Production of interleukin 27 by human neutrophils regulates their function in response to bacterial infection

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Abstract

Septicemia is the most severe form of melioidosis caused by the Gram-negative bacterium, *Burkholderia pseudomallei*. Here, we showed that levels of IL-27p28 transcript and protein were both significantly elevated in patients with sepsis, particularly melioidosis and in patients with unfavorable disease outcome. Moreover, monocytes/macrophages and neutrophils were the major source of IL-27 during infection. Addition of exogenous IL-27 in vitro resulted in significantly increased bacterial survival, reduced *B. pseudomallei*-induced oxidative burst and enhanced IL-1beta and TNF-alpha production by purified neutrophils from healthy subjects. Finally, blockade of endogenous IL-27 in neutrophils using soluble IL-27 receptor antagonist prior to infection led to significantly reduced survival of bacteria and decreased IL-1beta, but not TNF-alpha production. These results indicate a potential role of IL-27 in suppression of antibacterial defense mechanisms that might contribute to disease severity in sepsis. The targeting of this cytokine may be beneficial in the management of human sepsis.

Keywords

IL-27; neutrophil; sepsis; Burkholderia pseudomallei; melioidosis

Introduction

Sepsis is a clinical syndrome with a severe infection in the body and bloodstream that most commonly originates in the lung, urinary tract, and abdomen. Severe sepsis causes systemic inflammatory response with infection and organ dysfunction [1, 2]. The mortality rate of patients with septicemia is particularly high, reaching up to 60–80% [3, 4]. Neutrophils and macrophages are the first line of innate immune response and elicit pro-inflammatory cytokine response via pathogen-associated molecular pattern receptors. This is a critical role

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in eradication of invading microorganisms. If these immune cells fail to control the infection, microorganisms may reach the bloodstream inducing an overwhelming systemic immune response via the production of pro-inflammatory mediators leading to shock or multi-organ failure [1, 2, 5, 6].

In Southeast Asia and Northern Australia the Gram-negative bacillus, *Burkholderia pseudomallei* is an important cause of community-acquired sepsis (septicemic melioidosis) [7, 8]. It commonly presents with pneumonia, and is frequently associated with bacterial dissemination to distant sites. Identifying high-risk patients with sepsis is a great challenge in the care of critically ill patients [9, 10]. Our previous study has identified blood biomarkers for human septicemic melioidosis by whole genome transcriptional profiling [11]. The majority of changes observed were genes related to inflammation, interferon response, neutrophils, cytotoxic cells and T cells. Of interest, this genome-wide screen identified interleukin 27 (IL-27) as being differentially expressed between controls and septicemic patients. Indeed, many other cytokines have been shown to be critically involved in both protective and pathogenic antimicrobial immune responses [5]. However, detailed mechanisms and host factors that determine the immune response during sepsis are still incompletely understood. As an imbalance in cytokine responses may result in persistent infections or destructive systemic inflammatory response leading to multi-organ failure and death [1, 5], a detailed understanding of local cytokine function during infections is of potential use as a therapeutic target in sepsis.

Interleukin 27 (IL-27) is a member of IL-12 cytokine family with heterodimeric subunits, compose of Epstein-Barr virus-induced gene 3 (EBI3), and p28 subunits with some similarities to IL-12p35 and IL-23p19, respectively [12, 13]. Antigen presenting cells such as dendritic cells (DCs), monocytes and macrophages have been identified as rapid sources of IL-27 subunits after LPS, Poly I:C and CpG activated Toll-like receptor (TLR) ligation [14–16], suggesting that IL-27 may act very early in Th1-mediated immunity [14]. IL-27 mediates its biological function via binding to a specific receptor on target cells consisting of WSX-1/TCCR and glycoprotein (gp) 130 [17], activated signal via Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) and mitogen activated protein kinase (MAPK) signaling, following NF-κB activation [14]. IL-27 receptor (IL-27R) is commonly expressed on DCs, macrophages, monocytes, B cells and highly expressed on naïve T cells, thus previous studies have primarily concentrated on the biological of IL-27 in T cells and adaptive immune response. Recently, it has been reported that IL-27R is also expressed on granulocytes (mast cells, eosinophil and neutrophils) [5, 17], indicating that IL-27 function is not restricted to T cells. In a murine caecal ligation and puncture model of peritonitis and sepsis, IL-27 acts as a negative regulator of innate immune responses by suppressing the oxidative burst production and the migration of granulocytes. Blockade of IL-27 function by antibody neutralization increased the survival rate of these septic mice [5]. However, it is not clear what role IL-27 may play in human septicemic melioidosis or other forms of sepsis.

Here, we report that IL-27 is clearly induced in human sepsis and it can regulate neutrophil responses to *B. pseudomallei* infection in vitro. This study provides the first evidence from human patients to support the potential of anti-IL-27 therapy in the management of sepsis.

Results

IL-27 transcript and protein were elevated and associated with disease severity in sepsis

Given our previous observation in transcriptional profiling of human sepsis [11], we measured IL-27 mRNA abundance in the blood of 24 patients with sepsis caused by *B. pseudomallei* and other bacteria. The expression of IL-27p28 and EBI3 subunits was

markedly elevated in patients with sepsis as compared to uninfected controls, including recovery samples and samples from patients with type 2 diabetes (T2D), which is a risk factor for sepsis (Figures 1A and 1B).

We then measured IL-27p28 mRNA levels by quantitative real time PCR to confirm the microarray data, supported by analysis of IL-27p28 protein levels in plasma samples of 90 septic patients and 17 uninfected controls. We found that the levels of IL-27p28 protein correlated with the degree of clinical severity in patients with sepsis (Supplement Figure S1). These parameters mainly were septic shock and organ dysfunction (e.g. renal or liver) [18]. Furthermore, blood samples from patients who did not survive, showed higher IL-27p28 protein concentrations than those who survived (Mann-Whitney test, $p < 0.001$) and similar results were observed in septic patients caused by other pathogens (Mann-Whitney test, $p < 0.05$) (Figure 1C). These results suggested that plasma IL-27p28 protein concentrations might not be specific to *B. pseudomallei* infection but rather associated with severity in human sepsis and melioidosis.

B. pseudomallei induced IL-27 production in whole blood and monocyte derived dendritic cells in vitro

To determine the cell types, which can produce IL-27 in response to *B. pseudomallei*, whole blood samples from a representative healthy subjects were infected with live *B. pseudomallei* with LPS as the positive control or with medium alone. The results revealed that production of IL-27p28 protein increased over the time, and the peak response was seen at MOI of 10 within 24 h of incubation (Figure 2A). We confirmed this observation in whole blood samples of 17 healthy subjects (Figure 2B). The results showed that *B. pseudomallei* significantly induced IL-27p28 production in all samples (Mann-Whitney, $p < 0.001$) and of a similar magnitude to that induced by *E. coli* LPS.

In other infections, dendritic cells (DCs) are the major source of IL-27 [19, 20]. Next, we investigated whether *B. pseudomallei* induced IL-27 production by human monocyte derived dendritic cells (MoDCs). The results showed that LPS or killed *B. pseudomallei* could induce MoDCs to produce IL-27p28 after culture for 18 h (Figure 2C) and live *B. pseudomallei* clearly also induced IL-27 production in a dose dependent manner (Figure 2D). Taken together, the findings showed that *B. pseudomallei* induced IL-27 production in whole blood and MoDCs in vitro.

Monocytes/macrophages and neutrophils were the major cellular sources of IL-27 in response to *B. pseudomallei*

To further define the cellular sources of IL-27, whole blood samples from 3 healthy subjects were incubated with medium alone or infected with live *B. pseudomallei* at a MOI of 10 for 6 h. Then neutrophils and CD14+, CD3+, CD4+ and CD8+ cells were isolated as described above in Materials and Methods. The results showed induction of mRNA for both *IL-27p28* and its receptor (*WSX-1*) on CD14+ cells and neutrophils by reverse transcriptase PCR (Figure 3A). The results from another subjects also showed increased expression of *IL-27p28* in neutrophils which was at a similar level to CD14+ (monocytes/macrophages) cells by real-time PCR, whereas *IL-27p28* and *EBI3* transcripts could not be observed above the medium control in CD4+ and CD8+ T cells (Figure 3B).

To ask whether the neutrophil response occurred directly following the uptake of *B. pseudomallei* or indirectly via other interactions within whole blood, purified neutrophils from ten healthy subjects were stimulated with LPS, live *B. pseudomallei*, *E. coli* and *S. aureus* for 6 h. When transcript levels of IL-27 subunits (*p28* & *EBI3*) and IL-27 receptor (IL-27R) subunits (*WSX-1* & *gp130*) were determined by real time-PCR, all *IL-27* and

IL-27R transcripts were found to be expressed at the average levels higher than 2-fold relative expression above the medium control whereas only *IL-27p28* transcript of *S. aureus* stimulated neutrophils was lower than that (Figure 3C). In addition, the supernatants from the same experiment with six healthy subjects confirmed that neutrophils were the cellular source of IL-27 and could be directly induced by *B. pseudomallei* and other Gram-negative bacteria (*E. coli*) but not by *S. aureus* infection (Figure 3D). Taken together, our results indicated for the first time that human neutrophils as well as monocytes/macrophages could express both IL-27 and IL-27 receptor in response to *B. pseudomallei*.

Effect of exogenous rIL-27 on neutrophil functions

To determine whether IL-27 could affect neutrophil function, purified neutrophils from seven healthy subjects were pretreated with 0–300 ng/ml of rIL-27 at 37°C for 30 min prior to infection with live *B. pseudomallei*. Addition of rIL-27 increased the number of intracellular bacteria surviving in neutrophils in a dose dependent manner (Figure 4A). Moreover, the oxidative burst of granulocytes in whole blood from five healthy subjects treated with rIL-27 before *B. pseudomallei* infection or LPS activation was significantly reduced (Figure 4B). The similar results were observed when neutrophils were treated with chloroquine (CQ) to reduce oxidative burst or reactive oxygen species-ROS (Supplement Figure S2) suggesting that IL-27 enhanced survival of *B. pseudomallei* in neutrophils at least in part by suppression of ROS production. When pro-inflammatory cytokines were assayed in culture supernatants from *B. pseudomallei* infected neutrophils, we found that exogenous IL-27 treatment clearly enhanced IL-1β and TNF-α production in a dose dependent manner (Figure 4C). IL-10 transcript and protein levels could not be detected in any *B. pseudomallei* infected human neutrophils (data not shown). Meanwhile, anti-IL-10 pretreatment did not affect ROS production and bacteria survival in human neutrophils suggesting that IL-10 has no role in this system (Supplement Figure S3).

IL-27 binds to its receptor and signals to the activation of Janus kinase (JAK)/signaling transducer and activation (STAT) in T cells and macrophages [21, 22]. Purified neutrophils were pretreated with tyrphostin AG490 (a JAK2 inhibitor that could affect both STAT1 and STAT3) before infection with *B. pseudomallei*. Blocking this pathway resulted in the reduction of oxidative burst production and enhanced the survival of intracellular bacteria (Supplement Figure S3).

Taken together, these results suggest that IL-27 could be produced and directly act on neutrophils, affecting both oxidative burst and cytokine production, and subsequently reducing their antibacterial activity.

Effect of neutralization of endogenous IL-27 on neutrophil function

To test whether neutralization of endogenous IL-27 could affect neutrophil activity, we used a high affinity WSX-1 soluble receptor (sIL-27RA) to block the biological activity of endogenous IL-27 induced by *B. pseudomallei* infection. Purified neutrophils were treated with 0–10 μg/ml of sIL-27RA at 37°C for 30 min prior to infection with live *B. pseudomallei*. The result showed that addition of sIL-27RA reduced the number of bacteria surviving in neutrophils in a dose dependent manner (Figure 5A). Cytokine analysis of culture supernatants collected from sIL-27RA pretreated and *B. pseudomallei* infected neutrophils of four subjects revealed that IL-1β, but not TNF-α production was significantly inhibited by sIL-27RA (ANOVA and Dunnett's post test, $p < 0.05$).

Furthermore, the effect of caspase I inhibition and anti-IL1R on ROS production or intracellular bacterial survival in human neutrophils revealed that blocking this pathway affected both bacterial survival and oxidative burst (Supplement Figure S4). Thus, IL-27

induced in response to *B. pseudomallei* actively regulates bacterial killing and IL-1β production by human neutrophils.

Macrophage and neutrophils were the major cellular source of IL-27 in patients with sepsis

Our next objective was to identify the main IL-27 producing cells directly ex vivo from patients with sepsis caused by *B. pseudomallei* or other bacteria. In the absence of an efficient antibody for intracellular IL-27, we examined the transcriptional expression of IL-27 by real time PCR. Firstly, neutrophils and CD14^{+} cells were isolated from fresh blood samples from 15 septicemic patients caused by Gram-positive (n=6) and Gram-negative bacteria (n=9, only one caused by *B. pseudomallei*) on the day of admission compared to healthy subjects (n=10) and analyzed for *IL-27* (*p28 & EBI3*) and *IL-27R* (*WSX-1& gp130*) expression by real-time PCR. The results revealed that *IL-27p28* mRNA expression of isolated CD14^{+} cells (Figure 6A) and neutrophils (Figure 6B) from sepsis caused by Gram-negative bacteria was significantly higher than healthy subjects (ANOVA and Dunnett's post test and Tukey's post test, $p < 0.05$). Moreover, *IL-27R* mRNA (*WSX-1* & *gp130*) was decreased in comparison to healthy control.

Taken together, these results indicated that monocyte/macrophage and neutrophils were the major sources of IL-27 during bacterial infection and it could be demonstrated both in vitro and ex vivo. IL-27 might play an important role to regulate the immune response, particularly in Gram-negative bacterial sepsis.

Discussion

Sepsis is generally characterized by an acute inflammatory response with the rapid production of cytokines. In this study, we have shown that both IL-27 transcript and IL-27p28 protein levels were significantly elevated in patients with sepsis and also correlated with disease severity. Previously, elevated IL-27 levels have been shown with increased mortality in an animal model of experimental septic peritonitis induced by caecal ligation and puncture [5]. Together with our observation in septicemic patients, these results suggest that overproduction of IL-27 is a clear feature of sepsis and may play a role in pathogenesis of sepsis and shock, especially in the early phase of the disease.

We studied IL-27 responses in vitro by incubating whole blood or purified cell populations with live *B. pseudomallei* as a model of infection. IL-27 production was substantially increased in culture supernatants of unfractionated human peripheral blood at 6 h after infection. In other systems, IL-27 is mainly secreted from activated antigen-presenting cells such as macrophages by following engagement of TLRs [23–25]. In this study, we found that IL-27p28 subunits were rapidly produced by both macrophages and neutrophils within 6 h after infection by *B. pseudomallei*. This is evidently due to augmented *EBI3* and *p28* gene transcription, as both promoters contain TLR-responsive NF-κB binding elements [14]. In mice, induction of IL-27 subunits by LPS in macrophages is mediated through a MyD88-dependent signaling pathway, a finding supported by the reduced expression of IL-27p28 mRNA observed in LPS-stimulated MYD88^{−/−} macrophages [15]. In humans, macrophages can be induced to express IL-27 subunits after engagement of TLR ligands LPS, poly I:C, and R848 to their receptors TLR4, TLR3, and TLR8, respectively [26]. In addition, stimulation of human monocyte-derived DCs with *Streptococcus pyogenes* or activation of human macrophages by Sendai virus can induce the expression of these two subunits [26, 27]. Conversely, TLR2 and TLR9 ligands, Pam3Cys and CpG, have no effect on the expression of IL-27 subunits [26, 28]. In other reports, strong induction of both IL-27 subunits could be observed in MoDC incubated with Gram-negative bacteria whereas expression of p28 subunit in response to Gram-positive bacteria was at similar levels to controls [16, 29]. Here, our data from in vitro stimulation experiments showed that IL-27p28

subunit was expressed at higher levels in Gram-negative bacteria stimulated neutrophils but not in Gram-positive bacteria stimulation. This was supported by our finding of significantly greater IL-27 p28 expression in cases of human sepsis caused by Gram-negative bacteria than those in Gram-positives. This might be explained by the fact that Gram-negative bacteria exclusively carry LPS that signaling through TLR4 [30] and that IFN-γ signaling also contributes to transcriptional activation of gene p28 due to Gram-negative bacteria infection strongly induced Th1 polarizing capacity compared to Gram-positive control [16]. Gram-negative bacteremia can induce greater inflammatory responses and of higher severity, compared to Gram-positive bacteremia [31] suggesting that the type of infecting microorganism should be considered in treatment of septicemic patients. Furthermore, both isolated neutrophils and CD14+ cells from these septic patients could produce IL-27 ex vivo but the relative contribution of these cell types to produce this cytokine may be different according to the time course and types of bacterial infection.

Previous studies have shown that IL-27 receptor is expressed on macrophages, neutrophils and T cells [5, 17, 32] and our data are consistent with those reports. However, the role of IL-27 in regulation of neutrophil functions during sepsis is not completely understood. Previously, it has been reported that IL-27 suppresses LPS-induced ROS production and enhanced IL-1β production [5, 32]. We have confirmed and extended these findings by showing that IL-27 also regulates neutrophil function in response to intact, living Gram negative bacteria. Furthermore, we now report IL-27 can act as an autocrine regulator of neutrophils since they produce IL-27 and express its receptor on the cell surface. We found that IL-27 suppressed ROS production via JAK/STAT pathways and that caused the enhancement of intracellular bacterial survival in human neutrophils after the infection. One possible explanation for this observation is that IL-27 may up-regulate Suppression of cytokine signaling (SOCS) 3 and prevent JAK/STAT phosphorylation [33, 34]. In addition, our data also establish that IL-27 induces IL-1β and TNF-α production by human neutrophils. This finding is consistent with data reported previously in an experimental model of sepsis, which IL-1β levels were shown to be significantly reduced in IL-27 EBI3−/− mice [5]. However, this study did not determine how IL-27 acted on which immune cells to up-regulate IL-1β. Our results here thus demonstrate a direct role of IL-27 on IL-1β production by human neutrophils that will in turn contribute to immune regulation. The clinical relevance of these observations is underlined in the observation that IL-27 increases the number of *B. pseudomallei* CFU in neutrophils in part by suppression of ROS production and leads to an increase of TNF-α and IL-1β production, known to contribute to the immunopathology of sepsis [35, 36]. These effects of IL-27 on neutrophils may be different from T cells in other studies that immunosuppressive effect of IL-27 largely depends on IL-10 [41].

In conclusion, our data indicate that production of IL-27 is a prominent component of the cytokine response to *B. pseudomallei* in humans and may play a key regulatory role during sepsis. Here, we show for the first time that IL-27 can be produced from neutrophils and acts as an auto-regulator of neutrophil functions. The presence of IL-27 results in suppression of reactive oxygen intermediate, significantly enhancing *B. pseudomallei* infection in neutrophils in vitro and promoting the release of pro-inflammatory cytokines, TNF-α and IL-1β. When these cytokines are produced in excess, they could contribute to organ dysfunction and overwhelming sepsis. The detection of IL-27 in the blood of patients confirms the potential value of this molecule as a potential predictive and therapeutic target. Measuring IL-27 levels in patients may prove useful in making appropriate treatment decisions and blockade of IL-27 function by sIL-27R could be a novel treatment modality for patients with sepsis, particularly those caused by Gram-negative bacteria such as *B. pseudomallei.*

Materials and Methods

Human subjects

The study was performed by recruitment of patients who were suspected of having hospital or community acquired infection. Clinical specimens were collected for bacterial culture within 24 h following the diagnosis of sepsis. All blood samples were obtained at the Khon Kaen Regional Hospital, Khon Kaen, Thailand as approved by Khon Kaen University Ethic Committee for Human Research (Project number HE470506). Within 48 h of the diagnosis of sepsis, enrolled patients had 7 ml of whole blood collected by which 4 ml were added into a container with lithium heparin preservative (BD Biosciences) and 3 ml were added into a Tempus vacutainer tube containing an RNA stabilization solution (Applied Biosystems). The diagnosis of sepsis for this study was defined as having a positive blood culture with two or more of criteria for the systemic inflammatory responses [37]. Informed consent was obtained for all subjects. A total of 114 blood samples from control subjects and septicemic patients that met the case definitions were analyzed, including 90 patients with sepsis (55 patients with septicemic melioidosis, 35 patients with sepsis caused by other pathogens) and 24 non-infected controls including 9 subjects recovered from melioidosis for more than 5 years, 7 subjects with type 2 diabetes (T2D), a risk factor to infection, and 8 healthy blood subjects. Demographic, clinical and microbiological data were recorded for all subjects (Table 1).

RNA preparation and microarray hybridization

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 (RIN) were assessed on an Agilent 2100 Bioanalyzer (Agilent). Labeled cRNA was hybridized overnight to Sentrix Human-6 V2 BeadChip arrays (Ilumina), washed, blocked, stained and scanned on an Illumina BeadStation 500 following the manufacturer's protocols.

Growth of bacteria

The prototype genome sequenced *B. pseudomallei* strain K96243, *Escherichia coli* ATCC 25921 and *Staphylococcus aureus* ATCC 25923 were grown in Luria-Bertani broth for 18 h at 37°C, washed twice with phosphate-buffered saline (pH 7.4). The number of bacteria was determined by determining the absorbance index of suspension bacteria prior to use. In general, absorbance index of 0.33–0.35 was equivalent to 10^8 CFU/ml of bacteria. Live *B. pseudomallei* was handled under the US Centers for Disease Control regulations for biosafety containment level 3.

Real time quantitative PCR

The expression of IL-27 subunits and its receptors, including *IL-27p28, IL-27EBI3, WSX-1,* and *gp130* of purified cells was analyzed by real time PCR, 7500 Fast Real-Time PCR System (Applied Biosystems). The sequences of PCR primers for IL-27 cytokine (*IL-27p28* and *EBI3*) and IL-27receptor, *WSX-1* [38] and *gp130* [17] were obtained from previous reports. Transcript levels were determined by normalizing the expression of each target to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) within triplicate samples. Subsequently, the relative expression of transcripts was calculated with the medium control.

Whole blood culture

Whole blood was obtained by venipuncture of healthy subjects into containers with preservative-lithium heparin (BD Biosciences). Each blood sample was then diluted to obtain 3×10^5 cells/ml of mononuclear cells. One hundred microliters of diluted blood samples were dispensed in duplicated into a sterile 96-well flat bottom tissue culture plates

(Nalgene, Nunc International) together with the medium alone or test stimuli and incubate at 37 °C with 5% CO_2. Cultured supernatants from duplicated wells were collected and kept at −20 °C prior to cytokine analysis by sandwich ELISA.

Generation of monocyte-derived dendritic cells (MoDCs)

MoDCs were prepared as previously described by our study [39]. MoDCs were then seeded into 96 well tissue culture plates (Nalgene, Nunc International) at 1×10^5 cells/ml and rested for 24 h before addition of medium alone, 10 µg/ml of *E. coli* LPS, formaldehyde killed or live *B. pseudomallei* at MOI of 1, 10 or 100 for 18 h. The culture supernatants were assayed for IL-27p28 protein by ELISA.

Human neutrophil isolation

Human neutrophils were isolated from heparinized venous blood by 3.0% dextran T-500 sedimentation and Ficoll-Paque PLUS centrifugation (Amersham Biosciences) as previously described by Chanchamroen et al [40]. The purity of isolated cells was generally more than 95% as determined by flow cytometry (Becton Dickinson FACSCalibur). The other less than 5% was mainly eosinophils and basophils since isolated cells were stained for T cell and monocyte makers (anti-CD3 and anti-CD14 staining) and the results showed that anti-CD14 was negative and less than 1 % was positive for anti-CD3 (Supplement Figure S5).

Isolation of cells from whole blood samples

To identify cell types responsible for IL-27 production in response to *B. pseudomallei* and other bacteria, fresh whole blood was obtained from patients with sepsis at the day admission to Khon Kaen Regional Hospital, Thailand or whole blood samples from healthy subjects were infected with live *B. pseudomallei* at a multiplicity of infection (MOI) of 10 for 6 h in vitro. Then neutrophils were isolated as previously described [40] and $CD14^+$, $CD4^+$ and $CD8^+$ cells were separated by using immune-magnetic bead sorting (Miltenyi Biotec), according to the manufacturer's instruction.

Phagocytosis and oxidative burst in human neutrophils

Whole blood samples from healthy subjects were stimulated in vitro with *B. pseudomallei* at MOI of 10 or with 100 ng/ml lipopolysaccharide of *Escherichia coli* (*E. coli* LPS) (Sigma Aldrich) in the presence of recombinant human IL-27 (rIL-27, R&D Systems) at 0, 10, 30 or 100 ng/ml for 30 min at 37°C; then analyzed for oxidative burst by flow cytometry (Supplement Figure S6) as previously described by Chanchamroen et al. [40].

Enumeration of bacteria in human neutrophils

Purified neutrophils were treated with 0, 30, 100 or 300 ng/ml of rIL-27 before co-culturing with *B. pseudomallei* at a MOI of 10 at 37°C for 30 min. The intracellular survival of *B. pseudomallei* in neutrophils was determined by bacterial colony count after the extracellular bacteria were killed with 250 µg/ml kanamycin at 37°C for 30 min and culture supernatants were checked for sterility by plating on Luria-Bertani agar plates.

Cytokine analysis

IL-27 cytokine in each culture supernatant was measured in duplicate by MaxDiscovery™ Human IL-27p28 ELISA test kits (Bio Scientific). Tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-10 and IL-12p70 were determined using a cytometric bead array (CBA) multiplex assay in accordance with the manufacturer's instructions (BD Biosciences).

Statistical analysis

Statistical analysis (one way-ANOVA, Mann-Whitney test, unpaired t-test and paired t-test) was performed using Graphpad Prism version 5 software (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviation

Bps   Burkholderia pseudomallei

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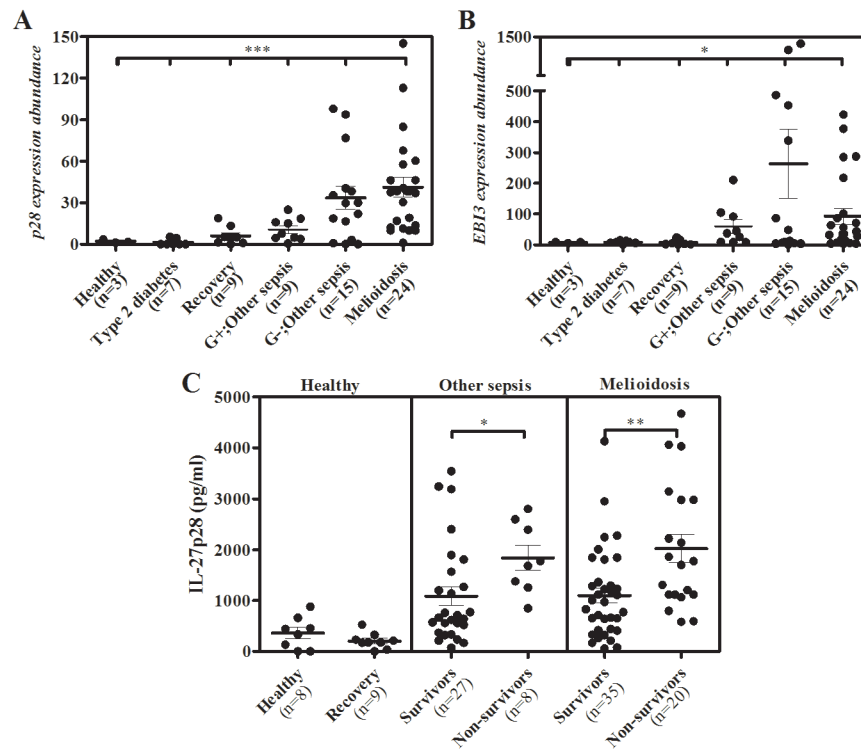
Figure 1. Enhanced expression of *IL-27p28* and *EBI3* mRNA and IL-27p28 protein in sepsis
mRNA expression levels for IL27p28 (A) and EBI3 (B) subunits were measured by microarrays in whole blood obtained from patients with sepsis caused by *B. pseudomallei* (n=24) or other pathogens including Gram-negative (n=15), Gram-positive bacteria (n=9). Blood samples obtained from healthy subjects (n=3), subjects with type 2 diabetes (n=7), and subjects who had recovered from sepsis caused by *B. pseudomallei* infection (n=9) were used as uninfected controls. IL-27p28 protein (C) was measured by ELISA in 55 plasma samples of patients with septicemic melioidosis (35 survivors and 20 non-survivors), 35 plasma samples of patients with sepsis caused by other pathogens (27 survivors and 8 non-survivors), and 17 plasma samples of uninfected controls (9 subjects who recovered from melioidosis and 8 healthy subjects). Statistical significance was determined using ANOVA and Tukey's post test (A and B) and Mann Whitney test (C); ns, not significant, $*$ $p < 0.05$, $**$ $p < 0.01$ and $***$ $p < 0.001$. The horizontal lines indicate mean $\pm$ standard errors (SE).
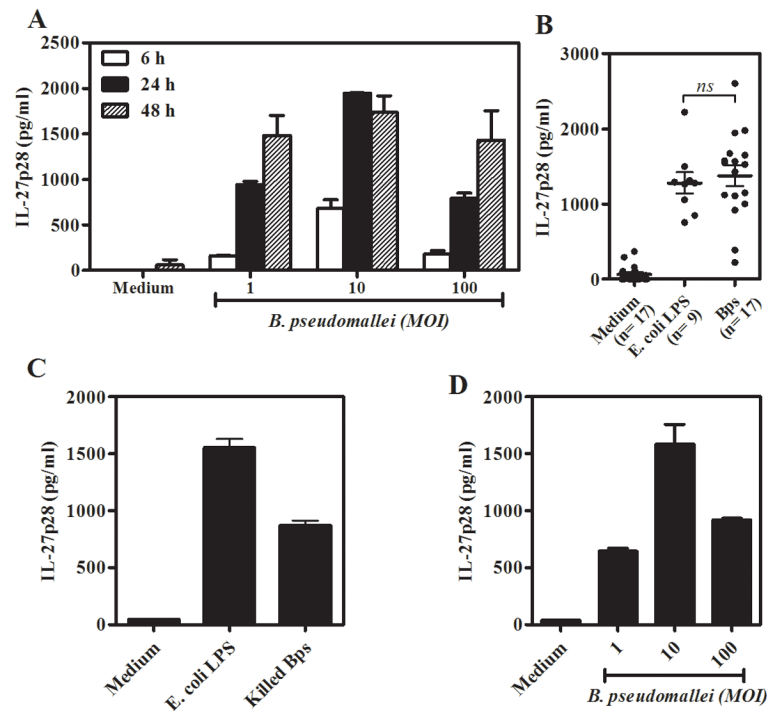
Figure 2. *B. pseudomallei* induced IL-27 production in whole blood and monocyte derived dendritic cells in vitro

Whole blood samples obtained from one representative healthy subject were co-cultured with live *B. pseudomallei* at MOI of 1, 10 and 100 for 6, 24 and 48 h in duplicate wells and quantified for IL-27p28 by ELISA (A). Distribution of IL-27p28 levels in the culture supernatants of whole blood samples from 17 healthy subjects incubated with 10 µg/ml of *E. coli* LPS (*E. coli* LPS; n=9) or live *B. pseudomallei* (Bps; n=17) at a MOI of 10 for 24 h (B). Monocyte-derived derived dendritic cells from three healthy subjects were incubated with medium alone, 10 µg/ml of *E. coli* LPS or 1×10^6 CFU/ml killed *B. pseudomallei* (C) or live *B. pseudomallei* at MOI of 1, 10 and 100 (D) for 18 h and IL-27p28 quantified in cultured supernatants by ELISA. All results represent the mean ± standard errors (SE) of duplicate measurement. Statistical significance was determined using Mann Whitney test; ns, not significant.
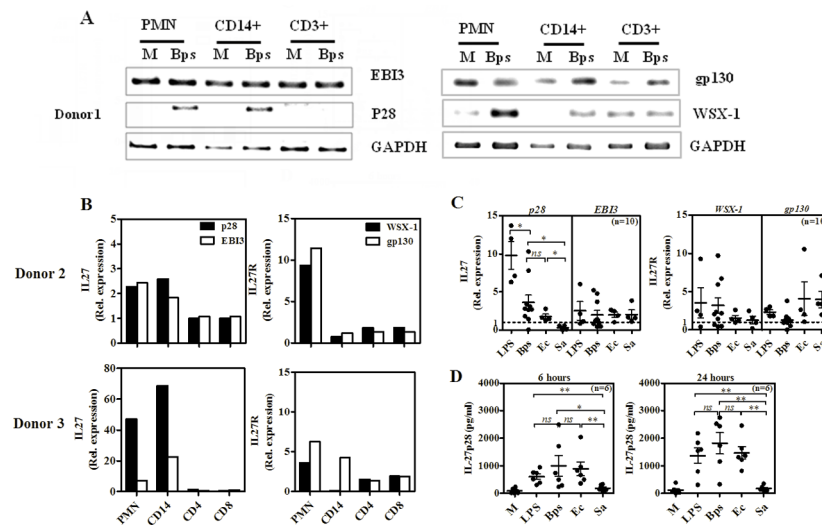
Figure 3. Expression of *IL27 (p28/EBI3)* and *IL27 receptor (WSX-1/gp130)* subunits by isolated human neutrophils, CD14^{+} cells and T cells following incubation of whole blood with live *B. pseudomallei*

Whole blood samples from healthy subjects were incubated with medium alone (M) or live *B. pseudomallei* (Bps) at a MOI of 10 for 6 h. Then, neutrophils, CD14^{+}, CD3^{+}, CD4^{+} and CD8^{+} cells were isolated by immunomagnetic beads, extracted for RNA and assayed for *IL27* and its receptor transcripts by reverse transcriptase PCR (A) and real-time PCR (B). Purified neutrophils from ten healthy subjects incubated with medium alone (M), *E. coli* LPS (LPS; n=4), live *E. coli* (Ec; n=4), live *S. aureus* (Sa; n=4) or live *B. pseudomallei* (Bps; n=10) at a MOI of 10 for 6 h, and *IL27* and *IL27R* expression determined by real-time PCR (C). The PCR values were normalized to the mean expression of *GAPDH* within triplicate samples and relative expression was calculated with the medium control. Dashed line indicates relative expression = 1. Purified neutrophils from six healthy subjects were incubated with medium alone, *E. coli* LPS, live *E. coli*, live *S. aureus* or live *B. pseudomallei* at a MOI of 10 for 6 and 24 h, and cultured supernatants assayed for IL-27p28 by ELISA (D). Experiments were performed in triplicate for real-time PCR and replicate for IL-27p28 measurement and results represent mean ± standard errors (SE). Statistical significance was determined using ANOVA and Tukey's posttest; ns, not significant, * $p < 0.05$ and ** $p < 0.01$.
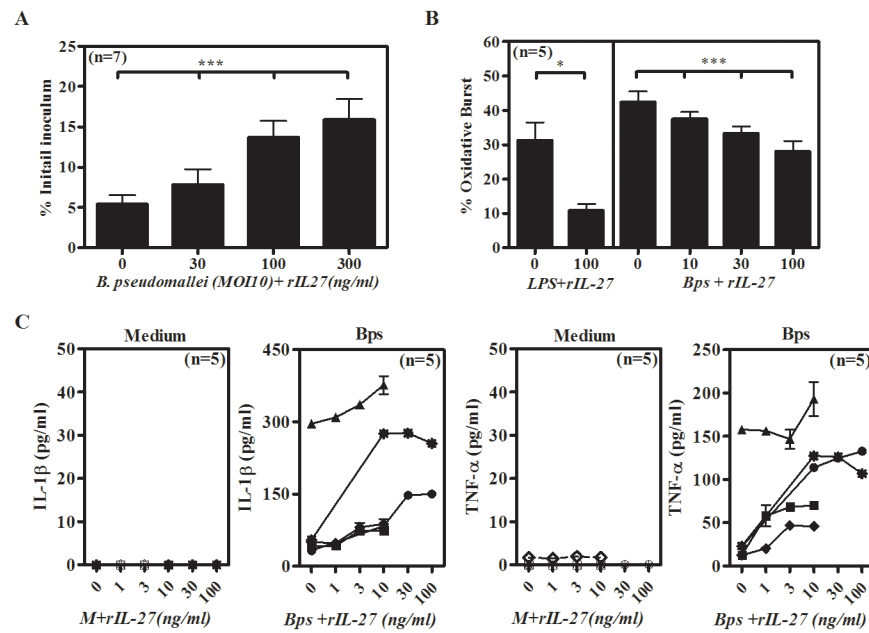
Figure 4. Effect of exogenous recombinant IL-27 on neutrophil functions
Purified neutrophils of seven healthy subjects were incubated with medium alone or with 30, 100 or 300 ng/ml rIL-27 for 15 min before addition of live *B. pseudomallei* at a MOI of 10 at 37°C for 30 min. The intracellular survival of *B. pseudomallei* in neutrophils was determined by bacterial colony count after the extracellular bacteria were killed with 250 µg/ml kanamycin at 37°C for 30 min (A). Oxidative burst production in whole blood samples from five healthy subjects incubated with medium alone, 100 ng/ml of *E. coli* LPS, or live *B. pseudomallei* at a MOI of 10 was analyzed by flow cytometry (B). Purified neutrophils of five healthy subjects were incubated with medium alone or with 1, 3, 10, 30, or 100 ng/ml of rIL-27 for 15 min before incubation with *B. pseudomallei* (Bps) at a MOI of 10 and medium control (Medium) at 37°C for 24 h and cytokine production in culture supernatants was determined by cytometric bead array (C). Experiments were performed in replicate measurement and results represent mean ± standard error (SE) of seven and five subjects in Panel A and B, respectively and individual subject (C). Statistical significance was ANOVA and Dunnett's post test; ns, not significant, * $p < 0.05$.
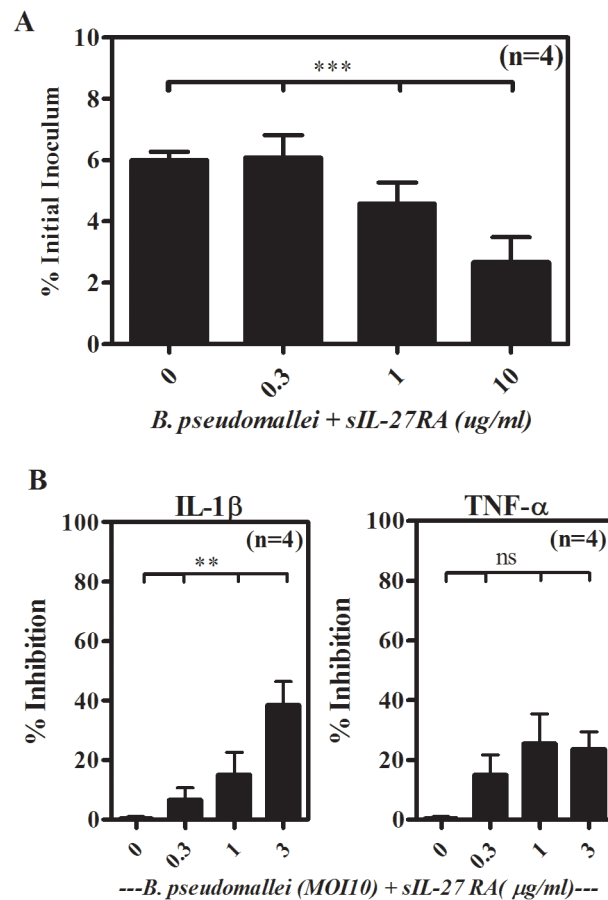
Figure 5. Neutralization of endogenous IL-27 and neutrophil activities against *B. pseudomallei*
Purified neutrophils of four subjects were untreated or treated with 0.3, 1 or 10 μg/ml of soluble IL-27 receptor antagonist (sIL-27RA) for 15 min before incubation with *B. pseudomallei* at a MOI of 10 for 30 min at 37°C. Intracellular survival of *B. pseudomallei* in neutrophils was determined by bacterial colony count (A). Purified neutrophils of four healthy subjects were incubated with medium alone or with 0.3, 1, or 3 μg/ml of sIL-27RA for 15 min before incubation with *B. pseudomallei* at a MOI of 10 and medium control at 37°C for 24 h, cytokine production in culture supernatants was determined by cytometric bead array and expressed as % inhibition (B). Experiments were performed by duplicate measurement and results represent mean ± standard errors (SE) of four subjects. Statistical significance was determined ANOVA and Dunnett's post test and Tukey's post test; * $p < 0.05$ and ** $p < 0.01$.
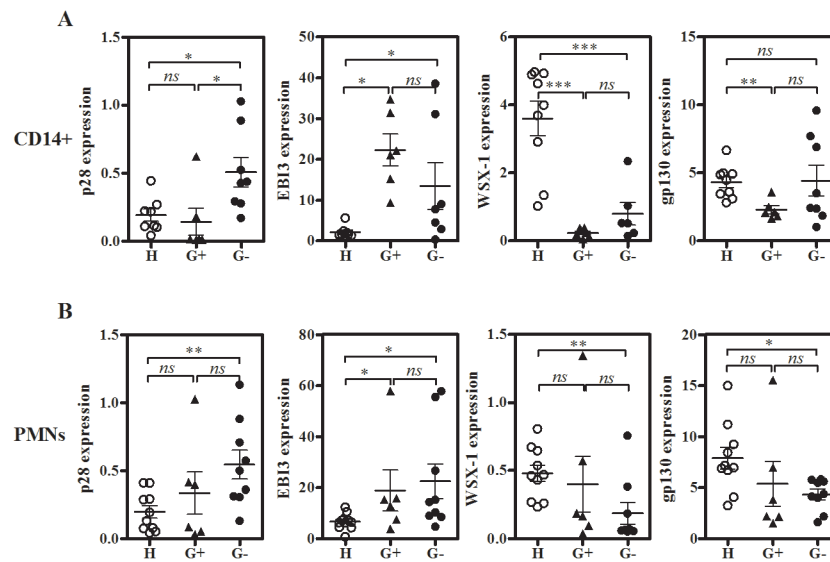
Figure 6. Expression of *IL27* (*p28/EBI3*) *and IL27* receptors (*WSX-1/gp130*) subunits by isolated human neutrophils and CD14+ from whole blood samples of patients with sepsis versus healthy subjects

Four ml of whole blood obtained from patient with sepsis caused by Gram-positive (G+, n=6) and Gram-negative bacteria (G-, n=9) or healthy subjects (H, n=10) were isolated for CD14+ cells (A) and neutrophils (B), extracted for RNA and analyzed for IL-27 and its receptors by real-time PCR. Experiments were performed in triplicate measurement and expression levels were determined by normalizing the expression of each target to *GAPDH* within triplicate sample. Subsequently, the expression of transcripts was calculated by $2^{-\Delta Ct} \times 1000$. The results represent means ± standard errors (SE). Statistical significance was determined using ANOVA and Dunnett's post test and Tukey's post test; ns, non significant, * $p < 0.05$ and ** $p < 0.01$ and *** $p < 0.001$.

Table 1

Demographic, clinical and microbiological data of subjects

	Melioidosis	Other sepsis	Recovery	Healthy
Number of subjects	55	35	9	17
Mean age (year, range)	52.4 (18–85)	57 (24–81)	47.4 (39–65)	46.6 (35–68)
Sex (Male/Female)	62/31	18/20	6/3	1/16
With Type 2 diabetes	33	20	4	-
Survivors/non-Survivors	27/8	35/20	-	-
Organisms (n)	*B. pseudomallei* (55)	*A. baumannii* (1), *Aromonas hydrophila* (1) *Bacillus spp.* (1), *Corynebacterium* spp. (2) *C. albicans* (4), *Citrobacter freundii* (1) *E. coli* (7), *Enterococcus* spp. (1) *K. pneumoniae* (3), *P. aeruginosa* (1) *Streptococcus* Non gr. A, gr. B or gr. D (1) *Staphylococcus* coagulase negative (6)* *S. aureus* (3), *Salmonella* serotype B (2) *Salmonella* spp. (1), *S. pneumoniae* (1)		

*
3/6 patients were positive in 2-blood culture.