegative healthy donors in response to purified *B. pseudomallei* proteins. The moDCs of seropositive and seronegative healthy donors were pulsed with heat-killed *B. pseudomallei* or its proteins for 18 h and then cocultured with $CD4^+$ T cells. Culture supernatants were collected at 48 h, and IFN-γ levels in culture supernatants were measured by ELISA. (A) The negative-control set consisted of moDCs plus T cells, moDCs alone, and T cells alone that were cultured in medium. Positive controls included mitogen (PHA) and heat-killed *B. pseudomallei*. (B) Responses to purified proteins. **, $P < 0.01$; *, $P < 0.05$ (by Mann-Whitney test between IFN-γ production of autologous $CD4^+$ T cells in response to antigen-pulsed moDCs from seropositive and seronegative donors).

alone, and unstimulated $CD4^+$ T cells (Fig. 2A). The results for the *B. pseudomallei* protein LolC showed that 4 of 7 seropositive individuals had a modest response to LolC, 3 had a response that was higher than the average, and 1 had a strong response; a similar response was observed with the Rp2 protein. The mean quantities of IFN-γ produced by $CD4^+$ T cells from seropositive donors in response to LolC and Rp2 were higher than those for the seronegative group (Fig. 2B) ($P < 0.01$ and $P < 0.05$, respectively). The other proteins also elicited elevated levels of IFN-γ from some, but not all, individuals in the seropositive group (3/9 seropositive donors), but this heterogeneity resulted in no statistically significant differences when the means between seropositive and seronegative donors were compared. Notably, $CD4^+$ T cells from these 3 seropositive donors responded strongly to all 10 *B. pseudomallei* proteins, while the other 6 individuals were low responders.

Heat-killed *B. pseudomallei* and its proteins are recognized by effector memory $CD4^+$ T cells through a contact-dependent pathway. The previous result indicated the dominant response of $CD4^+$ T cells of seropositive healthy donors to heat-killed *B. pseudomallei* and the *B. pseudomallei* proteins LolC and Rp2. We previously demonstrated that production of IFN-γ by T cells in response to *B. pseudomallei* occurs predominately via T-cell receptors but can also occur via a cytokine-dependent, cyclosporine A-insensitive pathway (20, 29). To investigate whether the stimulation of $CD4^+$ T cells by pulsed moDCs observed here occurred via the T-cell receptor pathway, a Transwell culture method was used to prevent cell-to-cell contact but permit diffusion of soluble factors during coculture. moDCs from seropositive healthy donors were pulsed separately with heat-killed *B. pseudomallei* or recombinant LolC or Rp2, plated into 24-well culture plates, and incubated for 18 h. Autologous purified $CD4^+$ T cells were then plated on a polycarbonate insert well at a moDC/$CD4^+$ T-cell ratio of 1:10 and cocultured for 48 h, and the IFN-γ concentration in culture supernatants was measured by ELISA. The amount of IFN-γ produced by $CD4^+$ T cells cocultured with but separated from moDCs pulsed with heat-killed *B. pseudomallei*, LolC, or Rp2 was reduced to the baseline level compared to cocultures in direct contact (Fig. 3A) ($P < 0.05$). This indicates that antigen-pulsed moDCs stimulate $CD4^+$ T cells to produce IFN-γ predominantly through a contact-dependent pathway rather than as a consequence of bystander cytokine-mediated effects.

To identify IFN-γ-producing $CD4^+$ T-cell subsets, memory phenotypes of T cells were characterized. Our previous study classified T-cell phenotypes based on expression of CD45RA and CCR7, whereby $CD45RA^+$ $CCR7^+$ denotes naive or resting T cells, $CD45RA^-$ $CCR7^+$ denotes T_{CM} (central memory) cells, $CD45RA^-$ $CCR7^-$ denotes T_{EM} (effector memory) cells, and $CD45RA^+$ $CCR7^-$ denotes T_{EMRA} (terminally differentiated effector memory RA) cells (2). In this study, purified $CD4^+$ T cells from seropositive donors were cocultured with moDCs primed separately with heat-killed *B. pseudomallei* or LolC for 24 h, and IFN-γ-producing $CD4^+$ T cells were analyzed by flow cytometry. The results showed that the majority of IFN-γ-producing $CD4^+$ T cells cocultured with moDC-pulsed heat-killed *B. pseudomallei* or LolC were T_{EM} cells (76% and 73% of IFN-γ-producing $CD4^+$ T cells, respec-

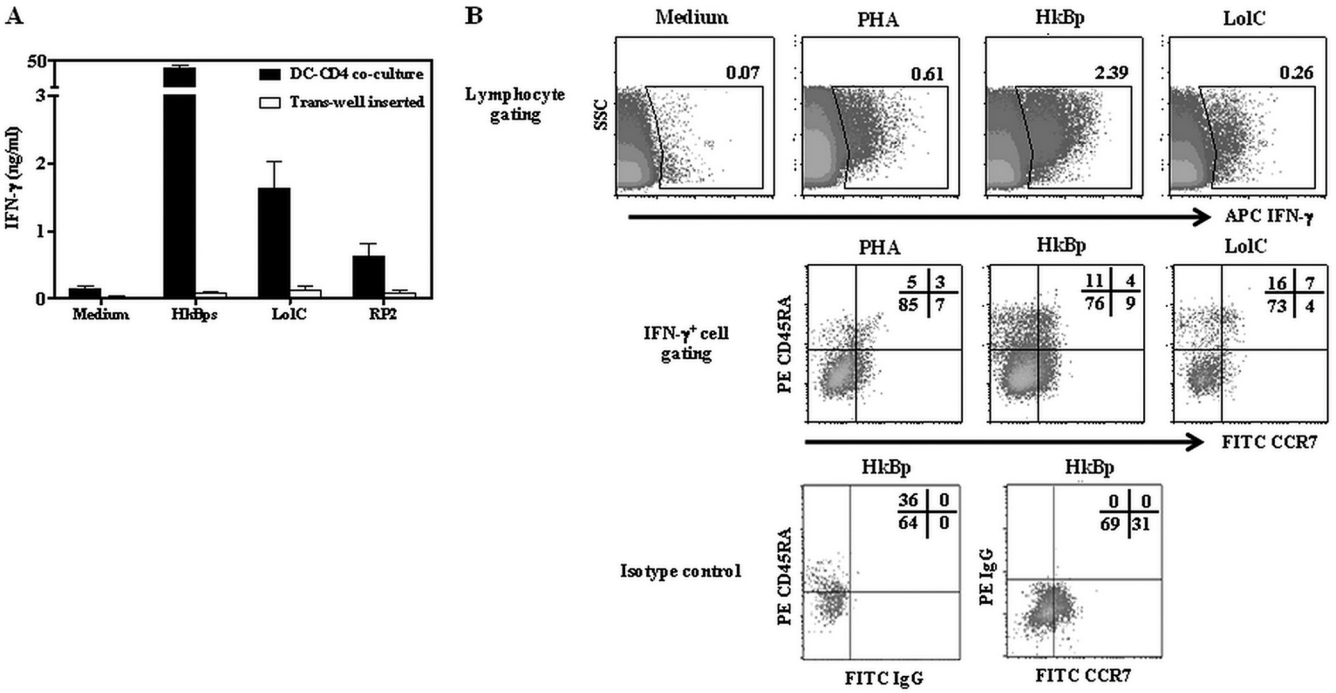

FIG. 3. IFN-γ response of $CD4^+$ T cells from exposed healthy donors to heat-killed *B. pseudomallei*- or purified *B. pseudomallei* protein-pulsed moDCs requires cell contact. (A) $CD4^+$ T cells isolated from seropositive healthy donors were cocultured with moDCs pulsed with heat-killed *B. pseudomallei*, LolC, or Rp2 in the presence (white bars) or absence (black bars) of polycarbonate insert wells to separate moDCs from T cells. The culture supernatants were collected, and IFN-γ levels were measured by ELISA. (B) Phenotypic flow cytometry analysis of intracellular IFN-γ-producing $CD4^+$ T cells from a single seropositive healthy donor in response to moDCs pulsed with heat-killed *B. pseudomallei* and LolC. $CD4^+$ T cells were stained with APC–anti-IFN-γ, FITC–anti-CCR7, and PE–anti-CD45RA prior to analysis by gating on lymphocyte cells (first row) and IFN-γ^+ cells (second row). Isotype-matched controls were also included in the analysis (third row).

tively). The other $CD4^+$ T-cell phenotypes detected in response to moDCs pulsed with heat-killed *B. pseudomallei* and LolC were 9% and 4% T_{CM} cells, 11% and 16% T_{EMRA} cells, and 4% and 7% resting T cells, respectively (Fig. 3B). Thus, in healthy seropositive donors, effector memory $CD4^+$ T cells are the major contributors to the IFN-γ response under these culture conditions.

Heat-killed *B. pseudomallei* and its purified proteins activate cytotoxic $CD8^+$ T cells to produce GrB. GrB is a serine protease that is an important mediator of target cell apoptosis by cells such as natural killer cells and cytotoxic $CD8^+$ T cells (25). To evaluate whether moDCs pulsed with heat-killed *B. pseudomallei* or purified proteins can stimulate *B. pseudomallei*-specific cytotoxic T lymphocytes (CTLs), we measured the expression of GrB in $CD8^+$ T cells from seropositive subjects in response to moDCs pulsed separately with heat-killed *B. pseudomallei* or the 10 *B. pseudomallei* proteins described above. The number of cells producing GrB was evaluated by ELISPOT assay. The results revealed that moDCs pulsed with heat-killed *B. pseudomallei*, LolC, and Hsp60 could activate $CD8^+$ T cells to produce GrB (Fig. 4). The other *B. pseudomallei* proteins tended to stimulate $CD8^+$ T cells of certain seropositive subjects even though GrB secretion was not significantly different from that with the medium negative control. Thus, of the 10 antigens tested, only LolC was recognized by both reactive $CD4^+$ T cells and $CD8^+$ T cells.

Prediction of T-cell epitopes of *B. pseudomallei* tandem repeat sequence-containing proteins (Rp proteins). Our data indicate that *B. pseudomallei* Rp2 significantly stimulates T-cell responses when presented by moDCs (Fig. 2). It was therefore of interest to identify the immunodominant epitopes of Rp proteins. Seven peptides were analyzed for their hypothetical strength of binding and range of affinity (Table 1). It was predicted that peptides 1, 2, 5, and 6 would be recognized most efficiently by HLA-DR04. Peptide 5 was predicted to be recognized by HLA-DR03, -DR04, -DR11, and -DR15. Peptide 7 would be recognized by HLA-DR13 according to ProPred prediction, but it would not be recognized by any HLA-DR according to IEDB prediction. Peptides 3 and 4 were predicted not to be bound by any type of HLA-DR; however, they may be recognized by other MHC class II molecules.

Rp peptides elicit responses from PBMC of healthy donors from an area where melioidosis is endemic. To investigate the immune response to Rp proteins and its peptides in healthy individuals in an area where melioidosis is endemic, PBMC from 6 volunteers were stimulated separately with LolC, Rp proteins, and the 7 Rp peptides predicted above (peptides 1 to 7). IFN-γ production was then measured by ELISPOT assay. The IFN-γ responses to both Rp1 and Rp2 were statistically significant; moreover, significantly greater numbers of cells responded to peptides 1, 3, 4, 5, and 6 than to the medium control (Fig. 5). To map the T-cell epitopes recognized by healthy subjects, typing of HLA-DRB1 was performed for all donors. HLA-DR typing of the donors responding to the peptides demonstrated that they represented a heterogeneous group with respect to HLA, expressing DR15,12, DR13,07,

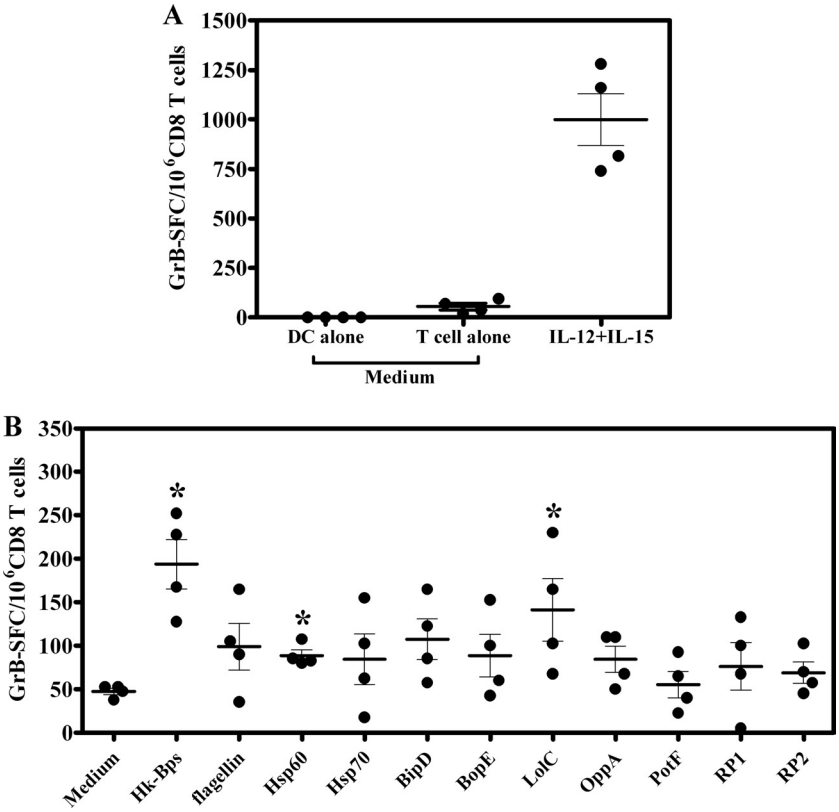

FIG. 4. Granzyme B production from purified $CD8^+$ T cells in response to moDCs pulsed with heat-killed *B. pseudomallei* or recombinant proteins. moDCs were pulsed with heat-killed *B. pseudomallei* or recombinant proteins for 18 h and then cocultured for 48 h with $CD8^+$ T cells (ratio of moDCs to T cells of 1:10), and GrB-producing cells were enumerated by ELISPOT assay. (A) Numbers of GrB-producing $CD8^+$ T cells with medium alone or in the presence of IL-12 and IL-15. (B) Numbers of GrB-producing $CD8^+$ T cells in response to heat-killed *B. pseudomallei* or 10 purified proteins. SFC, spot-forming cells. *, $P < 0.05$ by Mann-Whitney test between GrB secretion of autologous $CD8^+$ T cells in response to antigen-pulsed moDCs and that for the medium negative control (moDCs plus T cells) in the same panel.

DR15,14, DR15,04, DR03,04, and DR04,10 (Table 2). The ability of donor PMBC to respond to Rp peptides was largely consistent with the computational prediction of HLA-restricted epitopes, whereby peptides 1, 2, 5, 6, and 7 were predicted to bind HLA-DR types (Table 1).

DISCUSSION

B. pseudomallei causes severe invasive infections with a high mortality rate in areas where melioidosis is endemic. Recent studies have begun to unravel the bacterial constituents and host responses required for control of melioidosis. For example, we identified protein targets of human antibody responses by using protein microarrays (13). However, the development of effective vaccines for *B. pseudomallei* likely requires cell-mediated immunity owing to the ability of the pathogen to persist for protracted periods in an intracellular niche. In a previous study, we applied a whole-blood assay to explore human cellular responses to the pathogen, demonstrating the priming of *B. pseudomallei*-reactive T cells in healthy individuals living in the region of endemicity of northeast Thailand. However, such assays do not examine the antigen-presenting cell–T-cell interactions required for successful immune responses and give high background levels of IFN-γ due to the

TABLE 1. HLA-DR-restricted epitopes of *B. pseudomallei* Rp proteins, predicted by IEDB and ProPred

Peptide no.	Sequence	IEDB[a]	ProPred[b]	HLA-DR restriction
1	SGVASNAVGTLTNLANNNPL	5.42	39.36	DR04
2	PGALSNAVGTLQNAATNNPL	3.56	26.6	DR04
3	SGTSSTATGQGSQATGSNSTATGQDATA			
4	SGESSTAMGQGSQATGNNSTATGQDATA			
5	STSVVGISISFTGISVSYTGLSVSFTGV	3.21, 4.25, 5.96, 8.65	41.05, 30, 38.46, 31.12	DR03, -04, -11, -15
6	SASFTGLSTSFTGVSTSFTGVSTSFTGV	3.87	24.44	DR04
7	AAAEAAAAQAVTDKTAAEALAMQAAAER		28.41	DR13

[a] Lower scores predict stronger affinities for respective HLA types.
[b] The higher the prediction rank, the higher the hypothetical affinity for each HLA type.

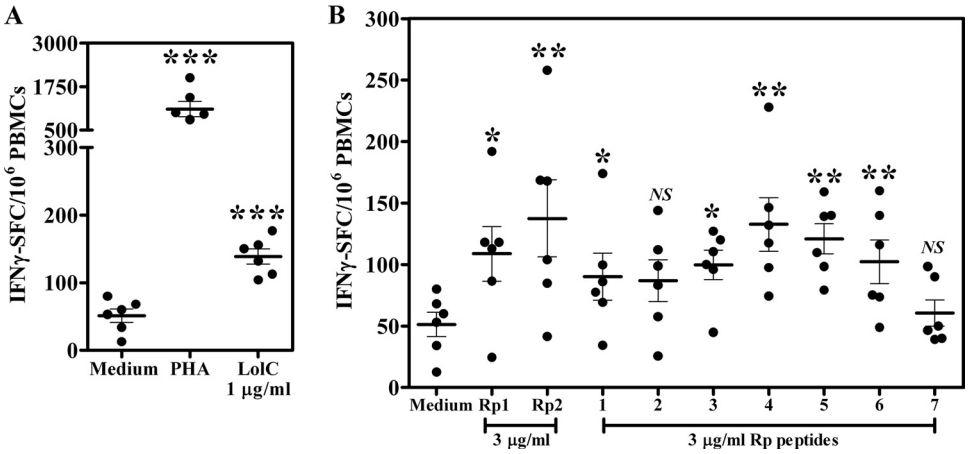

FIG. 5. Identification of immunogenic epitopes of *B. pseudomallei* Rp protein. PBMC were separated from leukocyte-rich samples from 6 donors. PBMC (5×10^5 cells/ml) were incubated with each stimulator for 42 h. (A) The controls included medium as a negative control, PHA as a mitogen positive control, and LolC as an antigen-specific control. (B) Rp proteins (Rp1 and Rp2) and seven Rp peptides were used as antigen stimulators. IFN-γ-secreting cells were enumerated under a stereomicroscope. Each horizontal line denotes the mean, and error bars show the standard error of the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant (by Mann-Whitney test between IFN-γ spot-forming cells of individual antigen proteins/peptides and the medium control).

presence of NK cells (29). In an effort to circumvent these issues, heat-killed bacteria and 10 purified proteins of the pathogen were evaluated for the ability to stimulate T-cell responses when presented by autologous moDCs.

DCs are professional antigen-presenting cells and play a central role in initiating T-cell responses. Evidence exists that *B. pseudomallei*-infected human moDCs are able to induce T-cell differentiation toward a Th1 population in a manner dependent upon cytokine production (3). Our study revealed that moDCs pulsed with heat-killed *B. pseudomallei* or recombinant protein antigens stimulated IFN-γ production predominantly by $CD4^+$ T cells rather than by $CD8^+$ T cells *ex vivo*. After heat-killed *B. pseudomallei* stimulation, IFN-γ-producing cells from seropositive subjects responded to a much greater extent than did those from seronegative subjects from the area of endemicity. In seropositive donors, IFN-γ production from $CD4^+$ T cells was considerably higher than that from $CD8^+$ T cells. This result is consistent with previous data showing that induction of IFN-γ in response to the organism is elevated in seropositive donors (1, 29).

The important role of $CD4^+$ T cells has been demonstrated in a mouse model of melioidosis, where antigen-specific $CD4^+$ T cells were induced by infection with a live-attenuated *B. pseudomallei* mutant and correlated with protection against secondary infection (16, 17). The role of $CD4^+$ T cells in human melioidosis is less clear, since HIV/AIDS is apparently not a risk factor for melioidosis (10). Nevertheless, our findings confirm that exposure to *B. pseudomallei* clearly results in priming of a robust T-cell response, as $CD4^+$ T cells from seropositive individuals responded to moDCs pulsed with heat-killed *B. pseudomallei* by producing high levels of IFN-γ. This was consistent with our previous study of whole-blood cultures that revealed an association between the secretion of IFN-γ from antigen-specific T cells in response to the pathogen or its ABC transporter proteins (LolC, OppA, and PotF) and increasing antibody titers (29). To further identify antigens that may be involved in this process, we confirmed the immunogenicity of ABC transporter proteins and screened new purified proteins, some of which are associated with *B. pseudomallei* virulence, for the ability to stimulate human T cells when presented by moDCs. This revealed that the newly defined Rp2 protein elicited significantly higher IFN-γ responses in primed $CD4^+$ T cells than in controls.

In addition, $CD4^+$ T cells of some responders from the seropositive group could produce IFN-γ at levels above the mean in response to the other proteins tested, including ABC

TABLE 2. Antigen-induced secretion of IFN-γ from PBMC of healthy donors in response to Rp peptides of *B. pseudomallei*, according to HLA class II allele

Donor	IHA titer	APC	No. of spot-forming cells secreting IFN-γ/10^6 PBMC in response to peptide[a]						
			1	2	3	4	5	6	7
EM026	1:10	HLA-DR12,15	69	83	96	97	98	73	39
EM029	1:40	HLA-DR07,13	86	**112**	**100**	**132**	**159**	**116**	98
EM031	1:320	HLA-DR14,15	**174**	**144**	**120**	**146**	**139**	**140**	50
EM032	1:80	HLA-DR04,15	77	57	**127**	**117**	**109**	75	46
EM033	1:40	HLA-DR03,04	34	25	44	74	79	48	90
EM034	1:40	HLA-DR04,10	99	98	**110**	**228**	**140**	**160**	40

[a] In the IFN-γ assay, a response was considered positive when the IFN-γ concentration in the culture stimulated with peptide was equal to or higher than the mean + 2 standard deviations for the medium control, comprising ≥100 spot-forming cells/10^6 PBMC. Statistically significant responses are indicated in bold.

transporter proteins (OppA and PotF), T3SS proteins (BipD and BopE), heat shock proteins (Hsp60 and Hsp70), the tandem repeat protein Rp1, and flagellin. These results suggested that there were 2 groups of seropositive individuals, those who were relatively high and low responders to *B. pseudomallei*. Meanwhile, $CD4^+$ T cells reactive to purified BipD and BopE have been detected following immunization of mice; however, in murine models, immunization with BipD or other predicted components of the Bsa type III secretion apparatus has not elicited significant protection against acute melioidosis (11, 27). Conversely, recombinant flagellin could provide limited protection in mice, albeit not as great as that when the protein was given as a plasmid DNA vaccine or with specific adjuvants such as CpG (5, 6, 30). Recombinant Hsp, especially Hsp70, can induce T-cell responses due to flagellin contamination (34). It remains possible that other T3SS-, flagellum-, or Hsp-related proteins may induce protective human cell-mediated immunity or that responses to existing antigens characterized to date may be improved rationally by using novel delivery or adjuvant strategies.

In a whole-blood assay, NK cells, $CD4^+$ T cells, and $CD8^+$ T cells from subjects exposed to *B. pseudomallei* respond to heat-killed *B. pseudomallei* by rapidly producing IFN-γ (29). In such assays, it is possible that the response could be cytokine dependent and independent of the TCR pathway. By using a Transwell system to prevent cell contact but permit diffusion of soluble mediators, we observed that the dominant response of the $CD4^+$ T-cell subset from exposed subjects to moDCs pulsed with heat-killed *B. pseudomallei*, LolC, or Rp2 relies on a cell-to-cell contact-dependent pathway.

Selected components of ABC transport systems, especially LolC, have been demonstrated to induce partial protection when mixed with adjuvant and used to immunize mice (18) as well as to induce human antigen-specific T-cell responses (29). Proteins of the tandem repeat family, of which Rp2 is a member, have been examined as a target for B cells to produce antibody in certain parasitic infections (15). We show here, for the first time, that Rp2 elicits human antigen-specific $CD4^+$ T-cell responses at least as effectively as LolC, and it will be of interest to determine if it is similarly effective in inducing partial protection against melioidosis in mice.

We used differential expression of CD45RA and CCR7 to characterize the IFN-γ-producing $CD4^+$ T cells as central memory, effector memory, and the more recently described effector memory RA populations (2). By these criteria, the majority of IFN-γ^+ $CD4^+$ T cells reacting to heat-killed *B. pseudomallei* and LolC were found to be T_{EM} cells (76% and 73%, respectively), with the remaining minority being T_{EMRA} and T_{CM} cells. While T_{CM} cells potentially could provide a rapid function by producing cytokines that aid in the generation of T_{EMRA} cells (14, 33), it is likely that the whole-blood assay involves distinct T-cell interactions from those detected with antigen-pulsed moDCs.

Although $CD4^+$ T cells were the dominant producers of IFN-γ in these assays, heat-killed bacteria and *B. pseudomallei* Hsp60 were also able to stimulate CD8 T cells to secrete granzyme B, an indicator of potential cytotoxic activity. In mycobacterial infections, immunization with mycobacterial Hsp60 induced the cytotoxic function of $CD8^+$ T cells specific for mycobacterial Hsp60 (35).

Having provided the first evidence that the *B. pseudomallei* Rp2 protein is a target of human cell-mediated responses, we sought to identify Rp peptide epitopes and determine if they were recognized by coculture with PBMC of healthy donors of various HLA types living in northeast Thailand. Five of seven predicted MHC class II epitopes of Rp stimulated a significant number of IFN-γ-secreting cells. HLA typing of individuals revealed that 50% of subjects presented heterozygous alleles of HLA-DR04 and 50% of subjects presented heterozygous alleles of HLA-DR15. The most common alleles in the northeastern Thai population include DRB1*1202 and DRB1*1502 (23). Our results indicate that several Rp epitopes elicit strong IFN-γ responses from human cellular immunity across individuals with HLA-DR-restricted alleles in this small sample of the Thai population in an area where *Burkholderia* is endemic.

In conclusion, we evaluated the ability of moDCs pulsed separately with 10 virulence-associated recombinant proteins of *B. pseudomallei* to stimulate autologous human $CD4^+$ T-cell and $CD8^+$ T-cell responses. Our results demonstrate that antigen-specific $CD4^+$ T cells are generated following exposure of humans to *B. pseudomallei* in the environment, as they can be stimulated to produce IFN-γ after encountering moDCs pulsed with heat-killed *B. pseudomallei* or purified proteins, particularly LolC and Rp2. Antigen-specific $CD8^+$ T cells are also produced that are a potential source of IFN-γ and GrB, especially in response to LolC. Our ability to detect human antigen-specific T-cell responses to defined *B. pseudomallei* proteins informs the selection of candidates for inclusion in protein or protein-polysaccharide conjugate subunit vaccines against this important and emerging infection. Moreover, the Rp epitope sequences analyzed here are conserved across *B. pseudomallei*, *Burkholderia thailandensis*, and the causative agent of equine glanders, *Burkholderia mallei*, suggesting that they would have broad vaccine potential for both human and veterinary use for melioidosis and glanders.

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