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Akt-Mediated Proinflammatory Response of Mononuclear Phagocytes Infected with *Burkholderia cenocepacia* Occurs by a Novel GSK3β-Dependent, IκB Kinase-Independent Mechanism

Thomas J. Cremer,* Prexy Shah,* Estelle Cormet-Boyaka,* Miguel A. Valvano,[†,‡] Jonathan P. Butchar,* and Susheela Tridandapani*

The environmental bacterium *Burkholderia cenocepacia* causes opportunistic lung infections in immunocompromised individuals, particularly in patients with cystic fibrosis. Infections in these patients are associated with exacerbated inflammation leading to rapid decay of lung function, and in some cases resulting in cepacia syndrome, which is characterized by a fatal acute necrotizing pneumonia and sepsis. *B. cenocepacia* can survive intracellularly in macrophages by altering the maturation of the phagosome, but very little is known on macrophage responses to the intracellular infection. In this study, we have examined the role of the PI3K/Akt signaling pathway in *B. cenocepacia*-infected monocytes and macrophages. We show that PI3K/Akt activity was required for NF-κB activity and the secretion of proinflammatory cytokines during infection with *B. cenocepacia*. In contrast to previous observations in epithelial cells infected with other Gram-negative bacteria, Akt did not enhance IκB kinase or NF-κB p65 phosphorylation, but rather inhibited GSK3β, a negative regulator of NF-κB transcriptional activity. This novel mechanism of modulation of NF-κB activity may provide a unique therapeutic target for controlling excessive inflammation upon *B. cenocepacia* infection. *The Journal of Immunology*, 2011, 187: 635–643.

Burkholderia cenocepacia is one of the members of the *Burkholderia cepacia* complex. This is a Gram-negative opportunistic respiratory pathogen that represents a threat to immunocompromised individuals, especially patients with cystic fibrosis (1–3). What is particularly troublesome is that these bacteria are also highly resistant to many antibiotics, making treatment difficult (4, 5). Because of this, it is critical to gain an understanding of host immune responses to this organism, as this may provide alternative means to overcoming the current limitations for treatment.

One major contributing factor to the morbidity and mortality caused by *B. cenocepacia* infection is an exacerbated inflammatory response, which causes collateral tissue damage (6, 7). Accordingly, administration of anti-inflammatory corticosteroids has been associated with favorable patient outcome during *B. cepacia* infection (8). It has also been demonstrated in vivo that TLR/MyD88-driven inflammation is detrimental to the host, as MyD88$^{-/-}$ mice show a survival advantage over wild-type mice after challenge with *B. cencoepacia* (9). Indeed, TLR5 plays a key role in promoting exacerbated inflammation in susceptible individuals (10, 11).

One of the key downstream effects of TLR/MyD88 pathway activation is TNF-α production. This cytokine has been shown to be a major mediator of mortality in an in vivo mouse model of *B. cenocepacia* infection, as TNF-α$^{-/-}$ mice were protected against a challenge lethal to wild-type mice (9). Hence, an understanding of how infection leads to TNF-α production may lead to newer, more effective treatments designed to regulate TNF-α production and other deleterious proinflammatory responses.

Numerous studies have highlighted the importance of macrophages during *B. cenocepacia* infection, as they are a site of bacterial replication much like lung epithelial cells (12, 13). It is interesting to note that CFTR-defective– or CFTR-inhibitor–treated wild-type murine macrophages show delayed phagolysosomal fusion compared with control (14). This helps to explain the persistence of *B. cenocepacia* in individuals with cystic fibrosis, as their macrophages would be less able to control the bacteria. Monocytes/macrophages are also major producers of inflammatory mediators such as TNF-α and IL-8 (7, 15–17), which contribute to the hyperinflammatory state following *B. cenocepacia* infection.

PI3K/Akt signaling is known to regulate various biological functions, including the proinflammatory response to TLR signaling. However, its effect on inflammatory response differs, depending upon several factors that remain to be fully understood (18). In this study, we have investigated the role of PI3K/Akt signaling on IκB kinase (IKK)/NF-κB activation and the ensuing proinflammatory response from mononuclear phagocytes infected with

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Abbreviations used in this article: IKK, IκB kinase; MOI, multiplicity of infection; PBM, peripheral blood monocytes.

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B. cenocepacia. We demonstrate that PI3K and Akt promote NF-κB activity and proinflammatory cytokine production, but not through IKK nor NF-κB p65 phosphorylation. Instead, PI3K/Akt serves to inactivate GSK3β, a downstream repressor of NF-κB. These findings suggest that targeting PI3K/Akt/GSK3β signaling may be of therapeutic value within the context of *B. cenocepacia* infection.

Materials and Methods

Cells and reagents

RAW 264.7 cells obtained from American Type Culture Collection were cultured in RPMI 1640 (Life Technologies-BRL, Rockville, MD) supplemented with 5% heat-inactivated FBS (HyClone, Logan, UT), L-glutamine, penicillin (10,000 U/ml), and streptomycin (10,000 μg/ml) (Invitrogen, Carlsbad, CA). The BAY 11-7085 (5 μM) IKK inhibitor was a generous gift from D. Guttridge (The Ohio State University). LY294002 (20 μM) PI3K inhibitor was obtained from Calbiochem (San Diego, CA). SB-216763 (2 μM) GSK3β inhibitor was obtained from Sigma-Aldrich (St. Louis, MO). DMSO vehicle control (0.2%) was obtained from Sigma-Aldrich. Akt inhibitor X (10 μM) was obtained from Calbiochem and dissolved in water. Abs against phospho-Akt-Ser^{473}, phospho-IKKα-Ser^{180}/β-Ser^{181}, phospho-NF-κBp65-Ser^{536}, phospho-GSK3α-Ser^{21}/β-Ser^{9}, phospho-GSK3β-Ser^{9}, and GSK3β were purchased from Cell Signaling (Beverly, MA). Abs against Akt and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Bone marrow-derived macrophages

Wild-type and MyrAkt-expressing mice were sacrificed according to institution-approved animal care and use protocols. Bone marrow cells were collected and differentiated, as previously described, with M-CSF (R&D Systems, Minneapolis, MN) and polymyxin-B (CalBiochem) (19–21).

Peripheral blood monocyte isolation

Human peripheral blood monocytes (PBM) were isolated by centrifugation through a Ficoll gradient, followed by CD14-positive selection by MACS (Miltenyi Biotec, Auburn, CA), according to manufacturer instructions, as previously described (22).

Bacterial infections

All monocyte/macrophage infections were conducted in 5 or 10% heat-inactivated FBS-containing RPMI 1640 without antibiotic. *B. cenocepacia* K56-2 isolate was grown in Luria Bertani medium (Sigma-Aldrich) for 12–14 h to postlogarithmic phase. OD at 600 nm was taken to assess the density of cultures and calculate the multiplicities of infection. Serial dilutions of cultures and plating on Luria Bertani agar to count CFU were done to verify accuracy of the multiplicity of infection (MOI) calculations. Prior to infection, cultures were centrifuged, washed, and resuspended in macrophage culture media.

ELISA cytokine measurements

Cell-free supernatants were assayed by sandwich ELISA. Human TNF-α, mouse TNF-α, mouse IL-6, and mouse RANTES ELISA kits were obtained from R&D Systems, and human IL-6 and human IL-8 ELISA kits were obtained from eBioscience (San Diego, CA). The manufacturer's instructions were followed, as previously described (19, 20).

Western blot analysis

Cells were lysed in T_N1 buffer (50 mM Tris [pH 8.0], 10 nm EDTA, 10 M $Na_4P_2O_7$, 10 nM NaF, 1% Triton X-100, 125 nM NaCl, 10 nM Na_3VO_4, and 10 μg/ml of both aprotinin and leupeptin). Whole-cell lysates were electrophoretically separated on 10% acrylamide gels, transferred to nitrocellulose membranes, and then probed with Ab of interest. Detections were performed using HRP-conjugated secondary Abs, followed by development with ECL Western blotting substrate (Pierce, Rockford, IL), as previously described (19, 20).

CFU assays

Assays using the aminoglycoside-sensitive strain of K56-2 were conducted as done by Hamad et al. (13). Primary macrophages were infected with *B. cenocepacia* and then placed on a rocker at room temperature for 5 min, followed by centrifugation at 400 × *g* for 4 min. After 1 h of infection, the media was removed and cells were washed with PBS. Media containing 50 μg/ml of gentamicin was added for 30 min. We found that under these conditions >98% of the aminoglycoside-sensitive K56-2 strain was killed, although <12% of the parental K56-2 strain was killed under the same conditions. After treatment, cells were again washed in PBS to remove antibiotic, then either lysed in 0.1% Triton X-100 (1-h infection time) or resuspended in culture media for later lysis at 8- and 24-h time points. Lysed samples were immediately serially diluted 10-fold and plated on Luria Bertani agar plates. At 24- to 36-h postplating, CFUs were counted. The dual-antibiotic CFU assays were conducted as above, with the exception that parental strain K56-2 was used and extracellular bacteria were killed with gentamicin (500 μg/ml) plus ceftazidime (250 μg/ml) for 1 h (12, 23). We found that this killed >98% of K56-2 *B. cenocepacia*.

Luciferase assays

Transfected macrophages were uninfected or infected for 5 h (time of robust NF-κB–luciferase activity; Supplemental Fig. 1) with *B. cenocepacia*. At the end of the infection period, cells were washed with PBS and then lysed in 200 μl Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI). Luciferase activity was then measured using Luciferase Assay Reagent (Promega), as previously described (20). Data are expressed as percentage of increase over matched uninfected control samples.

Transfection

A total of 5 μg pcDNA vector with or without wild-type human GSK3β, Addgene plasmid 14753 (24) (Addgene, Cambridge, MA), was used for each transfection with 10×10^6 RAW 264.7 cells. Amaxa solution V was used for electroporation using program U-14, as previously described (20). Infections were performed 14 h posttransfection. Overexpression was con-

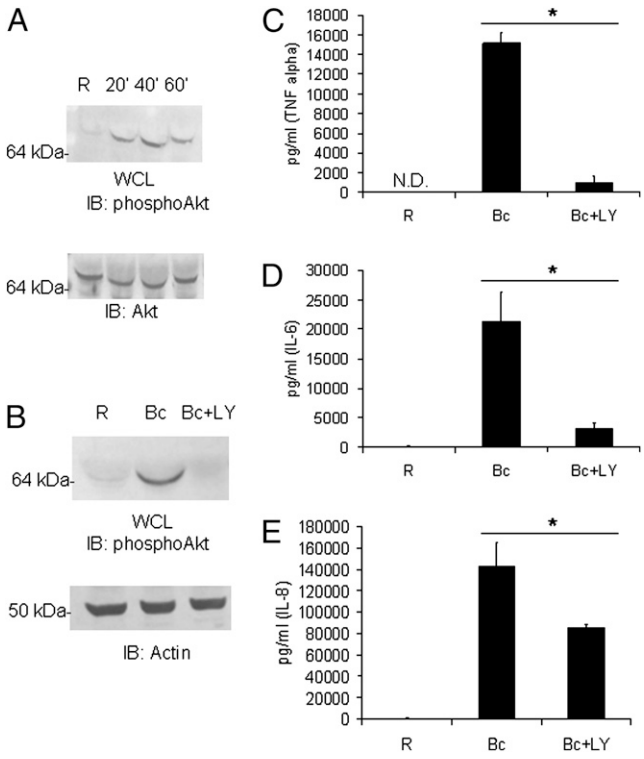

FIGURE 1. The PI3K/Akt pathway is activated by *B. cenocepacia*, and PI3K is required for inflammatory cytokine production. *A*, Primary human PBM were infected with *B. cenocepacia* (B.c.) at a MOI of 5 for 20, 40, or 60 min. Western blots on cell lysates were done to measure pSer^{473}-Akt, followed by reprobes for total Akt as a loading control. *B*, PBM were pretreated with DMSO vehicle control or the PI3K inhibitor LY294002 (20 μM) for 30 min, and then infected with *B. cenocepacia* (Bc) at a MOI of 5 for 8 h. To ensure inhibitor effectiveness, cells were lysed and pSer^{473}-Akt was measured by Western blotting, with actin reprobe as loading control. *C–E*, Proinflammatory cytokine production of PBM from *B* was measured by performing sandwich ELISAs on cell-free supernatants for TNF-α (*C*), IL-6 (*D*), or IL-8 (*E*). Three independent experiments were done. *$p < 0.05$, Student *t* test. R, resting or uninfected samples.

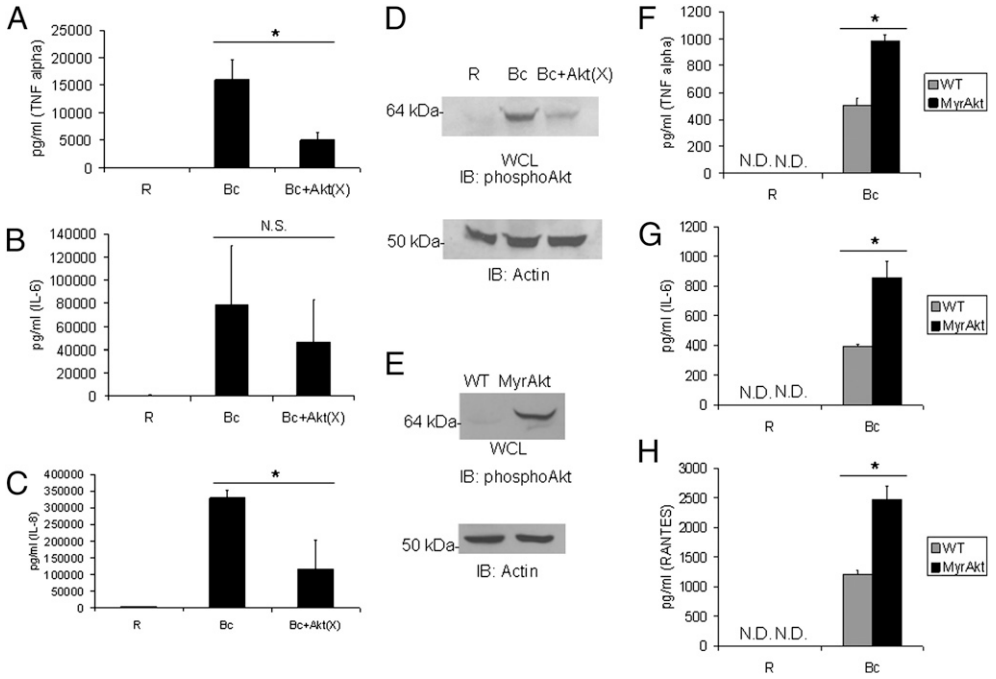

FIGURE 2. Akt promotes the proinflammatory cytokine production elicited by *B. cenocepacia. A–C*, PBM were pretreated with H_2O vehicle control or the Akt inhibitor Akt(X) (10 μM) for 30 min, and then infected with *B. cenocepacia* (Bc) at a MOI of 5 for 8 h. Sandwich ELISAs were done to measure TNF-α (*A*), IL-6 (*B*), or IL-8 (*C*) in cell-free supernatants. Three independent experiments were done. $*p < 0.05$, Student *t* test. *D*, Matched PBM samples run in parallel to that of *A* were lysed, and pSer-Akt was measured by Western blotting, followed by reprobe for actin as a loading control. *E*, Bone marrow-derived macrophages from wild-type mice and transgenic mice expressing a macrophage-specific myristoylated Akt were lysed, and pSer-Akt was measured by Western blotting. *F–H*, Bone marrow-derived macrophages from wild-type or MyrAkt-expressing mice were infected with *B. cenocepacia* (Bc) at a MOI of 5 for 8 h. Sandwich ELISAs were done to measure TNF-α (*F*), IL-6 (*G*), and RANTES (*H*). This represents a triplicate of biological samples. $*p < 0.05$, Student *t* test.

firmed by Western blotting of GSK3β. For NF-κB activity assays, 2 μg NF-κB luciferase reporter plasmid was transfected into RAW 264.7 cells, as described above. Transfection efficiency is routinely tested by flow cytometry analysis of enhanced GFP-transfected cells. We are able to achieve ≤80% transfection efficiency in Raw 264.7 cells with minimal effect on cell viability.

Results

PI3K signaling is required for the proinflammatory response to B. cenocepacia

PI3K signaling leads to generation of phosphatidylinositol-(3,4)-bisphosphate and phosphatidylinositol-(3,4,5)-trisphosphate, which

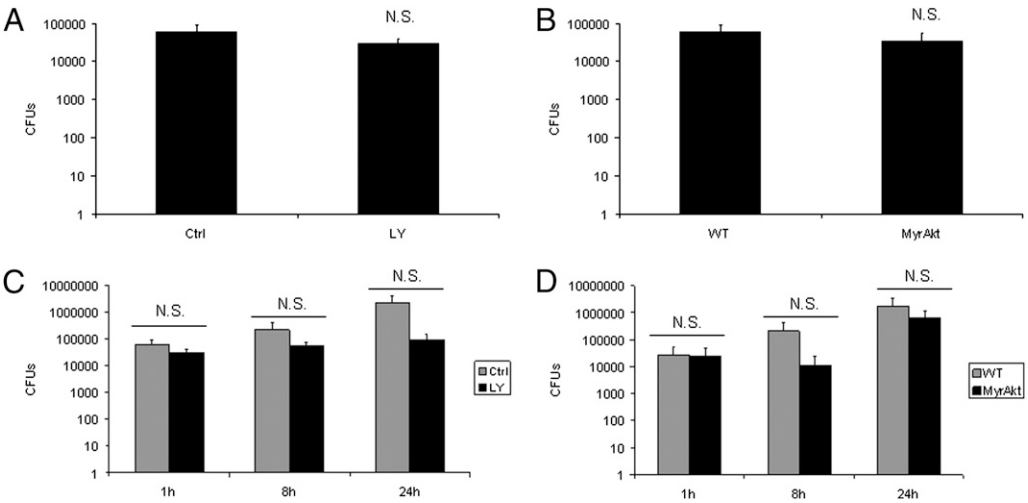

FIGURE 3. PI3K/Akt does not regulate phagocytosis or intracellular replication of *B. cenocepacia* within macropahges. *A*, 4×10^5 bone marrow-derived macrophages (BMM) were pretreated with DMSO or LY294002 for 30 min and then infected with *B. cenocepacia* at a MOI of 5 for 1 h. At the end of infection, cells were washed and treated with gentamicin to kill extracellular bacteria for 30 min. Cells were washed, lysed, serially diluted, and plated on Luria Bertani agar. Graphs represent the average and SD of recovered CFUs for three independently infected samples. A Student *t* test was done to determine significance. $p > 0.05$. *B*, Wild-type or MyrAkt BMM were infected with *B. cenocepacia* (*B.c.*) at a MOI of 5 for 1 h, as done in *A*. *C*, 4×10^5 BMM were pretreated with DMSO or LY294002 for 30 min, and then infected with *B. cenocepacia* at a MOI of 5 for 1 h. Samples were lysed at 1 h or cultured in fresh macrophage culture medium for 8 or 24 h and then lysed. Graphs represent the average and SD of recovered CFUs for three independently infected samples. A Student *t* test was done to determine significance. $p > 0.05$. *D*, 4×10^5 wild-type or MyrAkt BMM were infected and treated, as in *C*.

result in phosphorylation and activation Akt at the plasma membrane. This drives numerous cellular processes, including the production and release of proinflammatory mediators (18, 25). In this study, we examined PI3K/Akt signaling in primary human monocytes infected with *B. cenocepacia*. PBM were isolated and infected with *B. cenocepacia* at a MOI of 5 for time points of 20, 40, or 60 min. Whole-cell lysates were then probed for phospho-Akt levels by Western blotting, followed by reprobing for total Akt. It is apparent that Akt is robustly activated within 20 min of infection and is sustained through the first hour (Fig. 1*A*). We also tested the ability of killed *B. cenocepacia* to activate Akt and found that both live and dead bacteria induced this response within 1 h (data not shown). Therefore, this signaling event is not dependent upon bacterial viability.

To then investigate the function of PI3K on host-inflammatory response, PBM were pretreated with vehicle control or the PI3K inhibitor LY294002 (26) and infected with *B. cencoepacia* at a MOI of 5 for 8 h. The effectiveness of the inhibitor under these conditions was verified by Western blotting of phospho-Akt. PBM pretreated with vehicle showed high levels of Akt activation upon infection, whereas PBM given the PI3K inhibitor and infected did not (Fig. 1*B*).

Next, we examined the production of proinflammatory cytokines in the presence or absence of the PI3K inhibitor in PBM infected with *B. cenocepacia*. Matched supernatant from the samples in Fig. 1*B* was collected, and proinflammatory cytokine production was assayed by ELISA. Infected PBM showed strong induction of TNF-α, IL-6, and IL-8 compared with uninfected cells. However, PBM pretreated with LY294002 produced significantly less post-infection (Fig. 1*C–E*). Similar results were obtained with bone marrow-derived murine macrophages (data not shown). Therefore, the inflammatory response to *B. cenocepacia* by monocytes/macrophages requires PI3K signaling.

Akt promotes the proinflammatory response to B. cenocepacia

Given that class I PI3K activate Akt (25) and we find that PI3K is required for the monocyte/macrophage proinflammatory response to *B. cenocepacia*, we next examined whether Akt activation could be linked to the proinflammatory response in infected PBMs. Therefore, PBM were pretreated with the Akt inhibitor Akt (X) (27) or vehicle control and infected with *B. cenocepacia* for 8 h. Inhibition of Akt led to reduced production of TNF-α, IL-6, and IL-8 compared with cells pretreated with vehicle control (Fig. 2*A–C*). The reduction in IL-6 did not reach statistical significance, however, and this may be due to residual Akt activation even in the presence of Akt(X) following infection (Fig. 2*D*).

The above findings suggest that Akt activation is required for cytokine production following *B. cenocepacia* infection. To test whether Akt could drive production of these cytokines, we infected bone marrow-derived macrophages from wild-type mice or from mice expressing a macrophage-specific myristoylated Akt (MyrAkt) (28). We verified the constitutive activation of Akt in unstimulated macrophages by phospho-Akt Western blot (Fig. 2*E*). Following 8-h infection of wild-type and MyrAkt macrophages with *B. cenocepacia*, we performed ELISAs and found significantly enhanced production of TNF-α, IL-6, and RANTES in the MyrAkt-expressing cells (Fig. 2*F–H*).

PI3K/Akt do not regulate uptake nor intramacrophage replication of B. cenocepacia

PI3K signaling has been reported to influence both phagocytosis (29, 30) and intramacrophage survival (21) of other Gram-negative bacteria. To evaluate its role in macrophages infected with *B. cenocepacia*, we investigated bacterial uptake and replication in PBM after inhibition of PI3K. To overcome the natural antibiotic resistance of *B. cenocepacia* and to facilitate these experiments, we used an aminoglycoside-sensitive strain of *B. cenocepacia* (strain MH1K) that can be killed with a low concentration of gentamicin, but does not differ with the parental isolate in terms of intracellular survival and phagosomal trafficking (13). We pretreated primary murine macrophages with LY294002 and infected with *B. cenocepacia* MH1K. In parallel, we also infected MyrAkt-expressing macrophages. CFU were determined at 1 h of infection to estimate bacterial uptake (Fig. 3*A*, 3*B*) and at 8 and 24 h postinfection to estimate intracellular replication (Fig. 3*C*, 3*D*). We found no influence of PI3K/Akt activity on internalization or bacterial replication. We also performed these experiments with the parental *B. cenocepacia* strain plus the dual-antibiotic treatment of gentamicin (500 μg/ml) and ceftazidime (250 μg/ml), as done by Sajjan et al. (12), and the results also indicated no effect of PI3K/Akt on phagocytosis or intracellular replication of *B. cenocepacia* in mononuclear phagocytes (data not shown). These findings suggest that PI3K/Akt can be targeted to limit inflammation without altering the intramacrophage life cycle of *B. cenocepacia*.

IKK/NF-κB are activated by B. cenocepacia *and required for inflammatory responses*

We have shown that PI3K/Akt promotes inflammatory responses to *B. cenocepacia* without influencing uptake or replication in

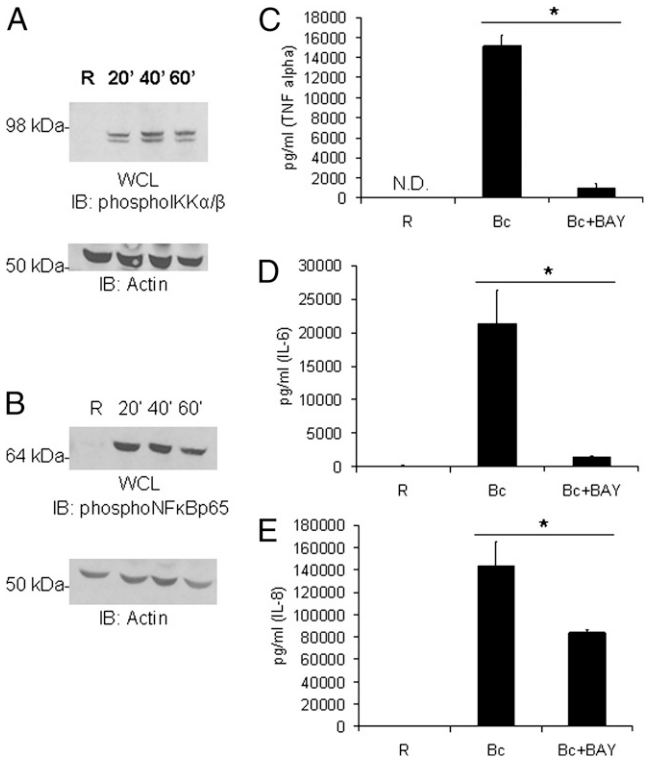

FIGURE 4. The IKK/NF-κB pathway is activated by *B. cenocepacia* and is required for proinflammatory cytokine production. *A*, PBM were infected with *B. cenocepacia* at a MOI of 5 for 20, 40, or 60 min. Cells were lysed and proteins analyzed by Western blotting for phospho-IKKα/β, followed by anti-actin as a loading control. *B*, $pSer^{536}$ of NF-κB p65 was measured in matched samples from Fig. 3*A* by Western blotting and reprobed with anti-actin as a loading control. *C–E*, PBM were pretreated with DMSO vehicle control or an IKK/NF-κB inhibitor, BAY7085 (5 μM), for 30 min, and then infected with *B. cenocepacia* (Bc) at a MOI of 5 for 8 h. Sandwich ELISAs were done to measure levels of TNF-α (*C*), IL-6 (*D*), or IL-8 (*E*) in cell-free media samples. Graphs represent three biological samples; $*p < 0.05$, Student *t* test. R, resting or uninfected samples.

macrophages. Next, to understand how PI3K and Akt promote this inflammatory response, we examined activation of the IKK/NF-κB pathway. NF-κB drives production of proinflammatory cytokines, and has been shown to be responsive to PI3K/Akt signaling (20). We infected PBM with *B. cenocepacia* for 20, 40, and 60 min, and measured IKK phosphorylation by Western blotting. Results show that there was phosphorylation of IKKα and β within the first 20 min of infection (Fig. 4*A*). Phosphorylation of the IKK complex triggers a number of events that lead to NF-κB nuclear translocation and gene transcription (31). The NF-κBp65 subunit itself can also be phosphorylated, which enhances its transcriptional activity (32). We examined the status of Ser^{536} phosphorylation on NF-κBp65 and found that it was also rapidly phosphorylated in response to *B. cenocepacia* infection (Fig. 4*B*).

To test the requirement of NF-κB for mediating proinflammatory responses to *B. cenocepacia*, we pretreated PBM with an IKK inhibitor, BAY 11-7085 (33), before infection. In the presence of this inhibitor, *B. cenocepacia*-driven production of TNF-α, IL-6, and IL-8 was greatly diminished compared with control following infection (Fig. 4*C–E*). These results indicate that IKK signaling to NF-κB is a major mediator of proinflammatory responses to *B. cenocepacia*. In light of our finding that PI3K/Akt promoted cytokine production postinfection, the possibility arose that PI3K/Akt drove IKK/NF-κB activation.

Activation of IKK/NF-κB and PI3K/Akt is independent of each other

Our data indicate that both PI3K/Akt and IKK/NF-κB are required for the monocyte/macrophage inflammatory response to *B. cenocepacia*. To determine whether the two pathways were linked, we examined their activation in the presence or absence of specific inhibitors. PBM were pretreated with vehicle control, PI3K inhibitor (LY294002), or IKK inhibitor (BAY 11-7085), and then infected with *B. cenocepacia* for the indicated time points. Surprisingly, the PI3K inhibitor did not impair IKKα/β phosphorylation (Fig. 5*A*) nor NF-κBp65-Ser^{536} phosphorylation (Fig. 5*B*) despite blocking Akt phosphorylation. Conversely, the IKK inhibitor blocked IKKα/β and NF-κBp65-Ser^{536} phosphorylation, but not Akt phosphorylation (Fig. 5*C*). These results show that each pathway is activated independently of the other following *B. cenocepacia* infection.

Because our earlier results (Fig. 2) showed that MyrAkt-expressing macrophages produced more proinflammatory cytokines than wild type upon infection, we also examined IKK/NF-κB phosphorylation in the MyrAkt-expressing macrophages. Wild-type and MyrAkt macrophages were infected with *B. cenocepacia* for the indicated time points. Consistent with results in Fig. 1*A*, Akt activation did not enhance phosphorylation of IKK or NF-κB (Fig. 5*D*, 5*E*). We have shown that PI3K/Akt are required for cytokine production (Figs. 1, 2), but these results suggest that either PI3K/Akt signaling is required in parallel to NF-κB for cytokine production, or alternatively, that any effect of PI3K/Akt on NF-κB would be downstream of NF-κB activation itself.

To directly test whether PI3K/Akt influenced NF-κB–driven gene transcription, we transiently transfected RAW 264.7 cells with the NF-κB luciferase reporter and pretreated with PI3K inhibitor (LY294002) or IKK inhibitor (BAY 11-7085). Cells were then infected, and NF-κB activity was measured by luciferase assay. NF-κB activity markedly increased upon infection, and as expected, the IKK inhibitor (BAY 11-7085) blocked this response. Strikingly, the PI3K inhibitor (LY294002) also strongly impaired NF-κB activity (Fig. 6*A*). These results confirm that PI3K signaling is required for NF-κB–driven gene trasncription following *B. cenocepacia* infection. Hence, we concluded from all of the results that in infected mononuclear phagocytes, PI3K influences NF-κB activity, but does so downstream of IKK.

PI3K/Akt regulates NF-κB activity through GSK3β

One strong candidate downstream target of Akt is GSK3β. This molecule has been reported to both promote (34) and repress (35–

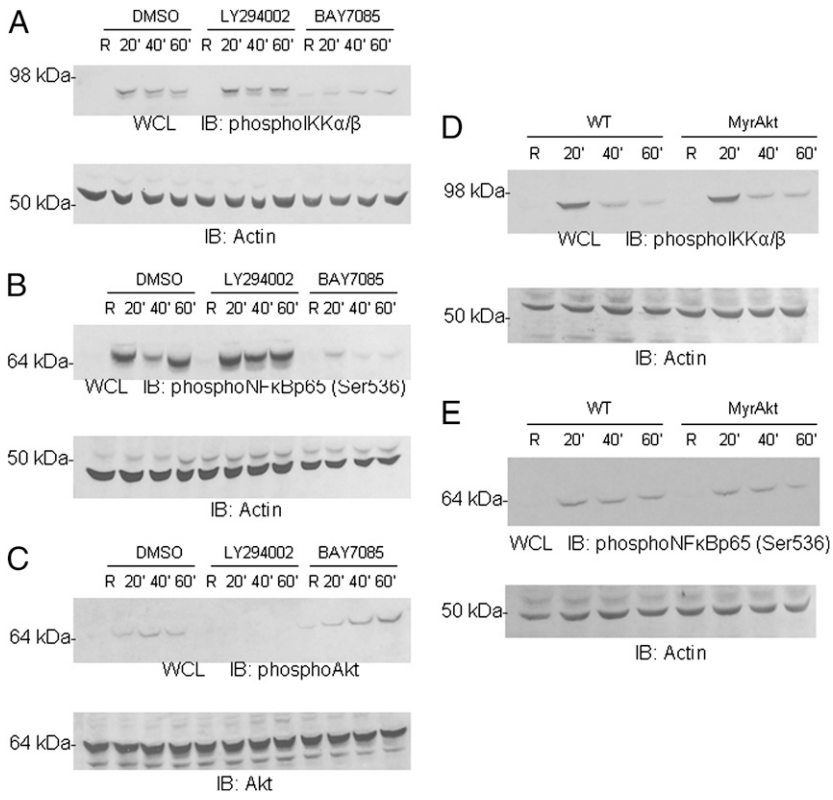

FIGURE 5. PI3K/Akt do not influence IKK/NF-κB phosphorylation. *A–C*, PBM were pretreated with DMSO vehicle control LY294002 (PI3K inhibitor) or BAY 11-7085 (IKK/NF-κB inhibitor) for 30 min and then infected with *B. cenocepacia* at a MOI of 5 for 20, 40, or 60 min. Cells were lysed, and Western blotting was done to measure phospho-IKKα/β (*A*), phospho-NF-κBp65 (Ser^{536}) (*B*), and phosphor-serine Akt (*C*). Actin and total Akt were probed as loading controls. *D* and *E*, Bone marrow-derived macrophages from wild-type or myrAkt-expressing mice were infected with *B. cenocepacia* at a MOI of 5 for 20, 40, or 60 min. Cells were lysed and Western blotting was done to measure phospho-IKKα/β (*D*) and phospho-NF-κBp65 (Ser^{536}) (*E*). Actin was used as a loading control. R, resting or uninfected samples.

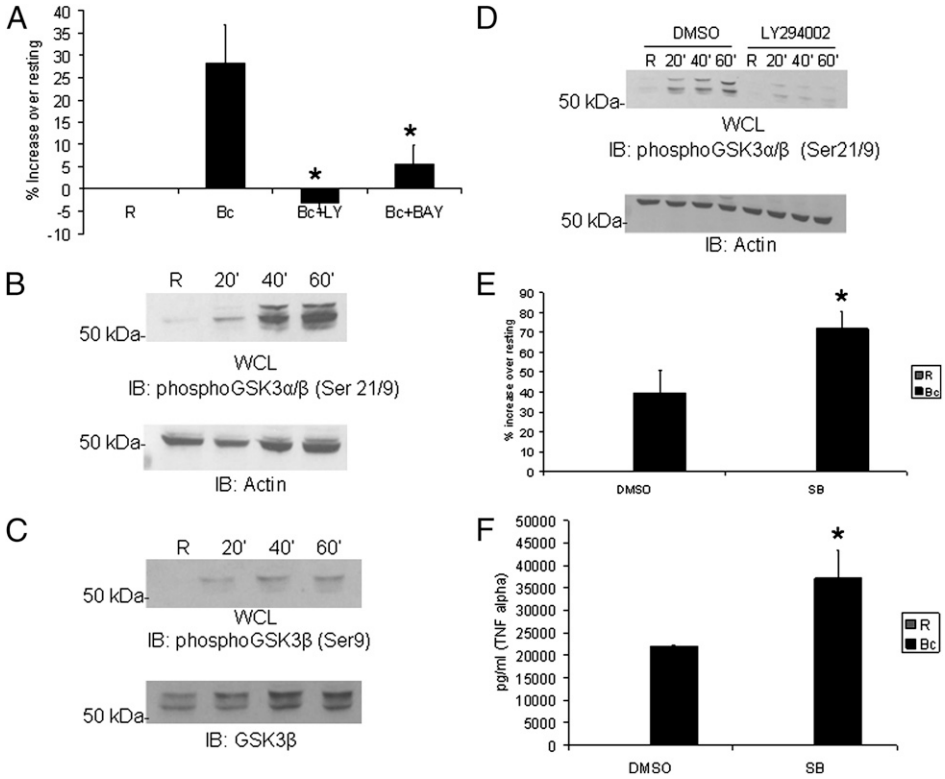

FIGURE 6. The PI3K/Akt pathway regulates NF-κB activity through GSK3β. *A*, RAW264.7 cells were transfected with a NF-κB luciferase reporter. Fourteen hours posttransfection, these cells were infected with *B. cenocepacia* (Bc) at a MOI of 5 for 5 h, the infection time yielding robust NF-κB–luciferase activity (Supplemental Fig. 1). Luciferase activity was measured by a luminometer, and values were converted into percentage of increase over matched resting/uninfected samples. *B*, PBM were infected with *B. cenocepacia* for 20, 40, or 60 min. Western blotting was done on cell lysates to measure phospho-GSK3α/β, and then reprobed with anti-actin as a loading control. *C*, Matched cell lysates from Fig. 5*B* were tested by Western blotting for phospho-GSK3β, and then reprobed for total GSK3β as a loading control. *D*, PBM were treated with DMSO vehicle control or the LY294002 for 30 min, and then infected with *B. cenocepacia* for 20, 40, or 60 min. Western blots were done on cell lysates for phospho-GSK3α/β, followed by reprobing for actin as a loading control. *E*, RAW264.7 cells were transfected with a NF-κB luciferase reporter plasmid. Transfected cells were pretreated with DMSO vehicle control or SB-216763 GSK3β inhibitor for 30 min, and then infected with *B. cenocepacia* (Bc) at a MOI of 5 for 5 h. Cells were lysed, and luciferase activity was measured by a luminometer. Data are expressed as percentage of increase in activity over matched uninfected control. *F*, RAW 264.7 cells were pretreated with DMSO vehicle control or SB-216763, and then infected with *B. cenocepacia* (Bc) at a MOI of 5 for 8 h. Cleared cell lysates were assayed for TNF-α production by ELISA. Data represent the average of three samples, and error bars denote SD. $*p < 0.05$, Student *t* test. R, uninfected cells.

37) NF-κB activity. This molecule is normally active until phosphorylation occurs on Ser^{21} of GSK3α (38) or Ser^{9} of GSK3β (39). We infected PBM with *B. cenocepacia* and examined both GSK3α and GSK3β phosphorylation by Western blotting. As shown in Fig. 6*B*, infection induces strong phosphorylation of GSK3α/β at Ser^{21} and Ser^{9}. Specific examination of Ser^{9} on GSK3β confirmed its phosphorylation following infection (Fig. 6*C*).

It is reported that Akt controls this inhibitory phosphorylation of GSK3β (40), so we tested whether PI3K activity during *B. cenocepacia* infection was required for GSK3α/β phosphorylation. PBM were pretreated with either the PI3K inhibitor LY294002 or vehicle control, followed by infection. Western blotting shows rapid phosphorylation of GSK3α/β with vehicle control. However, PI3K inhibition strongly attenuated this infection-mediated phosphorylation (Fig. 6*D*). This suggests that inhibition of GSK3β following *B. cenocepacia* infection is PI3K dependent. To determine whether GSK3β modulated phosphorylation of NF-κB, PBM were pretreated with either DMSO or the GSK3β inhibitor SB-21673 (41), and then infected for 20, 40, or 60 min. Western blots showed that inhibition of GSK3β did not lead to increases in NF-κB phosphorylation (Supplemental Fig. 2). This suggests that any effect of GSK3β would be at the functional level, downstream of NF-κB phosphorylation.

To test this, we examined the effect of GSK3β on NF-κB and inflammatory response during *B. cenocepacia* infection, using the NF-κB luciferase reporter, as in Fig. 6*A*. Reporter-transfected cells were treated with vehicle control or with the GSK3β-specific inhibitor SB-216763. Following this, cells were infected with *B. cenocepacia* for 5 h. Results showed that inhibition of GSK3β led to increased NF-κB reporter activity compared with vehicle control (Fig. 6*E*). Inhibitor treatment alone led to a modest, yet measurable increase in basal NF-κB activity (Supplemental Fig. 3), which also supports the findings (35–37) that GSK3β can repress NF-κB activity. Collectively, these results suggest that, within the context of *B. cenocepacia*-infected macrophages, GSK3β represses NF-κB activity, but not NF-κB phosphorylation. This is also in agreement with the findings that both PI3K and Akt promote NF-κB activity because these signaling molecules inactivate GSK3β. To test the functional outcome of this, we collected supernatants from infected macrophages and measured TNF-α production by ELISA. Results showed a significant increase in TNF-α with the GSK3β inhibitor (Fig. 6*F*).

Overexpression of GSK3β represses NF-κB and the proinflammatory response

To confirm the function of GSK3β as a repressor of NF-κB and inflammatory response to *B. cenocepacia*, we cotransfected

RAW264.7 macrophages with a NF-κB luciferase reporter and either vector control or wild-type GSK3β (24). Macrophages overexpressing GSK3β showed reduced NF-κB activity compared with vector control in response to *B. cenocepacia* infection (Fig. 7*A*). Consistent with our earlier results showing that GSK3β represses NF-κB activity in infected macrophages, GSK3β overexpression decreased basal NF-κB activity by 50%. We also found reduced production of TNF-α in overexpressing macrophages (Fig. 7*B*). Verification of GSK3β overexpression was done by Western blotting (Fig. 7*C*).

In summary, our results show that PI3K/Akt and NF-κB activation are required for proinflammatory responses to *B. ceoncepacia*. However, rather than driving NF-κB activation through IKK phosphorylation, PI3K/Akt signaling serves to inactivate the inhibitory GSK3β.

Discussion

We have found that PI3K/Akt signaling inactivates GSK3β to permit enhanced NF-κB activity and production of proinflammatory cytokines following *B. cenocepacia* infection. Akt has been shown to regulate NF-κB activity through its influence on IKK (42). Akt can directly phosphorylate IKKα to mediate NF-κB nuclear translocation and gene transcription (43) depending on the ratio of IKKα to IKKβ within the cell (44). PI3K can also lead to phosphorylation and activation of NF-κBp65 through Akt, independently of IκB degradation (45). Within the context of *B. cenocepacia* infection, however, it appears that the inactivation of GSK3β is the major mechanism by which PI3K/Akt modulated NF-κB activity. Of note, the PI3K pathway did not regulate expression of other negative regulators such as IRAK-M (Supplemental Fig. 4).

Studies of inflammatory responses to Gram-negative bacteria in intestinal epithelial cells showed that PI3K/Akt inhibition resulted in decreased NF-κBp65 phosphorylation (46, 47), which we did not observe. Both the species of bacteria and the type of host cell (epithelial cells versus monocytes/macrophages) may be responsible for these disparities. Inhibition of PI3K/Akt in vivo using gp91$^{phox-/-}$ mice (48) or vinblastine-immunosuppressed mice (9) may be required to determine the in vivo consequences of manipulating PI3K/Akt/GSK3β on bacterial growth, cytokine production, and overall host resistance to infection.

Our results are in agreement with Hii et al. (49), who found PI3K to be critical for proinflammatory responses of HEK293T cells infected with *Burkholderia pseudomallei*. However, it remains to be tested whether the mechanism of GSK3β inhibition by PI3K/Akt is also similar, as the nature of *B. cenocepacia* versus *B. pseudomallei* infection is quite different (50). Indeed, there is tight regulation of PI3K/Akt activity by factors such as phosphatases (19) and microRNAs (51); the possibility that *B. cenocepacia* differentially affects these regulators requires examination.

As well as differences in PI3K/Akt-mediated regulation of NF-κB, the role of GSK3β is also variable, depending upon a number of factors such as TLR activation and cell type (52). GSK3β has been shown to inhibit NF-κB activity (35–37), yet has also been shown to be required for NF-κB activation (34). Hence, it is conceivable that factors specific to *B. cenocepacia* influence not only the mode of PI3K/Akt-mediated NF-κB activation, but also the role of GSK3β.

In this study, we find that PI3K/Akt does not influence the uptake or replication of *B. cenocepacia* within macrophages. PI3K inhibition by wortmannin has been reported to inhibit intra-

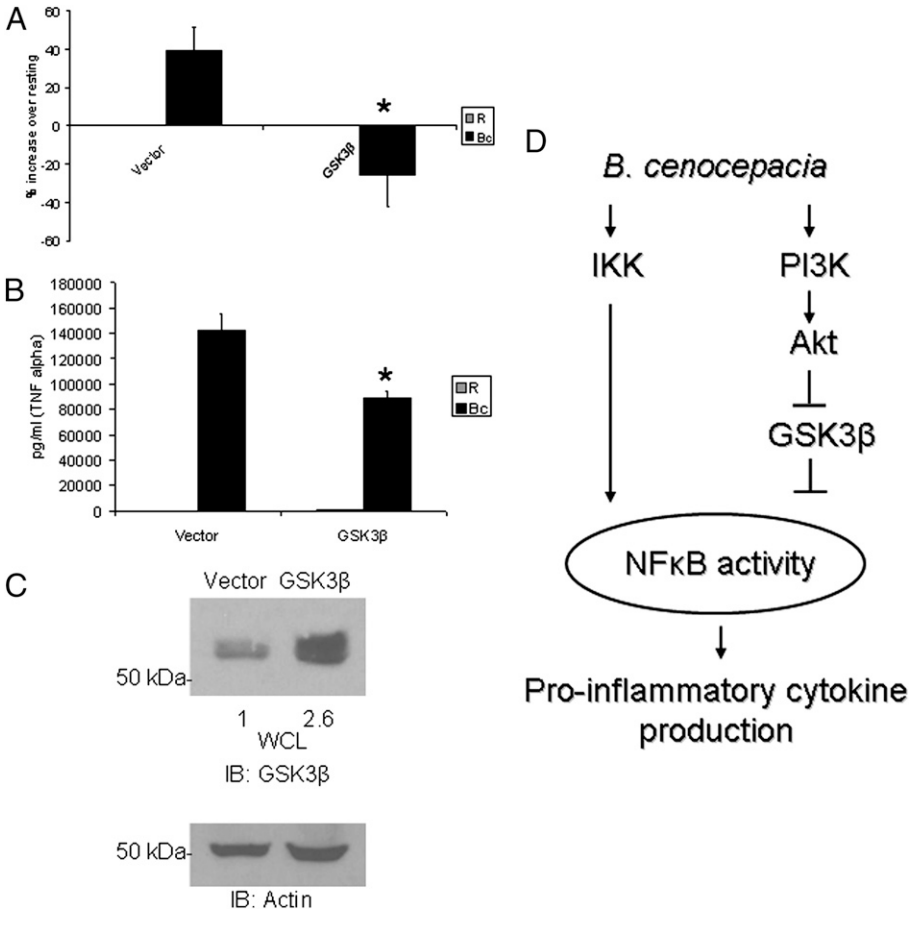

FIGURE 7. GSK3β overexpression inhibits *B. cenocepacia*-induced NF-κB activation and inflammatory response. *A*, RAW264.7 macrophages were transfected with a NF-κB luciferase reporter plasmid and either vector control or wild-type GSK3β plasmid. Fourteen hours post-transfection, macrophages were infected with *B. cenocepacia* at a MOI of 5 for 5 h. Cells were lysed, and luciferase activity was measured by a luminometer. Data are expressed as percentage of increase over matched uninfected control. *B*, RAW264.7 macrophages were transfected with vector control or wild-type GSK3β plasmid. Fourteen hours posttransfection, the macrophages were infected with *B. cenocepacia* at a MOI of 5 for 24 h. Cleared cell lysates were collected and assayed for TNF-α production by ELISA. Data represent the average of three samples, and error bars denote SD. *$p < 0.05$, Student *t* test. *C*, Western blots to measure GSK3β were done with cell lysates from uninfected macrophages transfected with either vector or GSK3β plasmid, followed by reprobes for actin as loading control. Densitometry quantification of GSK3β overexpression is shown and normalized by actin. *D*, Model of interactions among IKK, PI3K/Akt, and GSK3β pathways for regulating NF-κB and inflammatory response to *B. cenocepacia*.

cellular replication of *B. cenocepacia* within epithelial cells (23), presumably through an effect on autophagy. The difference between these findings may be reflective of macrophage versus epithelial cell or even LY294002 versus wortmannin pan-PI3K inhibition. However, we also find through genetic means that Akt does not influence macrophage phagocytosis or intramacrophage replication of *B. cenocepacia*. Thus, PI3K and Akt both strongly influence the macrophage inflammatory response without affecting *B. cenocepacia* uptake or survival.

One critical aspect related to this finding is that *B. cenocepacia* BC7, representing the virulent ET12 lineage that is associated with cepacia syndrome-related deaths, directly binds to the TNF receptor (TNFR1) to induce MAPK activation and IL-8 production (53). Importantly, one of the major effects of TNFR1 activation is activation of the proinflammatory transcription factor NF-κB (54). This current study was done with the K56-2 isolate, which too represents the highly virulent ET12 lineage (12). This strongly suggests that inhibition of PI3K/Akt may be an ideal therapeutic strategy to combat cepacia syndrome, as it regulates NF-κB at a point downstream of initial IKK activation. As such, it would be effective even against this TNFR-driven inflammatory response.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Valvano, M. A. 2006. Infections by *Burkholderia* spp.: the psychodramatic life of an opportunistic pathogen. *Future Microbiol.* 1: 145–149.
2. Sajjan, U., M. Corey, A. Humar, E. Tullis, E. Cutz, C. Ackerley, and J. Forstner. 2001. Immunolocalisation of *Burkholderia cepacia* in the lungs of cystic fibrosis patients. *J. Med. Microbiol.* 50: 535–546.
3. Jones, A. M., M. E. Dodd, and A. K. Webb. 2001. *Burkholderia cepacia*: current clinical issues, environmental controversies and ethical dilemmas. *Eur. Respir. J.* 17: 295–301.
4. McGowan, J. E. Jr. 2006. Resistance in nonfermenting Gram-negative bacteria: multidrug resistance to the maximum. *Am. J. Infect. Control* 34(Suppl. 1)S29–S37, discussion S64–S73.
5. Gibson, R. L., J. L. Burns, and B. W. Ramsey. 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 168: 918–951.
6. Govan, J. R., J. E. Hughes, and P. Vandamme. 1996. *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J. Med. Microbiol.* 45: 395–407.
7. Zughaier, S. M., H. C. Ryley, and S. K. Jackson. 1999. Lipopolysaccharide (LPS) from *Burkholderia cepacia* is more active than LPS from *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* in stimulating tumor necrosis factor alpha from human monocytes. *Infect. Immun.* 67: 1505–1507.
8. Kazachkov, M., J. Lager, J. LiPuma, and P. M. Barker. 2001. Survival following *Burkholderia cepacia* sepsis in a patient with cystic fibrosis treated with corticosteroids. *Pediatr. Pulmonol.* 32: 338–340.
9. Ventura, G. M., V. Balloy, R. Ramphal, H. Khun, M. Huerre, B. Ryffel, M. C. Plotkowski, M. Chignard, and M. Si-Tahar. 2009. Lack of MyD88 protects the immunodeficient host against fatal lung inflammation triggered by the opportunistic bacteria *Burkholderia cenocepacia*. *J. Immunol.* 183: 670–676.
10. De C. Ventura, G. M., R. Le Goffic, V. Balloy, M.-C. Plotkowski, M. Chignard, and M. Si-Tahar. 2008. TLR 5, but neither TLR2 nor TLR4, is involved in lung epithelial cell response to *Burkholderia cenocepacia*. *FEMS Immunol. Med. Microbiol.* 54: 37–44.
11. Blohmke, C. J., R. E. Victor, A. F. Hirschfeld, I. M. Elias, D. G. Hancock, C. R. Lane, A. G. Davidson, P. G. Wilcox, K. D. Smith, J. Overhage, et al. 2008. Innate immunity mediated by TLR5 as a novel antiinflammatory target for cystic fibrosis lung disease. *J. Immunol.* 180: 7764–7773.
12. Sajjan, S. U., L. A. Carmody, C. F. Gonzalez, and J. J. LiPuma. 2008. A type IV secretion system contributes to intracellular survival and replication of *Burkholderia cenocepacia*. *Infect. Immun.* 76: 5447–5455.
13. Hamad, M. A., A. M. Skeldon, and M. A. Valvano. 2010. Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in studies of intracellular bacteria with the gentamicin protection assay. *Appl. Environ. Microbiol.* 76: 3170–3176.
14. Lamothe, J., and M. A. Valvano. 2008. *Burkholderia cenocepacia*-induced delay of acidification and phagolysosomal fusion in cystic fibrosis transmembrane conductance regulator (CFTR)-defective macrophages. *Microbiology* 154: 3825–3834.
15. Saini, L. S., S. B. Galsworthy, M. A. John, and M. A. Valvano. 1999. Intracellular survival of *Burkholderia cepacia* complex isolates in the presence of macrophage cell activation. *Microbiology* 145: 3465–3475.
16. De Soyza, A., C. D. Ellis, C. M. Khan, P. A. Corris, and R. Demarco de Hormaeche. 2004. *Burkholderia cenocepacia* lipopolysaccharide, lipid A, and proinflammatory activity. *Am. J. Respir. Crit. Care Med.* 170: 70–77.
17. Palfreyman, R. W., M. L. Watson, C. Eden, and A. W. Smith. 1997. Induction of biologically active interleukin-8 from lung epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia* products. *Infect. Immun.* 65: 617–622.
18. Hazeki, K., K. Nigorikawa, and O. Hazeki. 2007. Role of phosphoinositide 3-kinase in innate immunity. *Biol. Pharm. Bull.* 30: 1617–1623.
19. Parsa, K. V., L. P. Ganesan, M. V. Rajaram, M. A. Gavrilin, A. Balagopal, N. P. Mohapatra, M. D. Wewers, L. S. Schlesinger, J. S. Gunn, and S. Tridandapani. 2006. Macrophage pro-inflammatory response to *Francisella novicida* infection is regulated by SHIP. *PLoS Pathog.* 2: e71.
20. Rajaram, M. V., L. P. Ganesan, K. V. Parsa, J. P. Butchar, J. S. Gunn, and S. Tridandapani. 2006. Akt/Protein kinase B modulates macrophage inflammatory response to *Francisella* infection and confers a survival advantage in mice. *J. Immunol.* 177: 6317–6324.
21. Rajaram, M. V., J. P. Butchar, K. V. Parsa, T. J. Cremer, A. Amer, L. S. Schlesinger, and S. Tridandapani. 2009. Akt and SHIP modulate *Francisella* escape from the phagosome and induction of the Fas-mediated death pathway. *PLoS One* 4: e7919.
22. Butchar, J. P., T. J. Cremer, C. D. Clay, M. A. Gavrilin, M. D. Wewers, C. B. Marsh, L. S. Schlesinger, and S. Tridandapani. 2008. Microarray analysis of human monocytes infected with *Francisella tularensis* identifies new targets of host response subversion. *PLoS One* 3: e2924.
23. Sajjan, U. S., J. H. Yang, M. B. Hershenson, and J. J. LiPuma. 2006. Intracellular trafficking and replication of *Burkholderia cenocepacia* in human cystic fibrosis airway epithelial cells. *Cell. Microbiol.* 8: 1456–1466.
24. He, X., J. P. Saint-Jeannet, J. R. Woodgett, H. E. Varmus, and I. B. Dawid. 1995. Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. *Nature* 374: 617–622.
25. Katso, R., K. Okkenhaug, K. Ahmadi, S. White, J. Timms, and M. D. Waterfield. 2001. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* 17: 615–675.
26. Vlahos, C. J., W. F. Matter, K. Y. Hui, and R. F. Brown. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269: 5241–5248.
27. Thimmaiah, K. N., J. B. Easton, G. S. Germain, C. L. Morton, S. Kamath, J. K. Buolamwini, and P. J. Houghton. 2005. Identification of N10-substituted phenoxazines as potent and specific inhibitors of Akt signaling. *J. Biol. Chem.* 280: 31924–31935.
28. Ganesan, L. P., G. Wei, R. A. Pengal, L. Moldovan, N. Moldovan, M. C. Ostrowski, and S. Tridandapani. 2004. The serine/threonine kinase Akt Promotes Fc gamma receptor-mediated phagocytosis in murine macrophages through the activation of p70S6 kinase. *J. Biol. Chem.* 279: 54416–54425.
29. Khelef, N., H. A. Shuman, and F. R. Maxfield. 2001. Phagocytosis of wild-type *Legionella pneumophila* occurs through a wortmannin-insensitive pathway. *Infect. Immun.* 69: 5157–5161.
30. Tachado, S. D., M. M. Samrakandi, and J. D. Cirillo. 2008. Non-opsonic phagocytosis of *Legionella pneumophila* by macrophages is mediated by phosphatidylinositol 3-kinase. *PLoS One* 3: e3324.
31. Karin, M., Y. Yamamoto, and Q. M. Wang. 2004. The IKK NF-kappa B system: a treasure trove for drug development. *Nat. Rev. Drug Discov.* 3: 17–26.
32. Sakurai, H., H. Chiba, H. Miyoshi, T. Sugita, and W. Toriumi. 1999. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J. Biol. Chem.* 274: 30353–30356.
33. Gross, S., and D. Piwnica-Worms. 2005. Real-time imaging of ligand-induced IKK activation in intact cells and in living mice. *Nat. Methods* 2: 607–614.
34. Hoeflich, K. P., J. Luo, E. A. Rubie, M. S. Tsao, O. Jin, and J. R. Woodgett. 2000. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. *Nature* 406: 86–90.
35. Saijo, K., B. Winner, C. T. Carson, J. G. Collier, L. Boyer, M. G. Rosenfeld, F. H. Gage, and C. K. Glass. 2009. A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. *Cell* 137: 47–59.
36. Escribano, C., C. Delgado-Martín, and J. L. Rodríguez-Fernández. 2009. CCR7-dependent stimulation of survival in dendritic cells involves inhibition of GSK3beta. *J. Immunol.* 183: 6282–6295.
37. Vines, A., S. Cahoon, I. Goldberg, U. Saxena, and S. Pillarisetti. 2006. Novel anti-inflammatory role for glycogen synthase kinase-3beta in the inhibition of tumor necrosis factor-alpha- and interleukin-1beta-induced inflammatory gene expression. *J. Biol. Chem.* 281: 16985–16990.
38. Sutherland, C., and P. Cohen. 1994. The alpha-isoform of glycogen synthase kinase-3 from rabbit skeletal muscle is inactivated by p70 S6 kinase or MAP kinase-activated protein kinase-1 in vitro. *FEBS Lett.* 338: 37–42.
39. Sutherland, C., I. A. Leighton, and P. Cohen. 1993. Inactivation of glycogen synthase kinase-3 beta by phosphorylation: new kinase connections in insulin and growth-factor signalling. *Biochem. J.* 296: 15–19.

40. Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378: 785–789.
41. Coghlan, M. P., A. A. Culbert, D. A. Cross, S. L. Corcoran, J. W. Yates, N. J. Pearce, O. L. Rausch, G. J. Murphy, P. S. Carter, L. Roxbee Cox, et al. 2000. Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem. Biol.* 7: 793–803.
42. Madrid, L. V., C. Y. Wang, D. C. Guttridge, A. J. Schottelius, A. S. Baldwin, Jr., and M. W. Mayo. 2000. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol. Cell. Biol.* 20: 1626–1638.
43. Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner. 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 401: 82–85.
44. Gustin, J. A., O. N. Ozes, H. Akca, R. Pincheira, L. D. Mayo, Q. Li, J. R. Guzman, C. K. Korgaonkar, and D. B. Donner. 2004. Cell type-specific expression of the IkappaB kinases determines the significance of phosphatidylinositol 3-kinase/Akt signaling to NF-kappa B activation. *J. Biol. Chem.* 279: 1615–1620.
45. Sizemore, N., S. Leung, and G. R. Stark. 1999. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. *Mol. Cell. Biol.* 19: 4798–4805.
46. Bandyopadhaya, A., S. Bhowmick, and K. Chaudhuri. 2009. Activation of proinflammatory response in human intestinal epithelial cells following *Vibrio cholerae* infection through PI3K/Akt pathway. *Can. J. Microbiol.* 55: 1310–1318.
47. Haller, D., M. P. Russo, R. B. Sartor, and C. Jobin. 2002. IKK beta and phosphatidylinositol 3-kinase/Akt participate in non-pathogenic Gram-negative enteric bacteria-induced RelA phosphorylation and NF-kappa B activation in both primary and intestinal epithelial cell lines. *J. Biol. Chem.* 277: 38168–38178.
48. Sousa, S. A., M. Ulrich, A. Bragonzi, M. Burke, D. Worlitzsch, J. H. Leitão, C. Meisner, L. Eberl, I. Sá-Correia, and G. Döring. 2007. Virulence of *Burkholderia cepacia* complex strains in gp91phox$^{-/-}$ mice. *Cell. Microbiol.* 9: 2817–2825.
49. Hii, C. S., G. W. Sun, J. W. Goh, J. Lu, M. P. Stevens, and Y. H. Gan. 2008. Interleukin-8 induction by *Burkholderia pseudomallei* can occur without Toll-like receptor signaling but requires a functional type III secretion system. *J. Infect. Dis.* 197: 1537–1547.
50. Wiersinga, W. J., T. van der Poll, N. J. White, N. P. Day, and S. J. Peacock. 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. *Nat. Rev. Microbiol.* 4: 272–282.
51. Cremer, T. J., D. H. Ravneberg, C. D. Clay, M. G. Piper-Hunter, C. B. Marsh, T. S. Elton, J. S. Gunn, A. Amer, T. D. Kanneganti, L. S. Schlesinger, et al. 2009. MiR-155 induction by *F. novicida* but not the virulent *F. tularensis* results in SHIP down-regulation and enhanced pro-inflammatory cytokine response. *PLoS One* 4: e8508.
52. Beurel, E., S. M. Michalek, and R. S. Jope. 2010. Innate and adaptive immune responses regulated by glycogen synthase kinase-3 (GSK3). *Trends Immunol.* 31: 24–31.
53. Sajjan, U. S., M. B. Hershenson, J. F. Forstner, and J. J. LiPuma. 2008. *Burkholderia cenocepacia* ET12 strain activates TNFR1 signalling in cystic fibrosis airway epithelial cells. *Cell. Microbiol.* 10: 188–201.
54. Chen, G., and D. V. Goeddel. 2002. TNF-R1 signaling: a beautiful pathway. *Science* 296: 1634–1635.