



# Defense mechanisms of hepatocytes against *Burkholderia pseudomallei*

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The Gram-negative facultative intracellular rod *Burkholderia pseudomallei* causes melioidosis, an infectious disease with a wide range of clinical presentations. Among the observed visceral abscesses, the liver is commonly affected. However, neither this organotropism of *B. pseudomallei* nor local hepatic defense mechanisms have been thoroughly investigated so far. Own previous studies using electron microscopy of the murine liver after systemic infection of mice indicated that hepatocytes might be capable of killing *B. pseudomallei*. Therefore, the aim of this study was to further elucidate the interaction of *B. pseudomallei* with these cells and to analyze the role of hepatocytes in anti-*B. pseudomallei* host defense. *In vitro* studies using the human hepatocyte cell line HepG2 revealed that *B. pseudomallei* can invade these cells. Subsequently, *B. pseudomallei* is able to escape from the vacuole, to replicate within the cytosol of HepG2 cells involving its type 3 and type 6 secretion systems, and to induce actin tail formation. Furthermore, stimulation of HepG2 cells showed that IFN $\gamma$  can restrict growth of *B. pseudomallei* in the early and late phase of infection whereas the combination of IFN $\gamma$ , IL-1 $\beta$ , and TNF $\alpha$  is required for the maximal antibacterial activity. This anti-*B. pseudomallei* defense of HepG2 cells did not seem to be mediated by inducible nitric oxide synthase-derived nitric oxide or NADPH oxidase-derived superoxide. In summary, this is the first study describing *B. pseudomallei* intracellular life cycle characteristics in hepatocytes and showing that IFN $\gamma$ -mediated, but nitric oxide- and reactive oxygen species-independent, effector mechanisms are important in anti-*B. pseudomallei* host defense of hepatocytes.

**Keywords:** *Burkholderia*, cytoskeleton, hepatocytes, interferon  $\gamma$ , iNOS, NADPH oxidase, secretion system

## INTRODUCTION

The Gram-negative saprophyte *Burkholderia pseudomallei* is the causative agent of melioidosis, an emerging infectious disease of humans and animals in certain areas of the tropics and subtropics. *B. pseudomallei* is an intracellular pathogen that can invade a variety of host cells (Jones et al., 1996). After invasion, *B. pseudomallei* can escape from the endocytotic vesicle of murine macrophage cells into the host cytosol depending on a functional type 3 secretion system-3 (T3SS-3; Stevens et al., 2002; Burtnick et al., 2008; Muangsombut et al., 2008; Gong et al., 2011). Within the cytosol bacteria can multiply and induce the BimA-dependent formation of actin tails, facilitating intracellular motility as well as spreading of *B. pseudomallei* into neighboring cells (Kespichayawattana et al., 2000; Breitbach et al., 2003; Stevens et al., 2005). A recent paper proposed that *B. pseudomallei*-induced cell fusion and the

formation of multinucleated giant cells represent the primary path for intercellular spread and plaque formation of this pathogen (French et al., 2011).

Several reports have shown that interferon  $\gamma$  (IFN $\gamma$ ) is essential for the early control of *B. pseudomallei* infection in mice (Santaniand et al., 1999; Breitbach et al., 2006). *In vitro* experiments also demonstrated a pivotal role for IFN $\gamma$  to eliminate intracellular *B. pseudomallei* in macrophages (Miyagi et al., 1997; Utaisincharoen et al., 2001). We recently demonstrated that the downstream effector molecule of IFN $\gamma$ , nitric oxide (NO), has a dual role among resistant and susceptible mouse strains after *B. pseudomallei* infection. NO had rather detrimental effects in innate resistant C57BL/6 mice in a murine model of melioidosis and was not involved in killing activity of C57BL/6 macrophages (Breitbach et al., 2006, 2011). In contrast, NO contributed to complete resistance in innate susceptible BALB/c mice and to growth restriction of *B. pseudomallei* in macrophages from those mice (Breitbach et al., 2011). In a previous study we revealed that NADPH oxidase-mediated mechanisms contribute to early resistance in bone marrow-derived macrophages and C57BL/6 mice (Breitbach et al., 2006).

The liver plays an essential role in the innate immune response, providing the first line of defense against microbes crossing the intestinal barrier (Crispe, 2009). Kupffer cells are important for the rapid clearance of microorganisms from the systemic circulation,

**Abbreviations:** AG, aminoguanidine hemisulfate; Apocynin, 4-hydroxy-3-methoxyacetophenone; BMM, bone marrow-derived macrophages; CAT, catalase; CFU, colony forming units; COX, cyclooxygenase; EPS, exopolysaccharide; IFN $\gamma$ , interferon  $\gamma$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; iNOS, inducible nitric oxide synthase; LAMP-1, lysosomal-associated membrane protein-1; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MOI, multiplicity of infection; NAC, N-acetyl-cysteine; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; T3SS, type three secretion system; T6SS, type six secretion system; TNF $\alpha$ , tumor necrosis factor.

and can facilitate the generation of a local inflammatory response leading to recruitment of inflammatory cells. Furthermore, hepatocytes can also secrete inflammatory cytokines and chemokines in response to cytokine activation and/or bacterial invasion (Rowell et al., 1997; Santos et al., 2005). However, to date little is known about the antimicrobial responses of hepatocytes. Only few studies indicate that IFN $\gamma$  can restrict growth of *Listeria monocytogenes* and *Salmonella typhimurium* in murine hepatocytes (Gregory and Wing, 1993; Lajarin et al., 1996).

In previous studies we and others revealed that the organotropism of *B. pseudomallei* for the spleen and liver in melioidosis patients can be mimicked by infection of mice (Hoppe et al., 1999; Santanirand et al., 1999; Liu et al., 2002). Electron microscopic investigations of the murine liver demonstrated that *B. pseudomallei*-containing phagosomes in hepatocytes fuse with lysosomes, leading to bacterial degradation (Hoppe et al., 1999). Therefore, the present study aimed to establish an *in vitro* hepatocyte infection model with human polarized HepG2 cells to study host defense mechanisms against *B. pseudomallei*. We investigated whether *B. pseudomallei* is able to invade and survive within hepatocytes and whether bacterial replication is dependent on the *B. pseudomallei* type 3 or type 6 secretion systems. Finally, we addressed the role of cytokines in enhancing anti-*B. pseudomallei* activity in HepG2 cells and a possible contribution of nitric oxide and reactive oxygen species in the bactericidal activity against *B. pseudomallei*.

## MATERIALS AND METHODS

### MATERIALS

Cytochalasin D (CytoD), latrunculin B (LatB), jasplakinolide (Jasp), and nocodazole (Noco) were obtained from Enzo Life Sciences (Lörrach, Germany). Catalase–polyethylene glycol (CAT–PEG), superoxide dismutase–polyethylene glycol (SOD–PEG), *N*-acetyl-cysteine (NAC), aminoguanidine (AG), and colchicine (Col) were from Sigma Aldrich (Taufkirchen, Germany). hIFN $\gamma$  was purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany), and both mIL-1 $\beta$  and mTNF $\alpha$  were from Roche (Mannheim, Germany). Apocynin (Apo) was obtained from Calbiochem (Darmstadt, Germany).

### BACTERIAL STRAINS

*Burkholderia pseudomallei* wild-type strain E8 comprises a soil isolate from the surrounding area of Ubon Ratchathani in northeast Thailand (Wuthiekanun et al., 1996) and was used throughout the study. T3SS-3 mutant  $\Delta$ BPSS1539 (16:48) and T6SS-1 mutant  $\Delta$ BPSS1509 (5:45) were generated by Tn5-OT182 mutagenesis of *B. pseudomallei* E8 as previously described (Pilatz et al., 2006). Bacteria were grown on Columbia agar at 37°C for 24 h and adjusted to the desired concentration in Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen, Darmstadt, Germany) or the respective cell culture medium.

### CELL CULTURE AND INFECTION OF HepG2 CELLS

Human hepatocellular carcinoma HepG2 cells were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Cells were cultured in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10% fetal calf serum (PAA Laboratories GmbH,

Cölbe, Germany) at 37°C in a humidified atmosphere containing 95% air and 5% CO $_2$ .

Twenty-four hours prior to infection, cells were seeded in 48 well plates ( $1.5 \times 10^5$  cells per well), grown to 80% confluence, and infected with *B. pseudomallei* strain E8 at the indicated multiplicity of infection (MOI). For invasion assays well plates were additionally centrifuged for 4 min at  $120 \times g$ . After infection for 30 min cells were washed twice with D-PBS and incubated in kanamycin (250  $\mu$ g/ml) containing medium to eliminate remaining extracellular bacteria. To minimize re-infection and extracellular replication the culture medium was replaced by fresh medium containing 125  $\mu$ g/ml kanamycin 6 h after infection. At indicated time points (time zero was taken 25 min after incubation under antibiotic-containing medium) the number of intracellular colony forming units (CFU) was determined. Consequently, cells were washed twice with D-PBS and subsequently lysed using 150  $\mu$ l D-PBS containing 0.5% Tergitol TMN (Fluka, Buchs, Switzerland) and 1% bovine serum albumin (BSA) per well. After 15 min of incubation appropriate dilutions of lysates were plated on Mueller–Hinton agar and incubated at 37°C for 48 h.

Activation of HepG2 cells was performed using 500 ng/ml IFN $\gamma$ , 50–200 U/ml IL-1 $\beta$ , or 10 ng/ml TNF $\alpha$  24 h prior to infection. For *in vitro* inhibition of inducible nitric oxide synthase (iNOS), NADPH oxidase, or ROS generation, HepG2 cells were treated by adding 2 mM aminoguanidine (24 h), 500  $\mu$ M apocynin, or 20 U/ml superoxide dismutase, 200 U/ml catalase, and 200  $\mu$ M *N*-acetyl-cysteine or corresponding vehicle into the culture medium 1 h (unless otherwise indicated) prior to infection and during the incubation with kanamycin-containing medium. For *in vitro* inhibition of the actin cytoskeleton or the microtubules, HepG2 were incubated for 1 h with 1  $\mu$ M cytochalasin D, 0.1  $\mu$ M latrunculin B, 0.5  $\mu$ M jasplakinolide, 10  $\mu$ M nocodazole, 5  $\mu$ M colchicine, or corresponding vehicle, followed by infection with *B. pseudomallei*. All inhibitors and vehicles were kept in the infection medium throughout the experiment.

### IMMUNOFLUORESCENCE STAINING

Twenty-four hours prior to infection, HepG2 cells were seeded on collagen type I coated cover slips in 24 well plates ( $2 \times 10^5$  cells per well) and infected with *B. pseudomallei* strain E8 at a MOI of 400 by centrifugation of the well plates for 4 min at  $120 \times g$ . After infection for 30 min cells were washed twice with D-PBS and incubated in kanamycin (250  $\mu$ g/ml) containing medium to eliminate remaining extracellular bacteria. At indicated time points HepG2 cells were washed with PBS, incubated for 10 min in ice-cold methanol, and washed three times with IF buffer [0.2% (w/v) BSA, 0.05% (w/v) saponin, 0.1% (w/v) sodium azide in PBS, pH 7.4]. To block non-specific antibody binding, cells were incubated for up to 1 h in IF buffer followed by an overnight incubation at 4°C in a humidity chamber with monoclonal mouse anti-*B. pseudomallei* 3015  $\gamma$ 2b (1:2000; Pilatz et al., 2006) and polyclonal rabbit anti- $\beta$ -actin (1:100; Cell Signaling, Frankfurt am Main, Germany) or monoclonal mouse anti-lysosomal-associated membrane protein-1 (anti-LAMP-1)  $\gamma$ 1 (1:400; BD Biosciences, Heidelberg, Germany) antibodies. After a wash with IF buffer, the immunoreacted primary antibodies were visualized with green

fluorescent Alexa Fluor 488 anti-mouse IgG2b (1:800; Invitrogen, Darmstadt, Germany) and red fluorescent Cy3-conjugated goat anti-rabbit IgG (1:400; Dianova, Hamburg, Germany) or red fluorescent Alexa Fluor 568 anti-mouse IgG1 (1:800; Invitrogen) by incubation for 1 h at room temperature in the dark. After another wash with IF buffer, slices were covered with Fluorprep (bioMérieux, Nürtingen, Germany) and observed by fluorescent microscopy with a BZ-9000 microscope (Keyence Corporation, Neu-Isenburg, Germany).

#### LDH ASSAY

To quantify the extent of cell damage after infection, release of lactate dehydrogenase (LDH) in cell culture supernatants was determined. HepG2 cells were seeded in 96 well plates ( $3.75 \times 10^4$  cells per well) and infected at the indicated MOI with *B. pseudomallei* for 30 min. Cells were washed twice with D-PBS, and 100  $\mu$ l of medium containing 250  $\mu$ g/ml of kanamycin was added to each well to eliminate extracellular bacteria. At the indicated time points, cell culture supernatant was collected, and LDH activity was detected by using the CytoTox-One homogeneous membrane integrity assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 50  $\mu$ l of supernatant was added to the kit reagent and incubated for 10 min. After addition of stopping solution, the fluorescence intensity was measured using the microplate reader Infinite M200 PRO (Tecan, Crailsheim, Germany) at excitation wavelength of 560 nm and emission wavelength of 590 nm.

#### REAL-TIME CELL ANALYSIS

HepG2 cells were seeded in 96 well E-plates ( $3 \times 10^4$  cells per well), grown for 24 h, and then infected at the indicated MOI with *B. pseudomallei* for 30 min followed by incubation in kanamycin (250  $\mu$ g/ml) containing medium. Cellular events were monitored in real-time using the xCELLigence system according to the manufacturer's instructions (Roche, Mannheim, Germany). The system measures electrical impedance across gold microelectrodes integrated into the bottom of tissue culture E-plates providing real-time, quantitative information about the biological status of the cells, including cell number, viability, morphology, and degree of cell adhesion. In the absence of cells on the electrode surface, the electrical impedance describes only the background. Changes in impedance are dependent on either the number of cells attached to the electrodes or the dimensional change of attached cells on the electrode surface. The xCELLigence system detects changes in impedance and calculates them as dimensionless parameter termed Cell Index:  $CI = (Z_i - Z_0)/15$  where  $Z_i$  is the impedance at an individual time point of the experiment and  $Z_0$  describes the background measurement at the beginning of the experiment. To evaluate the impact of *B. pseudomallei* on HepG2 cells, the normalized cell index ( $NCI_{ti}$ ) was used. Consequently, all selected wells were set on impedance value of 1 at a given time point (time of infection), and all further values were calculated as the cell index at a given time point ( $CI_{ti}$ ) divided by the Cell Index at the normalization time point ( $CI_{nml\_time}$ ).

#### DATA PRESENTATION AND STATISTICAL ANALYSIS

Figures and statistical analysis were performed using GraphPad Prism, version 4.0. Student's *t*-test was used to detect statistically

significant differences in the intracellular bacterial numbers. *p*-Values of  $<0.05$  were considered to be statistically significant.

## RESULTS

### BURKHOLDERIA PSEUDOMALLEI IS ABLE TO INVADE AND REPLICATE WITHIN HepG2 CELLS

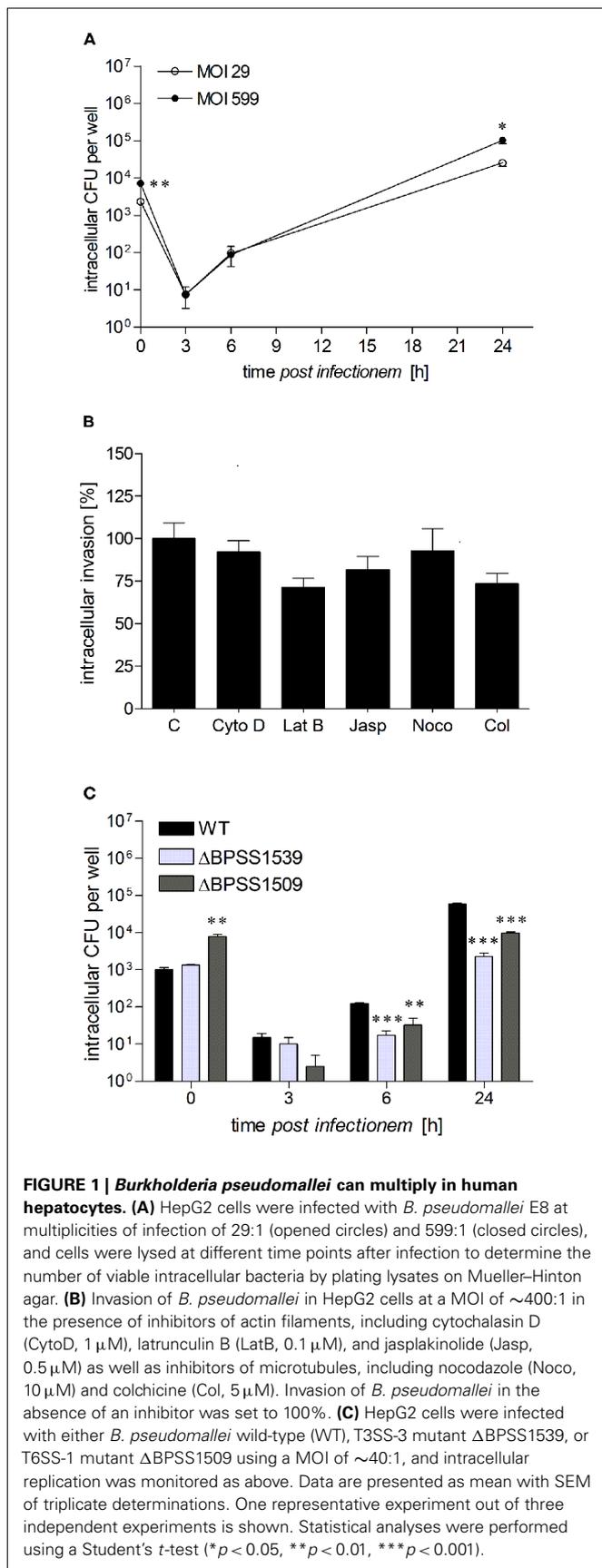
The invasion and intracellular replication of *B. pseudomallei* in hepatocytes or hepatocyte cell lines has not been investigated so far. Therefore, we first investigated the invasion and the course of the intracellular bacterial burden in human hepatocellular carcinoma HepG2 cells after infection with *B. pseudomallei* wild-type strain E8 at a MOI of 29 and 599, respectively. As shown in **Figure 1A**, *B. pseudomallei* was able to enter HepG2 cells. Within the first 3 h after infection, a strong reduction in *Burkholderia* counts could be observed which indicates that effective bactericidal defense mechanisms are activated in HepG2 cells in the early phase of infection. Six hours after infection the intracellular bacterial burden increased, suggesting that *B. pseudomallei* is able to multiply within cells. After 24 h 10-fold more bacteria were found in *Burkholderia*-infected HepG2 cells compared to the beginning.

To provide some information about the mechanism used by *B. pseudomallei* to enter HepG2 cells, we analyzed the role of the host actin cytoskeleton as well as microtubules by using well defined inhibitors. As shown in **Figure 1B**, there was a tendency of actin cytoskeleton inhibitors (1  $\mu$ M cytochalasin D, 0.1  $\mu$ M latrunculin B, 0.5  $\mu$ M jasplakinolide) as well as microtubule inhibitors (10  $\mu$ M nocodazole, 5  $\mu$ M colchicine) to reduce invasion of *B. pseudomallei*, as determined by enumeration of intracellular bacteria, although statistical significance was not reached. Therefore, our results indicate that the host actin cytoskeleton may contribute to *B. pseudomallei* uptake in HepG2 cells, but other invasion mechanisms may also play an important role.

As *B. pseudomallei* type 3 and type 6 secretion systems (T3SS, T6SS) are important for invasion of murine macrophages or human epithelial cells as well as for intracellular survival (Stevens et al., 2002, 2003; Burtneck et al., 2011), we analyzed whether they are also involved in the replication of *B. pseudomallei* in hepatocytes. Consequently, HepG2 cells were infected with *B. pseudomallei* wild-type strain E8, as well as a type 3 ( $\Delta$ BPSS1539) and type 6 ( $\Delta$ BPSS1509) secretion mutant, which have previously been shown to be attenuated *in vivo* (Pilatz et al., 2006). Surprisingly, mutant  $\Delta$ BPSS1509, but not  $\Delta$ BPSS1539 revealed a significantly higher invasion in HepG2 cells compared to the wild-type strain (**Figure 1C**). However, 24 h postinfection intracellular CFU counts were at least one log lower in cells infected with both mutant strains than in those infected with the wild-type strain. These results suggest that *B. pseudomallei* can infect and replicate in human hepatocytes and that the T3SS and T6SS contribute to optimal replication.

### BURKHOLDERIA PSEUDOMALLEI CAN ESCAPE FROM THE VACUOLE AND INDUCE FORMATION OF ACTIN TAILS IN HepG2 CELLS

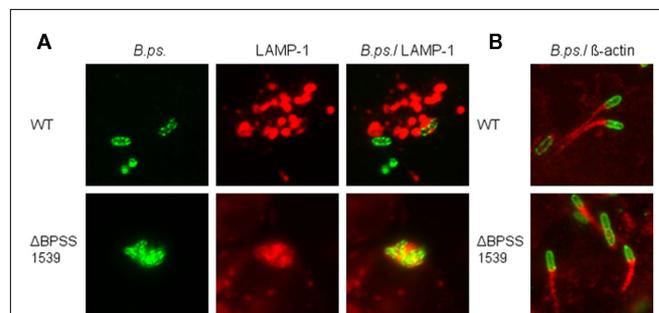
To further characterize the hepatocyte-pathogen-interaction we examined the intracellular localization of *B. pseudomallei* wild-type, T3SS, and T6SS mutant strains in HepG2 cells relative to LAMP-1 containing vacuoles as well as actin tail formation. Immunofluorescence microscopy revealed that 8 h after infection



the wild-type was rarely seen co-localized with regions of LAMP-1 staining (Figure 2A), however a part of the *B. pseudomallei* population was able to induce actin tails (data not shown). Sixteen hours after infection the majority of bacteria displayed long actin tails (Figure 2B). In contrast, T3SS mutant  $\Delta$ BPSS1539 was almost exclusively observed in association with LAMP-1, suggesting that the mutant is unable to escape from endocytotic vesicles 8 h after infection (Figure 2A). In accordance with our recent study using HeLa cells (Pilatz et al., 2006) this mutant was still able to multiply within the phagosomes of HepG2 cells, leading to large vesicles filled with bacteria. As *B. pseudomallei* was trapped within the phagosomes, no actin tail formation could be observed at this time point (data not shown). However, 16 h after infection some of these huge vacuoles seemed to become leaky and *B. pseudomallei*  $\Delta$ BPSS1539 could be detected within the cytosol in association with actin tails (Figure 2B). As the wild-type the T6SS mutant  $\Delta$ BPSS1509 was rarely seen associated with LAMP-1 8 h after infection, but actin tails could not be detected at this time point (data not shown). However, 16 h after infection the mutant was able to induce actin tails, but to a lesser degree compared to the wild-type (data not shown).

#### BURKHOLDERIA PSEUDOMALLEI INFECTION INDUCES CELL DAMAGE OF HepG2 CELLS

To determine whether *B. pseudomallei* affects the viability of hepatocytes, cytosolic LDH activity was measured in cell culture supernatants of infected and control HepG2 cells at 24 h postinfection as an indicator of cytotoxicity. Figure 3A demonstrates that infection of HepG2 cells with *B. pseudomallei* wild-type strain E8 induced only a small MOI-dependent release of LDH compared to uninfected cells. However, this slight cytotoxicity was not found



#### FIGURE 2 | *Burkholderia pseudomallei* is able to escape from the endocytotic vesicle and to form actin tails in hepatocytes.

HepG2 cells were infected with either *B. pseudomallei* wild-type (WT) or T3SS-3 mutant  $\Delta$ BPSS1539 using a MOI of ~400:1 and analyzed by immunofluorescence microscopy (magnification  $\times$ 1000). (A) Representative micrographs showing the association of intracellular *B. pseudomallei* with LAMP-1-containing vacuoles 8 h after infection. Bacteria were stained green with mouse anti-*B. pseudomallei* EPS (3015  $\gamma$ 2b) and Alexa Fluor 488 anti-mouse IgG2b antibodies. LAMP-1 was stained red with mouse anti-LAMP-1  $\gamma$ 1 and Alexa Fluor 568 anti-mouse IgG1 antibodies. (B) Representative micrographs showing the formation of actin tails by *B. pseudomallei* 16 h after infection. Bacteria were stained green with mouse anti-*B. pseudomallei* EPS (3015  $\gamma$ 2b) and Alexa Fluor 488 anti-mouse IgG2b antibodies. Actin tails were stained red with rabbit anti- $\beta$ -actin and Cy3-conjugated goat anti-rabbit IgG antibodies.

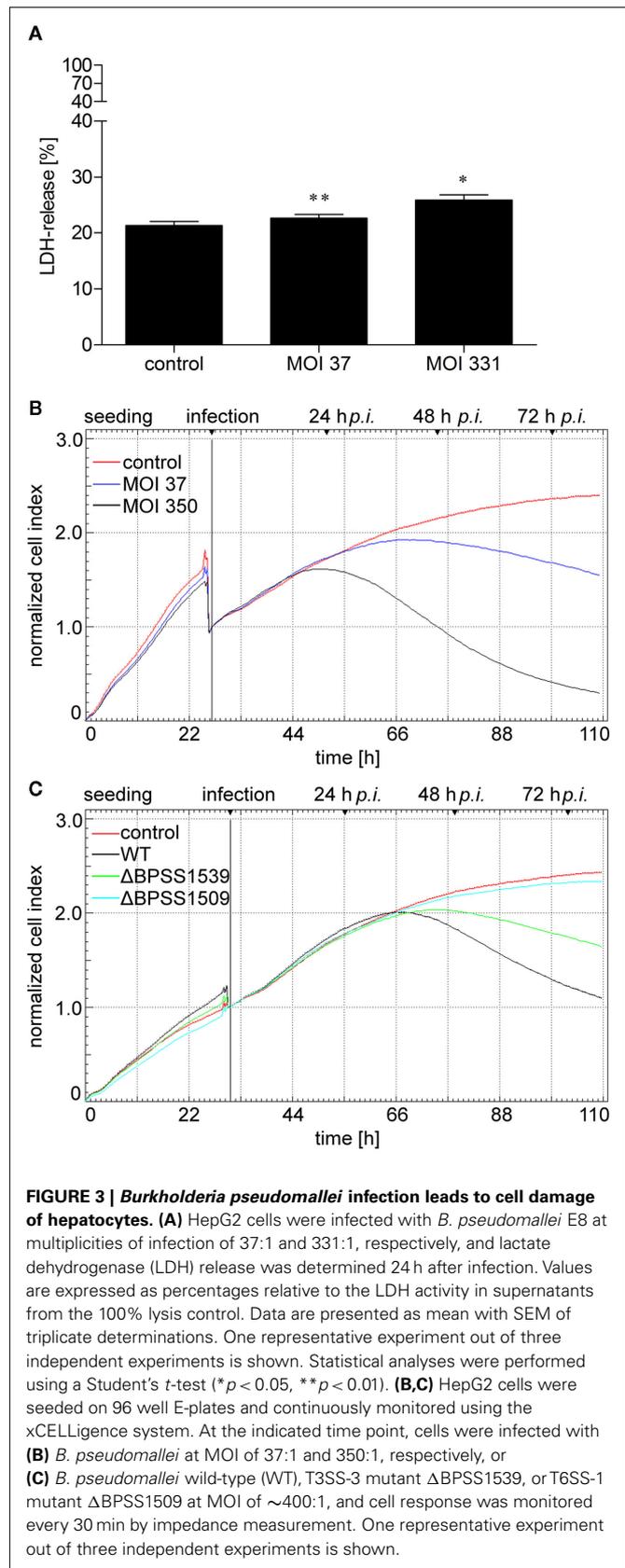
to be mediated by activation of apoptotic caspases 3 and 9 using Western blot analysis (data not shown).

Since *B. pseudomallei* infection led to detachment of hepatocytes after 24 h in a MOI-dependent manner as determined by microscopic analyses (data not shown), we aimed to quantify these cellular damages by performing electrical impedance measurements providing real-time information about the biological status of hepatocytes such as viability, morphology, and degree of cell adhesion. Such an experimental approach has recently been described for meningococcal infection in human brain microvascular endothelial cells (Slanina et al., 2011). HepG2 cells were grown for 24 h leading to an increase of the Normalized Cell Index (Figure 3B). Infection with *B. pseudomallei* wild-type strain E8 at MOI of 37 or 350 resulted in a transient decrease of the corresponding Cell Index caused by the cell culture medium exchange. Due to the proliferation and spreading of hepatocytes, non-infected cells led to enhanced electrical impedance within 110 h. In contrast, infection of HepG2 cells with *B. pseudomallei* at a MOI of 37 resulted in a loss of electrical impedance 36 h after infection, whereas infection at a MOI of 350 led to a loss of electrical impedance already 24 h postinfection (Figure 3B), correlating with the low LDH release after 24 h (Figure 3A). To exclude that the impedance was influenced by bacterial overload of the electrodes, electrical impedance was investigated in response to bacterial growth over the same period. However, no effect on the Cell Index could be observed when bacteria alone were grown in the wells (data not shown).

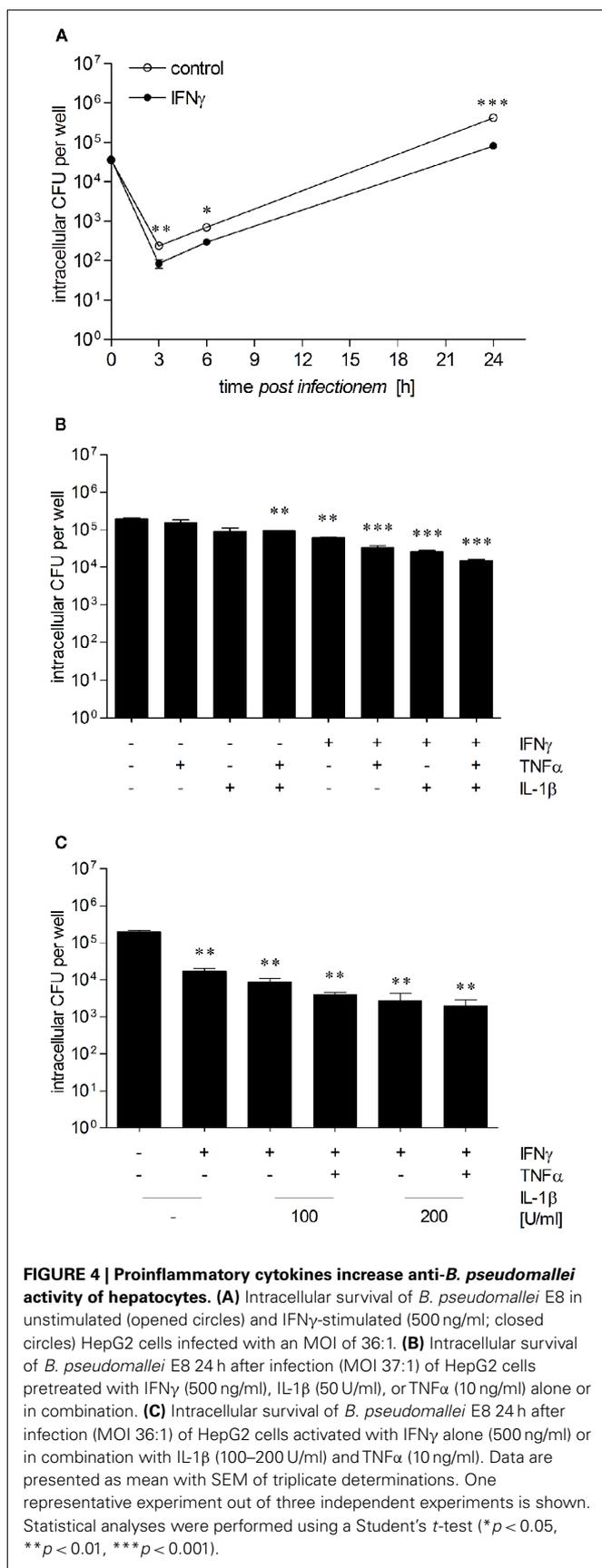
To determine whether reduced intracellular growth of T3SS and T6SS mutants correlates with an increased viability of hepatocytes, we measured the electrical impedance after infection of HepG2 cells with *B. pseudomallei* wild-type as well as the T3SS ( $\Delta$ BPSS1539) and T6SS ( $\Delta$ BPSS1509) mutants at a MOI of  $\sim$ 400. Figure 3C illustrates that the electrical impedance of HepG2 cells after infection with the T6SS mutant  $\Delta$ BPSS1509 did not differ significantly from the one of uninfected cells whereas infection with the wild-type strain resulted in a strong loss of electrical impedance. The decrease of the Normalized Cell Index observed in HepG2 cells infected with the T3SS mutant  $\Delta$ BPSS1539 was significantly delayed compared to wild-type infected cells. Our data indicate that *B. pseudomallei* induces cell damage of HepG2 cells depending on a functional T3SS and T6SS.

### INTERFERON $\gamma$ INCREASES ANTIBACTERIAL ACTIVITY OF HepG2 CELLS AGAINST *B. PSEUDOMALLEI*

IFN $\gamma$  is crucial for *in vivo* resistance against *B. pseudomallei* (Santanirand et al., 1999; Breitbach et al., 2006) and has been shown to restrict replication of *B. pseudomallei* in macrophages (Miyagi et al., 1997; Utasincharoen et al., 2001). We therefore compared the intracellular growth of *B. pseudomallei* between unstimulated or IFN $\gamma$ -stimulated (500 ng/ml) HepG2 cells. Although invasion of *B. pseudomallei* into HepG2 cells was comparable, already 3 and 6 h after infection intracellular bacterial load was reduced in IFN $\gamma$ -activated cells, which was most prominent after 24 h (Figure 4A). These data indicate that IFN $\gamma$  is able to increase the antibacterial capacity of HepG2 cells against intracellular *B. pseudomallei* in the early and late phase of infection.



**FIGURE 3 | *Burkholderia pseudomallei* infection leads to cell damage of hepatocytes. (A)** HepG2 cells were infected with *B. pseudomallei* E8 at multiplicities of infection of 37:1 and 331:1, respectively, and lactate dehydrogenase (LDH) release was determined 24 h after infection. Values are expressed as percentages relative to the LDH activity in supernatants from the 100% lysis control. Data are presented as mean with SEM of triplicate determinations. One representative experiment out of three independent experiments is shown. Statistical analyses were performed using a Student's *t*-test (\* $p$  < 0.05, \*\* $p$  < 0.01). **(B,C)** HepG2 cells were seeded on 96 well E-plates and continuously monitored using the xCELLigence system. At the indicated time point, cells were infected with **(B)** *B. pseudomallei* at MOI of 37:1 and 350:1, respectively, or **(C)** *B. pseudomallei* wild-type (WT), T3SS-3 mutant  $\Delta$ BPSS1539, or T6SS-1 mutant  $\Delta$ BPSS1509 at MOI of  $\sim$ 400:1, and cell response was monitored every 30 min by impedance measurement. One representative experiment out of three independent experiments is shown.



### PROINFLAMMATORY CYTOKINES ENHANCE ANTIBACTERIAL ACTIVITY OF IFN $\gamma$ -ACTIVATED HepG2 CELLS

IFN $\gamma$ , IL-1 $\beta$ , and TNF $\alpha$  have been detected in livers of BALB/c and C57BL/6 mice after *B. pseudomallei* infection (Ulett et al., 2000a,b), and numerous studies have shown that early cytokine responses are important for resistance to intracellular pathogens (Ehlers et al., 1992; Autenrieth et al., 1994; Bohn et al., 1994; Santanirand et al., 1999). To examine the impact of these inflammatory cytokines in controlling intracellular *B. pseudomallei* growth in hepatocytes, we analyzed the intracellular survival kinetics of *B. pseudomallei* in HepG2 cells in the presence of several cytokines. Therefore, HepG2 cells were stimulated with IFN $\gamma$  (500 ng/ml), IL-1 $\beta$  (50–200 U/ml), TNF $\alpha$  (10 ng/ml), or a combination of these cytokines for 24 h followed by infection with *B. pseudomallei*. As shown in **Figure 4B**, IL-1 $\beta$  or TNF $\alpha$  alone had no or only a marginal effect on intracellular bacterial replication, whereas the combination of both cytokines caused a small, but significant growth inhibition of *B. pseudomallei*. In contrast, IFN $\gamma$  alone reduced bacterial multiplication. This inhibitory effect was enhanced, when IFN $\gamma$  was combined with either IL-1 $\beta$  or TNF $\alpha$ . The combination of all three cytokines produced a maintained decrease in intracellular bacterial growth compared to that observed in unstimulated controls (**Figure 4C**), suggesting that IFN $\gamma$ , IL-1 $\beta$ , and TNF $\alpha$  are essential for the maximal activation of hepatocytes.

### INDUCIBLE NITRIC OXIDE SYNTHASE IS NOT ESSENTIAL TO ELIMINATE *B. PSEUDOMALLEI*

Previous studies provided evidence for a crucial role of NO in growth inhibition of *B. pseudomallei* in BALB/c macrophages, whereas NO did not play any role in killing activity of C57BL/6 macrophages (Breitbach et al., 2006, 2011). Thus, we investigated the role of iNOS-generated NO with respect to intracellular elimination of *B. pseudomallei* in HepG2 cells. First, we determined the amount of NO released from untreated and IFN $\gamma$ -treated HepG2 cells 24 h after infection with *B. pseudomallei*. However, we could neither detect NO in cell culture supernatants nor iNOS protein expression in lysates of infected HepG2 cells (data not shown). To assess the contribution of the NO pathway in the antibacterial effect of activated HepG2 cells, we used the iNOS inhibitor aminoguanidine (2 mM). However, after treatment with the iNOS inhibitor and infection of resting or IFN $\gamma$ -stimulated HepG2 cells, we could not observe any significant difference in intracellular killing of *B. pseudomallei* (**Figure 5**). This suggests that iNOS-derived NO is not necessary to eliminate *B. pseudomallei* in hepatocytes.

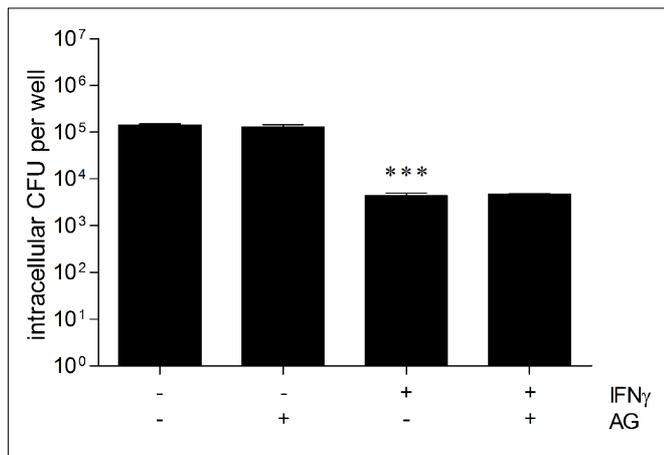
### NADPH OXIDASE IS NOT INVOLVED IN THE CONTROL OF *B. PSEUDOMALLEI* INFECTION

In a recent study we demonstrated that NADPH oxidase-mediated mechanisms contribute to early resistance in C57BL/6 mice *in vivo* and in BMM (Breitbach et al., 2006). Consequently, we investigated the role of NADPH oxidase in the intracellular *B. pseudomallei* elimination of hepatocytes. For this reason unstimulated and IFN $\gamma$ -stimulated HepG2 cells were pretreated with apocynin (500  $\mu$ M), a potent and selective inhibitor of NADPH oxidase, followed by infection with *B. pseudomallei*. In spite of a significantly higher invasion of apocynin-inhibited cells (data not shown), our

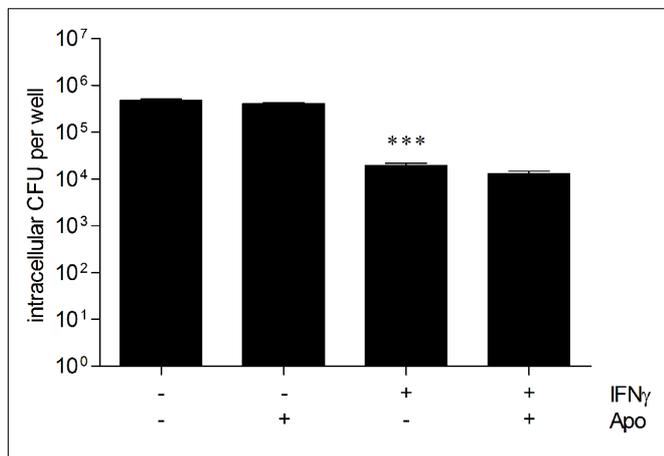
experiments indicated that bacterial growth of *B. pseudomallei* was not impaired by apocynin (Figure 6), suggesting that, in contrast to primary murine macrophages, NADPH oxidase does not contribute to antibacterial activity of human hepatocyte cells.

**SCAVENGERS/INHIBITORS OF REACTIVE OXYGEN SPECIES DO NOT INHIBIT THE ANTIBACTERIAL ACTIVITY**

To further study the involvement of reactive oxygen species as effector molecules of the antibacterial activity of hepatocytes, unstimulated, and IFN $\gamma$ -stimulated HepG2 cells were incubated



**FIGURE 5 | Inducible nitric oxide synthase (iNOS) is not necessary to eliminate *B. pseudomallei* in hepatocytes.** Intracellular replication of *B. pseudomallei* E8 24 h after infection (MOI 40:1) of resting and IFN $\gamma$ -activated (500 ng/ml) HepG2 cells in the presence of iNOS inhibitor aminoguanidine (AG, 2 mM). Data are presented as mean with SEM of triplicate determinations. One representative experiment out of three independent experiments is shown. Statistical analyses were performed using a Student's *t*-test (\*\*\*)  $p < 0.001$ .



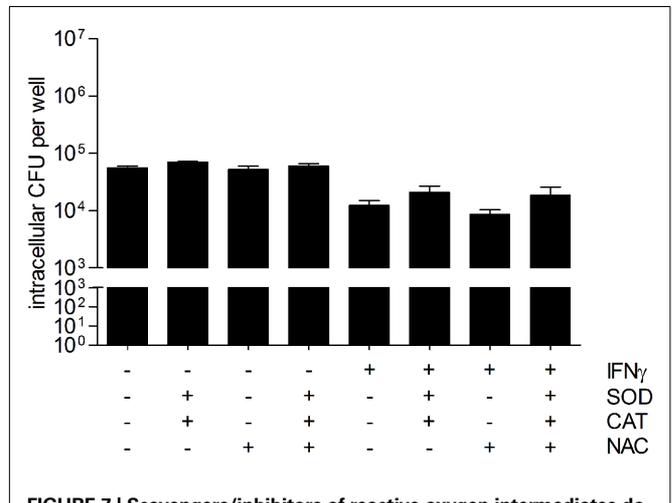
**FIGURE 6 | NADPH oxidase is not important for the control of *B. pseudomallei* hepatocyte infection.** Intracellular survival of *B. pseudomallei* E8 24 h after infection (MOI 31:1) of resting and IFN $\gamma$ -activated (500 ng/ml) HepG2 cells in the presence of NADPH oxidase inhibitor apocynin (Apo, 500  $\mu$ M). Data are presented as mean with SEM of triplicate determinations. One representative experiment out of three independent experiments is shown. Statistical analyses were performed using a Student's *t*-test (\*\*\*)  $p < 0.001$ .

with the free radical scavengers catalase (CAT, 200 U/ml), superoxide dismutase (SOD, 20 U/ml), and/or *N*-acetyl-cysteine (NAC, 200  $\mu$ M) prior to infection with *B. pseudomallei*. Figure 7 shows that treatment of IFN $\gamma$ -stimulated HepG2 cells with catalase and SOD slightly, but not significantly reversed the antibacterial activity. The effect of both antioxidant enzymes added at the same time was not further enhanced by addition of NAC. However, treatment of resting HepG2 cells did not increase the number of CFU recovered, indicating that basal, ROS-mediated antibacterial mechanisms do not exist in hepatocytes. Our results imply that reactive oxygen species do not play an important role in the control of *B. pseudomallei* replication.

**DISCUSSION**

The liver consists of both hepatocytes and different types of non-parenchymal cells including endothelial sinusoidal cells, Kupffer cells, and other immune cells. Although hepatocytes comprise 60% of cells in the liver and 80% of the hepatic volume (Malarkey et al., 2005), their role in antimicrobial defense is poorly understood. As the liver is among the most commonly affected organs in melioidosis, the present study was designed to examine the contribution of hepatocytes in host defense against *B. pseudomallei*.

To our knowledge, this is the first study showing that *B. pseudomallei* can infect and replicate within HepG2 cells (Figure 1A). The intracellular multiplication in hepatocytes is in accordance with the well-known ability of *B. pseudomallei* to proliferate inside several cell types, including phagocytic and non-phagocytic cells (Jones et al., 1996; Harley et al., 1998; Kespichayawattana et al., 2000). In addition, our results are consistent with other recently published data describing that intracellular *L. monocytogenes* (Gregory and Wing, 1993; Gregory et al., 1993; Wood et al., 1993; Szalay et al., 1995), *S. typhimurium* (Lajarin et al., 1996), and *Brucella*



**FIGURE 7 | Scavengers/inhibitors of reactive oxygen intermediates do not inhibit the antibacterial activity of hepatocytes.** Intracellular survival of *B. pseudomallei* E8 24 h after infection (MOI 42:1) of unstimulated and IFN $\gamma$ -stimulated (500 ng/ml) HepG2 cells in the presence of antioxidants superoxide dismutase (SOD, 20 U/ml), catalase (CAT, 200 U/ml), and *N*-acetyl-cysteine (NAC, 200  $\mu$ M). Data are presented as mean with SEM of triplicate determinations. One representative experiment out of three independent experiments is shown. Statistical analyses were performed using a Student's *t*-test.

*abortus* (Delpino et al., 2010) are able to invade and multiply within primary hepatocytes and hepatoma cells, respectively.

Several *in vitro* studies have demonstrated that *B. pseudomallei* T3SS-3 facilitates bacterial invasion of non-phagocytic cells (Stevens et al., 2003), contributes to escape from endosomes, intracellular survival, and replication (Stevens et al., 2002), and is necessary for the induction of caspase-1-dependent cell death in phagocytic cells (Sun et al., 2005). It has been reported that BsaU seems to be essential for control of needle length in T3SS-3 secretion apparatus (Sun and Gan, 2010) and important for the secretion of the T3SS-3 translocator protein BipD as well as the effector protein BopE (Hii et al., 2008). We report here that the intracellular growth of *B. pseudomallei* in hepatocytes is partially dependent on a functional T3SS, since mutant  $\Delta$ BPSS1539 (BsaU) exhibited reduced intracellular replication (**Figure 1C**) as well as decreased cell damage (**Figure 3C**) in HepG2 cells. In agreement with our previous study using HeLa cells (Pilatz et al., 2006), we found here that the transposon mutant of BsaU is unable to escape from the endocytotic vesicle after invasion, but is still able to proliferate within the vacuole (**Figure 2A**). We previously described that mutant  $\Delta$ BPSS1509, encoded within the *B. pseudomallei* T6SS-1 cluster, displays no intracellular growth defect in J774A.1 macrophages and HeLa cells, but is required for virulence in mice (Pilatz et al., 2006). In contrast, the present study indicates that mutant  $\Delta$ BPSS1509 reveals delayed and reduced induction of actin polymerization (data not shown) as well as decreased intracellular replication (**Figure 1C**) and reduced cell damage (**Figure 3C**) in hepatocytes compared to the wild-type. These results are in line with recent reports showing that different T6SS-1 mutants of *B. pseudomallei* exhibit reduced intracellular growth and cytotoxicity in RAW264.7 macrophages (Burtneck et al., 2011; Chen et al., 2011). Thus, our results suggest that BPSS1539 encoded by T3SS-3 and BPSS1509 encoded by T6SS-1 are potential virulence determinants that enable intracellular growth of *B. pseudomallei* and induce cell damage in hepatocytes. However, these factors are unlikely the main players responsible for intracellular survival in these cells, since HepG2 cells were not able to eliminate the corresponding mutants.

Hepatocytes have been shown to express a variety of cytokines (Rowell et al., 1997; Stonans et al., 1999) and to respond to bacterial or viral infections with enhanced secretion of inflammatory cytokines and chemokines (Rowell et al., 1997; Santos et al., 2005). Since it has been shown that numerous cytokines can modulate resistance to intracellular bacterial infections, primarily by stimulating antimicrobial activities (Ehlers et al., 1992; Autenrieth et al., 1994; Bohn et al., 1994; Santanirand et al., 1999), we investigated the impact of cytokine-activated hepatocytes in intracellular *B. pseudomallei* clearance. IFN $\gamma$  was previously shown to be a major factor in primary host defense to *B. pseudomallei* (Santanirand et al., 1999; Breitbach et al., 2006). In addition, several reports described that both primary murine hepatocytes and hepatocyte cell lines are efficiently activated by IFN $\gamma$  to kill intracellular *L. monocytogenes* or *S. typhimurium* (Gregory and Wing, 1993; Lajarin et al., 1996). This study, demonstrating that IFN $\gamma$  is capable to enhance the anti-*Burkholderia* activity of HepG2 cells (**Figure 4A**) argues for a potential role of activated hepatoma cells in host defense against this pathogen. Although IL-1 $\beta$  and

TNF $\alpha$  alone failed to significantly activate antibacterial activity of HepG2 cells, the combination of both cytokines together with IFN $\gamma$  caused the most prominent growth inhibition in hepatoma cells (**Figures 4B,C**). These results are in line with several reports indicating that murine hepatocytes and hepatoma cell lines stimulated with a combination of IFN $\gamma$ , TNF $\alpha$ , IL-6, IL-1 $\beta$ , or LPS exhibited the strongest antibacterial activity against *L. monocytogenes* (Szalay et al., 1995; Haponsaph and Czuprynski, 1996) and *S. typhimurium* (Lajarin et al., 1996, 1999). Therefore, our findings suggest that inflammatory cytokines, particularly IFN $\gamma$ , are important for resistance of hepatocytes against *B. pseudomallei*.

However, the mechanism responsible for the anti-*B. pseudomallei* activity of cytokine-activated hepatoma cells remains unclear. In a previous study we could show that iNOS-generated NO contributes to resistance in innate susceptible BALB/c mice in a murine model of melioidosis as demonstrated by enhanced mortality rates and higher bacterial loads in liver and spleen of aminoguanidine-treated mice compared to untreated control mice (Breitbach et al., 2011). Moreover, inhibition of iNOS in IFN $\gamma$ -stimulated and *B. pseudomallei*-infected BALB/c-derived macrophage cell lines revealed that reactive nitrogen species are involved in restricting the intracellular growth of *B. pseudomallei* (Miyagi et al., 1997; Utaisincharoen et al., 2001, 2003). Our present data indicate, however, that reactive nitrogen species do not play any role in controlling *B. pseudomallei* growth in hepatocytes since inhibition of NO production by aminoguanidine did not reverse the antibacterial activity of HepG2 cells (**Figure 5**). These results are in good accordance with reports demonstrating that elimination of *L. monocytogenes* (Gregory et al., 1993; Szalay et al., 1995; Haponsaph and Czuprynski, 1996) and *S. typhimurium* (Lajarin et al., 1996) from activated hepatocytes is not mediated by nitric oxide.

Besides the production of NO, the generation of ROS is another potential mechanism in the control of bacterial pathogens (Inoue et al., 1995; Vazquez-Torres et al., 2000; Breitbach et al., 2006). In a previous study we demonstrated that NADPH oxidase is able to restrict intracellular replication of *B. pseudomallei* in primary murine macrophages (Breitbach et al., 2006). But the present findings suggest that neither NADPH oxidase (**Figure 6**) nor other ROS-generating enzymes (**Figure 7**) are involved in the anti-*B. pseudomallei* activity of activated hepatoma cells. In contrast, some reports provided evidence of ROS-dependent mechanisms in both primary murine hepatocytes and hepatocyte cell lines against intracellular *L. monocytogenes* (Gregory and Wing, 1993) or *S. typhimurium* (Lajarin et al., 1996) since antibacterial activity was inhibited by the presence of scavengers or inhibitors of reactive oxygen species. In this context, Lajarin et al. (1999) indicated that cyclooxygenases (COX) are a potential source of ROS in *S. typhimurium* infected murine hepatocyte cell lines, and thus contribute to antibacterial host defense. However, several studies revealed that COX are involved in intracellular replication and survival of different pathogens (Uchiya and Nikai, 2004; Sadikot et al., 2007; Serezani et al., 2007).

In summary, the present study describes the *in vitro* invasion and intracellular replication of *B. pseudomallei* in human hepatocyte HepG2 cells involving type 3 and type 6 secretion systems.

We provide evidence that IFN $\gamma$  contributes to growth restriction of *B. pseudomallei* whereas the combination with IL-1 $\beta$  and TNF $\alpha$  is necessary for the maximal antibacterial activity of hepatocytes. As the IFN $\gamma$ -mediated effects are independent of NO and ROS production, we assume that other defense mechanisms are important to inhibit intracellular multiplication of *B. pseudomallei*. Thus, further investigations are required to elucidate the

NO- and ROS-independent mechanisms responsible for restricting intracellular growth of *B. pseudomallei* in cytokine-activated hepatocytes.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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