Programmed Death Ligand 1 on Burkholderia pseudomallei–Infected Human Polymorphonuclear Neutrophils Impairs T Cell Functions

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Programmed Death Ligand 1 on *Burkholderia pseudomallei*-Infected Human Polymorphonuclear Neutrophils Impairs T Cell Functions

Surachat Buddhisa,* Darawan Rinchai,* Manabu Ato, † Gregory J. Bancroft, ‡ and Ganjana Lertmemongkolchai*

Polymorphonuclear neutrophils (PMNs) are terminally differentiated cells that are involved in innate immune responses and form an early line of defense against pathogens. More recently, it has been shown that PMNs have immunosuppressive abilities on other immune cells. However, the effect of PMNs on T cell responses during bacterial infection remains to be determined. In this report, we examined the interaction of PMNs and T cells in response to infection with *Burkholderia pseudomallei*, the causative agent of human melioidosis. We observed that CD4+ T cell proliferation and IFN-γ production in response to polyclonal activators is significantly inhibited by uninfected PMNs, and to a greater extent *B. pseudomallei*-infected PMNs. Programmed death ligand 1 (PD-L1), a known regulator of T cell activation, is increased in mRNA expression in the blood of patients and upon infection of PMNs in vitro. The increased expression of PD-L1 was correlated with the degree of T cell inhibition in individuals with type 2 diabetes, a major risk factor of melioidosis. In vitro, addition of anti–PD-L1 Abs blocked this inhibitory activity and restored proliferation of CD4+ T cells and IFN-γ production, suggesting that PD-L1 on *B. pseudomallei*-infected PMNs is a regulatory molecule for the functions of T cells and may be involved in pathogenesis versus control of melioidosis. *The Journal of Immunology*, 2015, 194: 000–000.

PMNs secrete cytokines that influence CD4+ T cell differentiation into various effector Th subsets. IL-12 produced by PMNs in response to infection can drive Th1 differentiation in a murine model of *Legionella pneumophila* infection (2). In addition they induce the differential of human CD4+ naive T cells into Th17 cells in vitro (3). PMN-derived chemokines or granule proteins also recruit T cells to sites of infection (4, 5). More recently, it has been shown that PMNs have potent immunosuppressive abilities. Peripheral PMNs constitutively express and store large amounts of arginine-1 in their granules. T cell activation can be suppressed by the release of arginase-1 by PMNs in vitro, which causes depletion of L-arginine, a crucial molecule for T cell activation (3, 6, 7). PMNs also secrete mediators such as reactive oxygen species (ROS) that suppress T cell activation, proliferation, and viability (8, 9).

*Burkholderia pseudomallei* is a facultative intracellular Gram-negative bacterium that is the causative agent of melioidosis, an important cause of septic shock and death in Southeast Asia and Northern Australia (10, 11). In Northeast Thailand, the mortality rate of acute melioidosis can surpass 50%, and 9.7% of survivors relapse despite intensive and prolonged antibiotic treatment (12). Protective immunity against *B. pseudomallei* requires effective innate and adaptive immune responses. Experimental models of melioidosis in mice clearly demonstrated PMNs (13) and IFN-γ produced by NK cells and Ag-specific CD4+ T cells are important for protection against *B. pseudomallei* infection (14–16). However, genome array studies in the peripheral blood of patients with melioidosis suggest that T cell functions may be impaired, with underexpression of transcripts related to T cells and cytotoxic cells. In contrast, overexpression of transcripts corresponding to PMN functions including the abundance of immature neutrophils (e.g., DEF1A, DEF3A, FALL-39) and genes encoding neutrophil cell-surface markers (such as ITGAM [CD11b], FCGR1 [CD64], CD62L, and CSF3R) was observed in blood of patients with sepsis compared with uninfected control subjects (17). We believe that PMNs may have multiple cytokine or cell contact–mediated immune-regulatory interactions with other cells of the immune response during *B. pseudomallei* infection, but to date this has not been investigated.

The PD-1 (programmed death 1)/programmed death ligand (PD-L) pathway has been implicated as an important regulator of effector T cell functions. Interaction of PD-1 on T cells with its ligands, PD-L1/PD-L2, delivers inhibitory signals that impair many T cell functions, including proliferation, cytotoxic activity, and cytokine production (18–20). Recent findings demonstrated that...
the PD-1/PD-L1 pathway has a crucial role in the interaction between host and pathogenic microorganisms such as intracellular bacteria and viruses that evolved to resist immune responses. In tuberculosis, the PD-1/PD-L1 pathway suppresses *Mycobacterium tuberculosis*–specific IFN-γ production and cytotoxicity by T cells from TB patients (18). In HIV-1 infection, there is evidence that this pathway also suppresses IFN-γ production by T cells from HIV patients (21). Some studies have suggested a pathological role for PD-1/PD-L1 by reducing microbial clearance and innate inflammatory responses, but accelerating T cell apoptosis in sepsis (22–24). PD-L1 is reportedly expressed on a variety of different cell types, both hematopoietic and nonhematopoietic cells, including T cells and myeloid cells such as dendritic cells and monocytes, and its expression is highly upregulated during inflammatory states (20). Moreover, PD-L1 is overexpressed on neutrophils in blood of patients with HIV-1 infection (21) and active tuberculosis (25), and is associated with reduced success of therapy. A recent study has reported that PD-L1 is also expressed on some neutrophils and inhibits T cell function via the PD-1/PD-L pathway, after injection of a low dose of bacterial LPS (26).

The fact that mortality from *B. pseudomallei* infection remains high despite the use of antimicrobial agents that are highly active against organisms suggests that defects in host immunity may contribute to this pathogenesis. Our objective in this study was to determine the expression of PD-L1 on PMNs in response to *B. pseudomallei* and ask whether this has any effect on T cell functions.

### Materials and Methods

#### Human subjects

This study was approved by Khon Kaen University ethics committee for human research. Informed consent was obtained for all subjects. A total of 91 patients who were suspected of community-acquired sepsis at Khon Kaen Hospital, Thailand, were enrolled in this study. Clinical blood samples for the study and bacteria culture were collected within 24 h after the diagnosis of suspected sepsis. Whole blood (10 ml) was collected, of which 7 ml was added to lithium-heparin tubes (BD Biosciences) and 3 ml was added to Tempus tubes containing RNA stabilization solution (Applied Biosystems). The diagnosis of sepsis was defined as having a systemic inflammatory response syndrome with positive blood culture for microorganisms (27). Blood samples from control subjects and septicemic patients included 47 with sepsis (23 patients with septicemic melioidosis and 24 patients with sepsis caused by other microorganisms) and 44 uninfected control subjects (17 subjects with type 2 diabetes [T2D] and 27 healthy subjects). Demographic, clinical, and microbiological data were recorded for all subjects.

#### RNA preparation and processing for microarray

Total RNA was isolated from whole-blood lysates using the Tempus Spin Isolation kit (Applied Biosystems) according to the manufacturer’s instructions. RNA integrity numbers were assessed on an Agilent 2100 Bioanalyzer (Agilent). Labeled cRNA was hybridized overnight to Sentrix Human-6 V2 BeadChip arrays (Illumina), washed, blocked, stained, and...
scanned on an Illumina BeadStation 500 following the manufacturer’s protocol. More details were previously published (17).

Isolation of peripheral blood populations

Human PMNs, PBMCs, and CD4+ T cells were isolated from heparin-anticoagulated venous blood from healthy and diabetic subjects using the previously reported criteria and methods (28). In brief, PMNs were isolated by 3% Dextran T-500 sedimentation (Amersham) and separated by Ficoll-Hypaque density gradient centrifugation (Sigma Aldrich). PBMCs were collected, followed by hypotonic lysis to remove RBCs, and also isolated by Ficoll-Hypaque density gradient centrifugation. Purity of PMNs was >95%, as measured by differential count after Giemsa staining, and >99% viability, as determined by trypan blue exclusion assay. CD4+ T cells were isolated from PBMCs by positive selection using anti-CD4–coated magnetic beads (Miltenyi Biotec). Purity of isolated CD4+ T cells was >98% as analyzed by flow cytometry. Then PMCs and CD4+ T cells were labeled with CFSE (Molecular Probes Invitrogen) according to the manufacturer’s protocol.

Coculture of T cells with B. pseudomallei–infected PMNs

*B. pseudomallei* strain K96243 (29), *Escherichia coli* strain ATCC 25922, and *Salmonella typhimurium* strain ATC 13311 were cultured for 18 h at 37˚C in Luria-Bertani broth, washed twice with PBS, and resuspended in medium containing 10% FBS (BioWest). The number of bacteria was determined by measuring the OD at 600 nm. Uninfected, *B. pseudomallei*–, *E. coli*–, and *S. typhimurium*–infected PMNs were generated by exposing purified PMNs to either medium alone or live bacteria at a multiplicity of infection (MOI) of 3 for 30 min at 37˚C in 5% CO2. Extracellular bacteria were killed by 250 µg/ml kanamycin (Life Technologies) for 30 min. The cells were then washed twice in PBS and resuspended in medium containing 10% FBS, 25 µg/ml kanamycin, 100 µg/ml penicillin-streptomycin (Life Technologies), and 100 µg/ml gentamicin (Sigma-Aldrich).

CFSE-labeled PBMCs or CD4+ T cells were stimulated with PHA (Biochrom AG), or plate-bound anti-CD3 Ab (3 µg/ml; Pharmingen) and soluble anti-CD28 Ab (1 µg/ml; Pharmingen). CFSE-labeled PBMCs or CD4+ T cells (1 × 10^5) were cultured either alone or with variable numbers of uninfected or *B. pseudomallei*–infected autologous PMNs (PBMC or CD4+ T cell/PMN ratios are 1:1, 1:2, and 1:4). After 18 h, T cell and PMN apoptosis and PD-L1 expression on PMNs were determined by flow cytometry (as described later). Proliferation was investigated after coculture at 37˚C in 5% CO2 for 3 d by flow cytometry (gating strategy of CD4+ T cell proliferation is described in Supplemental Fig. 1). In other experiments, catalase (300 U/ml; Sigma-Aldrich) or N ω-hydroxy-nor-L-arginine (nor-NOHA, 30 µg/ml; Merck) was added into CFSE-labeled PBMCs–PMNs coculture. In different experiments, CFSE-labeled CD4+ T cells were incubated with or without blocking Ab against PD-L1 (1 µg/ml, MIH1; eBioscience) or isotype control Ab (10 µg/ml; eBioscience). After 3 d, T cell proliferation was investigated by flow cytometry and IFN-γ production by ELISA.

**FIGURE 2.** *B. pseudomallei* (Bp)-infected PMNs inhibit Ag-specific T cell responses. PBMCs from healthy subjects were stimulated with *B. pseudomallei* peptide in the presence of uninfected PMNs or *B. pseudomallei*–infected PMNs for 72 h; then IFN-γ producing CD4+ T cells were assessed by flow cytometry. A representative example of PMNs inhibiting specific T cell response by IFN-γ production is shown.
Transwell assay

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 mediators through the membrane but prevents contact interaction of the cells. CFSE-labeled PBMCs (5 × 10³) were plated in the lower chamber, and B. pseudomallei–infected PMNs were plated onto the upper chamber (PBMCs/PMNs, 1:2) in the presence of PHA (0.3 μg/ml). CD4⁺ T cell proliferation was investigated after coculture for 3 d as described earlier.

Flow cytometry

CFSE-labeled PBMCs or CD4⁺ T cells–PMNs cocultured in the presence of stimuli were stained for CD3 (Caltag), CD4 (Caltag), PD-L1 (eBioscience), and annexin V (eBioscience) expression using mAbs. Proliferation of T cells was determined by diluted CFSE content of CFSE⁺CD3⁺ CD4⁺ cells in the lymphocyte gate. In all samples, 10⁵ cells were acquired by FACSCalibur flow cytometer (BD Biosciences) and analyzed by FlowJo software (Tristar).

Statistical analysis

Statistical analysis was performed using tests appropriate to the dataset, as specified in the figure legends, using GraphPad Prism 5 software (GraphPad, San Diego, CA). The p values ≤0.05 were considered statistically significant.

Results

B. pseudomallei–infected PMNs inhibit CD4⁺ T cell functions

CD4⁺ T cells and PMNs are each key components of the immune response against B. pseudomallei, but their potential interaction has not been studied. To examine the effect of B. pseudomallei–infected PMNs on polyclonal T cell functions, we cocultured PBMCs with PHA either alone or in cultures with uninfected PMNs or B. pseudomallei–infected PMNs at different ratios. The addition of B. pseudomallei–infected PMNs significantly reduced CD4⁺ T cell proliferation compared with PBMCs alone or uninfected PMNs in a dose-dependent manner (Fig. 1A and 1B). Because there was variation in CD4⁺ T cell proliferation in response to PHA between individual donors, the percentage inhibition of proliferation was then analyzed. The results show that uninfected PMNs showed moderate inhibition of CD4⁺ T cell proliferation, whereas B. pseudomallei–infected PMNs showed a robust effect in all individuals, with an average of 48.9%, and up to 79% inhibition of proliferation in some donors (Fig. 1C). Moreover, the inhibition of CD4⁺ T cell proliferation by B. pseudomallei–infected PMNs was higher than other Gram-negative intracellular bacteria, such as S. typhimurium–infected PMNs (Supplemental Fig. 2). This was not due to differences in bacterial burden (data not shown). The suppressive effect of B. pseudomallei–infected PMNs was also observed in isolated CD4⁺ T cells activated by anti-CD3/CD28, which excluded a role for other leukocytes such as monocyte or B cells in this effect (Fig. 1D).

Because B. pseudomallei–infected PMNs had an effect on proliferation of CD4⁺ T cells, we also studied the result of infected PMNs on T cell cytokine production. Secreted IFN-γ was measured in culture supernatant after 72 h of coculture period. IFN-γ production was significantly decreased in cultures with uninfected or B. pseudomallei–infected PMNs compared with that in PBMCs or isolated CD4⁺ T cells culture alone (Fig. 1E and 1F). To test whether infected PMNs also inhibited Ag-specific T cell responses, we stimulated PBMCs with B. pseudomallei peptide in the presence of either uninfected PMNs or B. pseudomallei–infected PMNs, and the frequency of IFN-γ-producing CD4⁺ T cells was assessed by flow cytometry after 72 h. The results showed that both uninfected and B. pseudomallei–infected PMNs inhibit IFN-γ production by CD4⁺ T cells in response to a specific activator (Fig. 2). The inhibitory effect of PMNs in both groups was not explained by induction of lymphocyte or PMN apoptosis, because both PBMCs culture alone and cocultured with uninfected or B. pseudomallei–infected PMNs showed similar amounts of lymphocyte and PMN apoptosis in our cocultures (Supplemental Fig. 3). These data indicate that B. pseudomallei–infected PMNs have an inhibitory effect on both CD4⁺ T cell proliferation and IFN-γ production.

Inhibitory effect of B. pseudomallei–infected PMNs on CD4⁺ T cell function occurs by both contact-dependent and -independent mechanisms

To determine the mechanism(s) by which B. pseudomallei–infected PMNs inhibit CD4⁺ T cell proliferation, we used a Transwell system to investigate whether this required direct cell–cell interaction. PBMCs were cocultured with uninfected PMNs or B. pseudomallei–infected PMNs as described earlier or in a Transwell system in which PMNs were separated from PBMCs by a membrane, which allowed diffusion of soluble factors but prevented cell contact. In this system, B. pseudomallei–infected PMNs lost their suppressive capacity by 56% compared with that of PMNs that were not separated (Fig. 3A), indicating that cellular contact is at least partially needed between PMNs and T cells. Decreased CD4⁺ T cell proliferation was also partially contact independent, suggesting that the suppression can also be mediated by soluble factors released by PMNs. Other studies have previously shown that PMN-derived ROS and arginase-I could suppress T cell proliferation and cytokine production. To assess the contribution of ROS and arginase-I inhibition on T cell proliferation in response to B. pseudomallei, we added arginase inhibitor nor-NOHA or catalase (which catalyzes the decomposition of hydrogen peroxide to water and oxygen) to our coculture system. The result shows that addition of catalase, but not nor-NOHA, partially restored T cell proliferation (Fig. 3B).

T cell suppression by B. pseudomallei–infected PMNs is also mediated by the PD-L1/PD-1 pathway

We then addressed the possible mechanisms of contact-dependent effects of PMNs on T cell functions. The PD-1 pathway reportedly suppresses T cell proliferation and effector functions, through binding of PD-1 and its ligands (PD-Ls). To test whether B. pseudomallei can induce this regulatory pathway, we exposed freshly isolated PMNs of healthy subjects to live B. pseudomallei at an

![FIGURE 3](http://www.jimmunol.org/)
MOI of 3 for 30 min, then cultured them in medium alone, or with PBMCs, or with CD4+ T cells, and expression of PD-L1 on PMNs was investigated. *B. pseudomallei* could induce PD-L1 expression on PMNs as measured by flow cytometry (Fig. 4A), which was especially apparent on annexin V+ PMNs (Supplemental Fig. 4). Incubation with *B. pseudomallei* increased PD-L1 expression on PMNs after 12 h as compared with freshly isolated PMNs (baseline) or uninfected PMNs and reached a highest level at 18 h postinfection (Fig. 4B). Moreover, rapid expression of PD-L1 on PMNs after 6 h was observed after coculture with PBMCs as compared with freshly isolated PMNs (Fig. 4B and 4C).

To test whether the interaction of PD-1 on T cells and PD-L1 on PMNs was responsible for the inhibitory actions of infected PMNs in these cocultures, we incubated isolated CD4+ T cells with anti-CD3/CD28 in the presence or absence of blocking mAbs against PD-L1 and CD4+ T cell proliferation, and IFN-γ production was measured after 72 h. Blocking PD-L1 significantly restored both CD4+ T cell proliferation (Fig. 4D) and IFN-γ production (Fig. 4E) in the presence of *B. pseudomallei*-infected PMNs.

*FIGURE 4.* Suppressive effect of *B. pseudomallei*-infected PMNs on T cell function is mediated by the PD-L1/PD-1 pathway. (A) Uninfected and *B. pseudomallei*-infected PMNs were cocultured in medium alone or with PBMCs or with isolated CD4+ T cells. Cells were stained for PD-L1, assessed by flow cytometry and gated on live PMNs. A representative example of PD-L1 expression on uninfected PMNs and *B. pseudomallei*-infected PMNs is shown. (B) PD-L1 expression on PMNs cultured alone in medium, and PMNs-PBMCs cocultured in the presence of PHA after exposure to *B. pseudomallei* for 6, 12, 18, and 24 h (*n* = 5 individual donors). Data represent the mean ± SEM, and statistical significance was determined using paired *t* test. (C) PD-L1 expression on uninfected and *B. pseudomallei*-infected PMNs for 18 h. *n* = 8 individual donors. Data represent the mean ± SEM, and statistical significance was determined using paired *t* test. (D) Isolated CD4+ T cells were cocultured with uninfected PMNs and *B. pseudomallei*-infected PMNs in the conditions of absence (medium) or presence of isotype control Ab (10 μg/ml) or anti–PD-L1 (1 μg/ml). After 72 h, anti-CD3/CD28–induced T cell proliferation was measured by flow cytometry (*n* = 5 individual donors). Data represent the mean ± SEM from each Ab. Statistical significance was determined using paired *t* test (*p* < 0.05, **p** < 0.01, ***p*** < 0.001).

Because diabetes has been identified as the strongest risk factor of melioidosis, we investigated the expression of PD-L1 at the transcript level by microarray analysis of whole blood of diabetic patients with sepsis caused by *B. pseudomallei* and other bacteria. The results show that PD-L1 was significantly upregulated in whole blood of diabetic patients with sepsis caused by either...
B. pseudomallei or other bacteria when compared with nondiabetic patients or uninfected control subjects (Fig. 6A) (Table I).

Next, we investigated the expression of PD-L1 on surface of PMNs in response to B. pseudomallei infection in vitro. PD-L1 expression was significantly increased on PMNs from T2D patients after B. pseudomallei stimulation for 18 h when compared with healthy control subjects (Fig. 6B) and correlated with inhibition of T cell proliferation (Fig. 6C and 6D). Blocking PD-L1 on PMNs significantly restored CD4+ T cell proliferation (Fig. 6E). These data suggest that PD-L1 expressed on B. pseudomallei-infected PMNs play a role in T cell suppression in T2D.

Discussion
PMNs play an important protective role during innate immune responses against invading pathogens. However, there is increasing interest in the potential immunosuppressive activity of human PMNs on cells of the adaptive immune response (7, 8, 21). In this study, we demonstrated that the interaction of uninfected PMNs and T2D patients after B. pseudomallei stimulation for 18 h when compared with healthy control subjects (Fig. 6B) correlated with inhibition of T cell proliferation (Fig. 6C and 6D). Blocking PD-L1 on PMNs significantly restored CD4+ T cell proliferation (Fig. 6E).

In various murine models of cancer and sepsis, neutrophils were found to be part of myeloid-derived suppressor cells. This heterogeneous group of cells has suppressive effects on lymphocyte activation via arginase-I– or ROS-dependent mechanisms (30). In humans, arginase-I– or ROS-mediated suppression of lymphocytes has also been reported (3, 8). In our study, the suppressive neutrophils generated after exposure to B. pseudomallei inhibited T cell proliferation partially via production of ROS, whereas arginase-I was not involved. Several mechanisms have been suggested for T cell suppression by ROS, including changes in NF-kB activation and oxidation of the actin remodeling protein coflin (31, 32). This is consistent with a report of suppression of CD4+ T cells in response to protein Ags in adjuvants (33). Instead, we found a predominant role for the PD-1/PD-L1 pathway in response to B. pseudomallei. In other systems, PD-1 interacts with its ligand PD-L1 to deliver inhibitory signals that regulate T cell and other responses, thus helping to maintain the balance among effective immunity, tolerance, and immunopathology (20). We observed rapid induction of PD-L1 expression on PMNs after exposure to live B. pseudomallei in vitro. Furthermore, Ab-mediated blockade of PD-1/PD-L1 interactions completely and partially restored T cell proliferation and IFN-γ responses, respectively.

The mechanism(s) leading to the upregulation of PD-L1 in B. pseudomallei infection is likely mediated by a combined effect of several factors. The rapidity of the response suggests that PD-L1 is directly induced by the pathogen, consistent with previous reports in which inactivated HIV-1 virions directly upregulate neutrophil PD-L1 expression (21). Macrophages, dendritic cells, and neutrophils can be induced to express PD-L1 after engagement of TLR ligands LPS and R848 to their receptors TLR4 and TLR8 (21). PD-L1 upregulation on B. pseudomallei–infected PMNs might be explained by B. pseudomallei expressing LPS that signals through TLRs (34). However, other factors may contribute to this process. IFN-γ, a cytokine produced and secreted by inflammatory cells in response to infection, has been shown to increase expression of PD-L1 on various cells (20) and also neutrophils (26). This observation was supported by our finding in vitro of greater PD-L1 expression on PMNs after coculture with PBMCs than PMNs alone, where IFN-γ is produced and secreted by T cells in response to polyclonal activator. We have shown that IL-27 could be produced by PMNs during bacterial infection (35), and IL-27 priming of naive T cells upregulated expression of PD-L1 (36), suggesting that IL-27 from B. pseudomallei–infected PMNs may also contribute to PD-L1 expression on PMNs.

Numerous studies on PD-1/PD-L1 have focused on the role of this pathway in chronic diseases or stimulations including persistent infections, tolerance, and cancer (20). However, our findings of reduced T cell responses mediated by PD-L1 on B. pseudomallei–infected PMNs are consistent with the increasing involvement of this pathway in regulating antimicrobial responses to acute infections (24, 37). This is strongly supported by a report demonstrating that IFN-γ–stimulated neutrophils after acute inflammatory responses suppress T cell function via their expression of PD-L1 on neutrophils (21). In other systems, the key role of PD-1/PD-L1 pathway in T cell suppression has led to development of strategies to manipulate the interaction of PD-1 and PD-L1 for the reversal of exhausted T cells and improvement of pathogen control. In mice, blocking the PD-1 pathway with anti–PD-L1 Ab restored cytokine production, augmented the generation of LCMV-specific T cells, and led to a dramatic reduction in viral load (38). In addition to viral infections,
blockade of the PD-1 pathway decreased mortality in clinically relevant animal models of bacterial sepsis (24).

Patients with diabetes mellitus have an increased risk for development of infections and sepsis (39), including melioidosis (10). Our previous studies and those of others in diabetes mellitus patients have described defects in PMN phagocytosis, migration, and oxidative burst responses to kill *B. pseudomallei* (28), as well as reductions in proinflammatory cytokines (40) and dysfunction in Th1 cell responses to *B. pseudomallei* infection (41). We report in this article that increased PD-L1 expression on *B. pseudomallei*–infected PMNs correlated with increasing T cell dysfunction in type 2 diabetic patients. These data suggest that increased PD-L1 expression on *B. pseudomallei*–infected PMNs from type 2 diabetic patients than healthy individuals may be associated with increased susceptibility to infection in diabetic patients. In contrast, PMN-mediated reduction of T cell–derived IFN-γ via expression of PD-L1 may be of some benefit in diabetes patients, by reducing the excessive tissue damage caused by excessive proinflammatory responses, and contributing to the reduced mortality of diabetic patients with melioidosis (42).

In conclusion, our study indicates that *B. pseudomallei* infection can impair efficient T cell responses by promoting PD-L1 expression on PMNs. We show that expression of PD-L1 on PMNs can be induced by *B. pseudomallei* and that interaction of PD-L1 expressed on either uninfected or infected PMNs with PD-1 on T cells results in inhibition of T cell proliferation and cytokine production. Our data suggest that neutrophils recruited to the site of *B. pseudomallei* infection have the potential to inhibit potentially protective T cell responses in these lesions even if they do not contain *B. pseudomallei*, but this is even more potent after their uptake of live bacteria. This may explain why elimination of this organism is so difficult and is consistent with the presence of both lymphocytes and neutrophils in human lesions of individuals who have died of this disease where the bacterial growth has clearly not been controlled. This study enhances our understanding of T cell dysfunction in *B. pseudomallei* infection and highlights the need to consider targeting PD-1/PD-L1-immuno-
suppressive pathways in novel therapeutic approaches for patients with *B. pseudomallei* infection.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


Supplemental Figure S1: CD4$^+$ T cell proliferation assay gating strategy. 

A, after 3 days of co-culture, lymphocytes were gated on the basis of their FSC/SSC. 

B, CD4$^+$ T cells were selected based on CD3 and CD4 positive cells. 

C, the percentage of CD4$^+$ T cell proliferation was selected versus that of unstimulated CD4$^+$ T cells. All gates were identical in all samples within each experiment.
**Supplemental Figure S2: Bacterial infected PMNs inhibit CD4\(^{+}\) T cell proliferation.** Purified PMNs of healthy donors were exposed to *B. pseudomallei*, *E. coli* or *S. typhimurium* at MOI of 3 for 30 minutes, then co-cultured with PBMCs in the presence of 0.3 µg/ml PHA for 3 days. Effect of *B. pseudomallei* and other bacteria infected PMNs on inhibition of CD4\(^{+}\) T cells proliferation in 1:2 PBMCs: PMNs culture condition was analyzed by flow cytometry. Data represents the mean ± SEM of percentage inhibition of CD4\(^{+}\) T cell proliferation. Statistical significance was determined using unpaired t test (*p < 0.05, **p < 0.01).
Supplemental Figure S3: Lymphocyte apoptosis in *B. pseudomallei* infected PMNs cultures. Uninfected and *B. pseudomallei* infected PMNs were added to PBMC at 2:1 ratio in culture conditions for T cell proliferation. At 18 h of co-culture, lymphocyte and PMNs were stained with annexin-V, and apoptosis was measured by flow cytometry (n=8 individual donors). Horizontal line indicates the mean ± SEM. Statistical significance was determined using paired *t* test (ns, non significant).
Supplemental Figure S4: PD-L1 expression on live PMNs. Uninfected and *B. pseudomallei* infected PMNs were co-cultured in medium alone or with PBMCs. PD-L1 expression on live and apoptotic PMNs was measured by flow cytometry after 18 h of cultured. Data represents the mean ± SEM of MFI. Statistical significance was determined using paired t test (*p < 0.05, **p < 0.01, ***p < 0.001).