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The tyrosine kinase Syk promotes phagocytosis of *Francisella* through the activation of Erk

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Abstract

Francisella tularensis is a highly infectious, Gram-negative intra-cellular pathogen that can cause the zoonotic disease tularemia. Although the receptors critical for internalization of *Francisella* by macrophages are beginning to be defined, the identity of the downstream signaling pathways essential for the engulfment are not yet identified. In this study we have tested the role of Syk in the phagocytosis of *Francisella*. We report that Syk is activated during *Francisella* infection and is critical for the uptake of the organisms. Pharmacologic inhibition of Syk almost completely abrogated uptake, whereas the overexpression of Syk significantly enhanced uptake. However, Syk appears to be dispensable during initial host-pathogen contact. Further analyses of the molecular mechanism of Syk influence on *Francisella* uptake revealed that the MAPK Erk but not the PI3K/Akt pathway is the downstream effector of Syk. Thus, the inhibition of Erk in Syk-overexpressing cells or the inhibition of Syk in Erk-overexpressing cells led to a significant attenuation of uptake. Collectively, these data identify Syk and Erk as key players in the phagocytosis of *Francisella*.

Keywords

Francisella; Syk; PI3K/Akt; Erk; phagocytosis

Introduction

The Gram-negative intra-cellular pathogen *Francisella tularensis* is the causative agent of the zoonotic disease tularemia. Four sub-species of *Francisella tularensis* exist- *tularensis* (Type A), *holarctica* (Type B), *novicida* and *mediasiatica. Francisella novicida* is virulent in mice but not in humans[1]. However, the intra-cellular life style of *F. novicida* is similar to that of the highly virulent Type A strain[2]. Thus, *F. novicida* is a frequently used experimental model for tularemia in the murine system. Because the infectious dose of *F. tularensis tularensis* is very

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low and the organism can be easily aerosolized, it is currently considered a potential biological weapon[1].

Francisella tularensis primarily infects immune cells such as macrophages, monocytes and neutrophils. After bacterial internalization, the *Francisella*-containing phagosomes fail to fuse with the lysosome and the bacteria escape into the cytosol. In the cytosol, the bacteria replicate and subsequently trigger apoptosis of the host cell[3,4]. The host response to *Francisella* infection is beginning to be delineated. Host response involves key processes such as phagocytosis, production of inflammatory mediators and generation of toxic metabolites. The molecular mechanisms leading to the production of inflammatory mediators has received a lot of attention. The activation of MAP Kinases and NFκB has been reported in *Francisella* infected cells[5-9] and we have recently reported that in addition to the MAP Kinase pathway, the phosphatidylinositol 3 kinase (PI3K)/Akt pathway is also activated and plays a critical role in the production of inflammatory cytokines[7-9]. Further, activation of the PI3K/Akt pathway and the subsequent generation of inflammatory cytokines are negatively regulated by the inositol phosphatase SHIP[8]. Recent reports also demonstrate that activation of the inflammasome complex is mediated by *Francisella* that escape into the cytosol, leading to the processing and release of IL-1β[10,11].

In contrast to the large body of information on host cell inflammatory response, the mechanisms underlying phagocytosis of *Francisella* are currently unknown. Several host cell receptors including complement receptor3 (CR3)[12-14], mannose receptor[14,15], class A scavenger receptor[16], Toll-like receptor 2[17,18] and Fcγ receptors[14] have been implicated in the recognition of *Francisella*. However, the downstream signals that orchestrate phagocytosis of *Francisella* have not been identified.

Syk is a tyrosine kinase that has been shown to be critical for various immune cell functions, including cytoskeletal rearrangements and phagocytosis[19,20]. Thus, in this study we specifically examined the role of Syk in the uptake of *Francisella*. Our data demonstrate that Syk is phosphorylated in *Francisella*-infected cells. Inhibition of Syk markedly attenuates uptake of *Francisella*, whereas overexpression of Syk enhances uptake. Interestingly, this Syk-dependent uptake is mediated via Erk but not through the PI3K/Akt pathway. These data suggest an indispensable role for Syk during the phagocytosis of *Francisella*.

Materials and Methods

Cells, antibodies and reagents

Raw 264.7 and THP-1 cells were obtained from ATCC and maintained in RPMI 1640 with 5% heat-inactivated fetal bovine serum (FBS). Antibodies specific for phospho-tyrosine, Syk, Erk and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pErk and pSer Akt were obtained from Cell Signaling Technology (Beverly, MA). Mouse anti-*Francisella* lipopolysaccharide primary antibody was from Immune-Precise Antibodies Limited (Victoria, B.C., Canada). Piceatannol, UO126 and LY294002 were purchased from Calbiochem (San Diego, CA). *F. novicida* U112 (JSG1819), a generous gift of Dr. John Gunn (The Ohio State University, OH) were used in all experiments. Bacteria were streaked and grown overnight on Chocolate II agar plates (Becton, Dickinson and Company, MD) at 37°C.

Cell stimulation, lysis, and Western blotting

Macrophages were infected with plate-grown *F. novicida* (grown on Chocolate II agar plates for 16-18 h at 37°C) as previously described[8] at a multiplicity of infection (MOI) of 100. Briefly, RAW 264.7 cells were plated in 12-well or 6 well plates and allowed to adhere. *F.*

novicida resuspended in RPMI medium containing 5% heat inactivated FBS was added to the adherent macrophages and then incubated at 37°C and 5% CO_2 for the indicated time points. In parallel, the viability of bacteria was tested by plating the inoculum on Chocolate II agar plates and bacterial numbers in the inoculum were quantified using the Petroff-Hauser chamber. These data indicate that >98% of bacteria in the inoculum were viable. During the infection, cells were not washed at any point unless indicated otherwise. Where indicated, before infection, cells were incubated with specific pharmacological inhibitors for 30 minutes. Post-infection, the cell supernatant was aspirated and uninfected and infected cells were lysed in TN1 buffer (50mM Tris pH 8.0, 10mM EDTA, 10mM $Na_4P_2O_7$, 10mM NaF, 1% Triton-X 100, 125mM NaCl, 10mM Na_3VO_4, 10μg/ml each aprotinin and leupeptin). Post-nuclear lysates were boiled in Laemmli Sample Buffer and were separated by SDS/PAGE, transferred to nitrocellulose filters, probed with the antibody of interest and developed by enhanced chemiluminescence (ECL).

Western blot data quantitation

The ECL signal was quantitated using a scanner and a densitometry program (Scion Image) as previously described[7,9]. To quantitate the phospho-specific signal in the activated samples, we first subtracted background, normalized the signal to the amount of actin or total target protein in the lysate, and plotted the values as arbitrary units (a.u). Statistical analysis was performed by unpaired Student's *t-test.* $p<0.05$ was considered significant.

Colony forming unit (CFU) assay

CFU assays were performed as we have previously reported[8] with few modifications. Briefly, RAW 264.7 cells were infected with 100 MOI and then centrifuged at 650g for 4 minutes. Infection was allowed to occur for a total of 60 minutes, next infected cells were washed two times with sterile PBS and treated with 50 μg/ml of gentamicin for 30 min at 37°C and 5% CO_2. Cells were then washed twice and subsequently lysed in 0.1% SDS for 5 minutes. Immediately, 10 fold serial dilutions were made and appropriate dilutions were plated on Chocolate II agar plates. Assays were performed in triplicate for each test group. Statistical analysis was performed by unpaired Student's *t-test.* $p<0.05$ was considered significant.

Microscopy analysis of Francisella association with macrophages

Phagocytosis of *Francisella* was measured by microscopy as previously described[7], with a few modifications. In brief, 60 minutes post-infection, cells were washed (with PBS) and fixed in 4% paraformaldehyde for 20 minutes. The cells were washed again and one of the two sets of samples was permeabilized with 100% methanol for 10 minutes and the other set was left non-permeabilized. Immunostaining was then performed with mouse anti-*F. novicida* LPS antibody (diluted 1/100; Immune Precise Antibodies) and the bacteria were visualized by anti-mouse Alexa Fluor 488 secondary antibody. Bacteria binding to macrophages were counted on non-permeabilized samples whereas total number of bacteria associated (both attached and phagocytosed) with the cells were counted on methanol permeabilized samples using the X100 oil immersion objective of a BX40 Olympus fluorescence microscope. The number of bacteria phagocytosed was obtained by subtracting bacterial numbers adhering to macrophages from the total number of bacteria associated with the cell. At least 100 cells per sample were examined and three separate sets of infection were analyzed. Phagocytic and binding indices are defined as the number of bacteria phagocytosed or adherent to 100 macrophages, respectively. Statistical analysis was performed by unpaired Student's *t-test.* $p<0.05$ was considered significant.

Plasmids and transient transfection

The construct encoding Syk was a kind gift from Dr. Axel Ullrich (Max-Planck Institute of Biochemistry, Germany). GST-Erk2 was a generous gift from Dr. Mark Coggeshall (Oklahoma Medical Research Foundation, OK). RAW 264.7 cells were transfected with the appropriate plasmid DNA using the Amaxa Nucleofector apparatus (Amaxa biosystems, Germany) as previously described[8]. Briefly, 7×10^6 cells were resuspended in 100 μl Nucelofector Solution V and were nucelofected with 8 μg of appropriate plasmid. Immediately post- nucleofection, 500 μl of pre-warmed RPMI was added to the transfection mix before transferring to 12-well plates containing 1.5 ml pre-warmed RPMI per well. Plates were incubated for 16 hours at 37° C before infections were performed.

Results

Syk is phosphorylated during *Francisella* infection

The activation of Syk is accompanied by autophosphorylation, so we used tyrosine phosphorylation of Syk as a measure of its activity. To examine whether Syk is activated during *Francisella* infection, RAW 264.7 cells were infected with *F. novicida* (MOI of 100) for different time points indicated in Figure 1A and the induction of Syk phosphorylation was examined. For this, Syk was immunoprecipitated from uninfected and infected cells and Western blotting was performed using phospho-tyrosine (pY) antibody (Figure 1A, upper panel). The same membrane was reprobed with Syk antibody to ensure equal loading in all lanes (Figure 1A, lower panel). Results indicated that Syk is phosphorylated as early as 30 seconds in cells infected with *Francisella*. We next examined whether the phosphorylation of Syk was dose-dependent. To test this, RAW 264.7 cells were infected with 1, 10 or 100 MOI of *F. novicida* and phosphorylation of Syk was assessed as described above. Results shown in Figure 1B indicate that Syk phosphorylation is minimal at 1 MOI but clearly evident at 10 MOI. Further, Syk was also phosphorylated when cells were infected with *F. tularensis* LVS, the vaccine strain of *F. tularensis* (Figure 1C).

Host responses in human and mouse cells may differ considerably during *Francisella* infection. Thus, to test if Syk is phosphorylated in human cells, THP1 cells (human monocytic cell line) were infected for different time points and the phosphorylation of Syk was assessed by Western blot analysis. Results shown in Figure 1D indicate that Syk is also phosphorylated in infected human monocytic cells. Collectively, these data demonstrate that Syk is activated when cells are infected with *Francisella*, suggesting a potential role for Syk in macrophage/monocyte response to *Francisella*.

Syk promotes the phagocytosis of *Francisella*

Having established that Syk is activated early in infection, we next examined whether Syk is required for phagocytosis of *Francisella*. For this, RAW 264.7 cells were incubated with DMSO (vehicle control) or piceatannol, a specific Syk inhibitor, and then infected with 100 MOI of *F. novicida* for 1 hour. Phagocytosis of the bacteria was assessed by colony forming unit (CFU) assay or by immunofluorescence microscopy and the results are shown in Figure 2. Inhibition of *Francisella* phagocytosis by the actin polymerization inhibitor cytochalasin-D served as our positive control. As shown in Figure 2A, inhibition of Syk activation significantly decreased the phagocytosis of *Francisella*. As expected, treatment of cells with cytochalasin-D also suppressed the engulfment of *Francisella*. At the concentrations used, piceatannol was not toxic to the cells as assessed by trypan blue exclusion (data not shown). To test whether Syk activation was inhibited by piceatannol, RAW 264.7 cells were treated with DMSO or piceatannol prior to infection with 100 MOI of *F. novicida* for 1 minute and the phosphorylation of Syk was measured as described in Figure 1. Results, shown in Figure

2B, indicate that *Francisella*-induced Syk phosphorylation was inhibited by pre-treatment of cells with piceatannol.

As an additional approach, we tested the role of Syk in *Francisella* phagocytosis by immunofluorescence microscopy and the results are shown in Figure 2C and 2D. Consistent with the results obtained with CFU assays, inhibition of Syk significantly decreased *Francisella* phagocytosis (Figure 2C). However, the attachment of the bacteria to macrophages remained unaffected by Syk inhibition (Figure 2D).

To validate the findings obtained above with piceatannol, the role of Syk in the phagocytosis of *Francisella* was assessed using a genetic approach. Here, RAW 264.7 cells were transiently transfected with empty vector or a Syk encoding construct. Sixteen hours after transfection, cells were infected with 100 MOI of *F. novicida* for 1 hour and phagocytosis was assessed by CFU assay. The results shown in Figure 3A indicate that over-expression of Syk significantly increased the phagocytosis of *Francisella*. In parallel experiments, the transfectants were analyzed by Western blotting with Syk antibody to test the over-expression of Syk. The data shown in Figure 3B verify that Syk is indeed over-expressed in cells transfected with the Syk construct. Collectively, these experiments demonstrate that Syk promotes the phagocytosis of *Francisella*.

Syk is upstream of the Erk and Akt pathways

Having established that Syk is essential for the engulfment of *Francisella*, we then examined the molecular mechanism underlying Syk-dependent phagocytosis. We have reported previously that in addition to the activation of Erk, the PI3K/Akt pathway is also activated during *F. novicida* infection[7-9]. Thus, we examined whether Syk were upstream of either the Erk pathway and/or the PI3K/Akt pathway. For this, cells were treated with vehicle control or piceatannol, infected for 15 minutes and the cell lysates were examined for the phosphorylation status of Erk and Akt by Western blotting with phospho-specific antibodies (Figures 4A and B, upper panels). The same membranes were reprobed with Actin antibody to ensure equal loading (Figures 4A and B, middle panels). The phosphorylation signals were quantitated and plotted (Figures 4A and B, lower panels). Results show a significant decrease in the phosphorylation of Erk and Akt when Syk is inhibited with piceatannol.

To test the role of Syk in the activation of Erk and PI3K/Akt using an alternate approach, cells were transfected with either an empty vector or a plasmid encoding Syk. The transfectants were subsequently infected and cell lysates were analyzed by Western blotting with phospho-specific antibodies. The results are shown in Figures 4C and D and indicate that phosphorylation of Erk and Akt is significantly enhanced in the Syk-overexpressing cells compared to cells transfected with vector alone. Taken together, these data demonstrate that Syk is upstream of and required for the activation of Erk and PI3K/Akt.

Erk but not PI3K/Akt is required for the phagocytosis of *Francisella*

Having established that Syk is upstream of Erk and PI3K/Akt, we next examined which of these pathways is required for the phagocytosis of *Francisella*. To test this, cells were incubated with vehicle control or U0126 (MEK/Erk pathway inhibitor) or LY294002 (PI3K/Akt inhibitor), infected and phagocytosis of *Francisella* was assessed by CFU and immunofluorescence assays. The results shown in Figures 5A and B indicate that pre-treatment of cells with the Erk but not the PI3K/Akt inhibitor significantly decreased phagocytosis of *Francisella* by macrophages. However the attachment of *Francisella* with the host macrophages was not altered (Figure 5C). This observation was consistent with the unchanged pathogen-host attachment when Syk is inhibited (Figure 2D). In parallel experiments, we tested the efficacy of Erk and PI3K/Akt inhibitors by Western blotting analysis (Figure 5D).

Treatment of cells with U0126 specifically blocked the phosphorylation of Erk proteins where as incubation of cells with LY294002 inhibited the phosphorylation of Akt. Inhibition of Erk also significantly suppressed the phagocytosis of *Francisella* at a lower MOI, suggesting that the decrease in the phagocytosis from Erk blockade is dose independent (data not shown).

We next examined the role of Erk in the phagocytosis of *Francisella* using an over-expression system. For this, cells were transfected either with vector alone or a construct encoding GST-tagged Erk2. The transfectants were infected and phagocytosis of *Francisella* was analyzed by CFU assays. Results shown in Figure 6A indicate that GST-Erk2 transfected cells ingest a significantly higher number of bacteria than cells transfected with vector alone. In parallel, the over-expression of GST-Erk2 protein was tested by Western blotting (Figure 6B). Collectively these data demonstrate that Erk but not PI3K/Akt is critical for the phagocytosis of *Francisella* by macrophages.

Syk-dependent increase in phagocytosis of *Francisella* is abrogated by Erk inhibition

We next examined if the enhancement of phagocytosis due to Syk over-expression can be inhibited by blocking the downstream Syk-effector Erk. For this, cells transfected with vector alone or a plasmid encoding Syk were incubated with vehicle control, Erk inhibitor or PI3K/Akt inhibitor. The cells were subsequently infected and the phagocytosis of *Francisella* was examined by CFU assay. The results shown in Figure 7A indicate that the increase in phagocytosis due to Syk over-expression was suppressed Erk when the Erk pathway was inhibited but not when the PI3K/Akt pathway was inhibited. In parallel experiments, the effectiveness of the inhibitors and Syk over-expression were tested by Western blotting (Figures 7B and C).

The Erk-dependent increase in phagocytosis of *Francisella* is abrogated by Syk inhibition

Finally, we tested whether the Erk-dependent phagocytosis of *Francisella* is blocked if signaling through upstream Syk is inhibited. To test this, vector- or GST-Erk2-transfected cells were treated with DMSO or the Syk inhibitor piceatannol. Cells were subsequently infected and phagocytosis was assessed by CFU assays (Figure 8A). Erk2 overexpression significantly increased the phagocytosis of *Francisella*, but treatment of Erk2-overexpressing cells with Syk inhibitor significantly decreased the phagocytosis of *Francisella* to control levels. In parallel experiments, the effect of Syk inhibition on the phosphorylation status of both exogenous and endogenous Erk was monitored by Western blotting with phospho-Erk antibody. Results shown in Figure 8B indicate that piceatannol inhibited phosphorylation of both exogenous and endogenous Erk proteins. Collectively, the data obtained in this study demonstrate that Syk promotes the phagocytosis of *Francisella* via Erk but not through the PI3K/Akt pathway.

Discussion

Phagocytosis is one of the earliest host immune responses against a pathogen. However, the mechanisms of phagocytosis differ between pathogens. The molecular mechanisms governing the uptake of *Francisella* are not understood. Our current study demonstrates a critical requirement for Syk activation in the engulfment of *Francisella*. These findings are consistent with the widely accepted role of Syk in phagocytosis of various agents such as opsonized zymozan and erythrocytes. Attesting to the importance of Syk during phagocytosis, inhibition of Syk by RNAi suppressed the reorientation of actin around the forming phagosomes in HL60 cells fed with opsonized zymosan[19]. Likewise, Syk-deficient macrophages were found to exhibit defective phagosomal closure[19,20]. However, Syk did not influence binding of either C3bi-opsonized zymosan[19] nor IgG-coated erythrocytes[20] to macrophages in these studies indicating that Syk is not involved in particle binding.

The requirement of Erk activation for the phagocytosis of *Francisella* is mechanistically consistent with other studies. For example, Kugler et al. reported that overexpression of an Erk phosphatase, MKP-1, significantly decreased the phagocytosis of *Listeria monocytogenes* by macrophages indicating that Erk is critical for the phagocytosis of this organism[21]. Further, Erk was reported to be crucial for the phagocytosis of IgG-coated erythrocytes by macrophages[22]. Both Erk and Syk were also found to be essential for the phagocytosis of *E. coli* by haemocytes of *Manduca Sextata*, a lepidopteran insect[23]. However, Erk was reported to be dispensable for the phagocytosis by monocytes and microglia suggesting that Erk requirement for phagocytosis may differ depending upon the target and the immune cell type[24,25].

Although Erk is reported to be critical, its precise role in the phagocytosis of pathogenic organisms or IgG-coated erythrocytes is poorly understood. It is possible that Erk promotes phagocytosis via indirect and direct mechanisms. Though less likely, Erk may induce the transcriptional synthesis of proteins involved in the engulfment of *Francisella*. The more conceivable explanation for Erk-dependent phagocytosis is that Erk, similar to Syk, may modulate the actin cytoskeletal rearrangements that are critical for the uptake of *Francisella*. Several pieces of information support this hypothesis. First, MAPKs are reported to be important for the activation of phoshpolipaseA_2 which via profilin can modulate the actin cytoskeleton[26]. Second, Erk2 phosphorylation is required for the polarization of the microtubule organizing center in natural killer cells[27]. Third, a recent study showed that leukocyte specific protein-1, an F-actin binding cytoskeletal protein, interacts with Erk2 and targets it to peripheral actin filaments[28].

It is noteworthy that the uptake of *Francisella* is not affected by inhibition of the PI3K pathway. The activation of PI3K has been shown to be critical for phagocytosis initiated through various receptors such as FcγR[29], complement receptor[30] and CD44[31]. The lack of PI3K requirement for phagocytosis of *Francisella* is consistent with our recent findings that SHIP, an inositol phosphatase that negatively regulates *Francisella*-induced PI3K/Akt, does not affect the uptake of the *Francisella*[8]. PI3K activation has been shown to be dispensable for the phagocytosis of other intra-cellular pathogens such as *Salmonella*[32,33], *Shigella*[33] and Type II *Helicobacter pylori*[34]. This reiterates the observation that intracellular signaling events that modulate the functional responses of host cells are dependent upon the cellular context and the nature of the stimulus.

Since the Syk/Erk axis is important for the phagocytosis of *Francisella*, how Syk activates Erk deserves attention. Syk is able to activate Erk through at least two independent pathways. First, Syk phosphorylates the Ras adapter Shc which can then associate with the Grb2/SoS complex, leading to the activation of Ras, an upstream activator of Erk[35]. Second, autophosphorylation of Syk can result in the activation of protein kinase C (PKC), ultimately leading to the phosphorylation of Erk. Specifically, using constitutively active and dominant-negative mutants of PKCδ, a key component in the phagocytic pathway of neutrophils, Ueda et al have shown that PKCδ mediates phorbol ester-induced activation of Erk in a Raf and MEK dependent but Ras-independent manner[36]. We are currently investigating the precise mechanism by which Erk is activated during *Francisella* infection.

In this study we have demonstrated that Syk is required for the activation of PI3K/Akt pathway. We previously reported an indispensable role for PI3K/Akt pathway in modulating *Francisella*-induced inflammatory response[8]. Also, in other model systems, Syk is critical for the induction of iNOS, which is important for innate immune responses against invading pathogens[37]. Thus, Syk may be important in regulating additional aspects of the innate immune response to *Francisella*. We are currently testing these possibilities.

In summary, we have identified that Syk regulates the activation of Erk and PI3K/Akt, and we further demonstrate that Erk axis but not PI3K is critical for the phagocytosis of *Francisella*. To our knowledge, this is the first study that reports the identity of molecular signals coordinating the phagocytosis of *Francisella*.

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Abbreviations used in this paper

CFU
: colony forming unit

MAPK
: mitogen activated protein kinase

MOI
: multiplicity of infection

PI3K
: Phosphatidylinositol 3 kinase

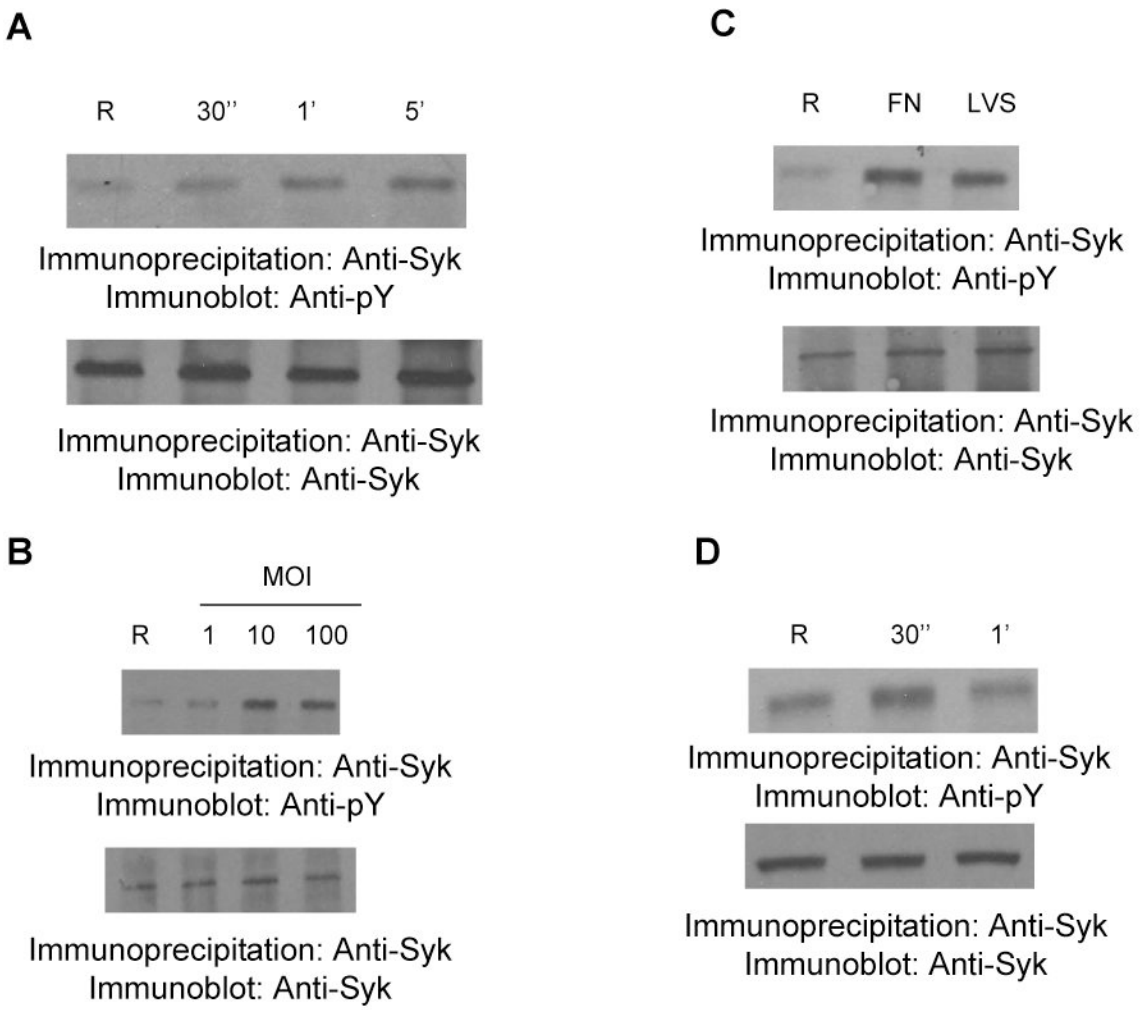

Figure 1. Syk is phosphorylated during *Francisella* infection. A&B

RAW 264.7 cells were infected with 100 MOI of *F. novicida* for the indicated time points (**A**) or with indicated MOI for 1 minute (**B**). Syk protein was immunoprecipitated, resolved by SDS/PAGE and analyzed by Western blotting with phospho-tyrosine (pY) antibody (upper panel). The same membrane was reprobed with Syk antibody to ensure equal loading (lower panel). **C.** RAW 264.7 cells were infected with 100 MOI of *F. novicida* (FN) or *F. tularensis LVS* (LVS) for 1 minute and phosphorylation of Syk was examined by Western blotting. **D**. THP1 cells were infected with 100 MOI (FN) for indicated time points and Syk phosphorylation was assessed. These data are representative of three independent experiments. R, resting, uninfected cells.

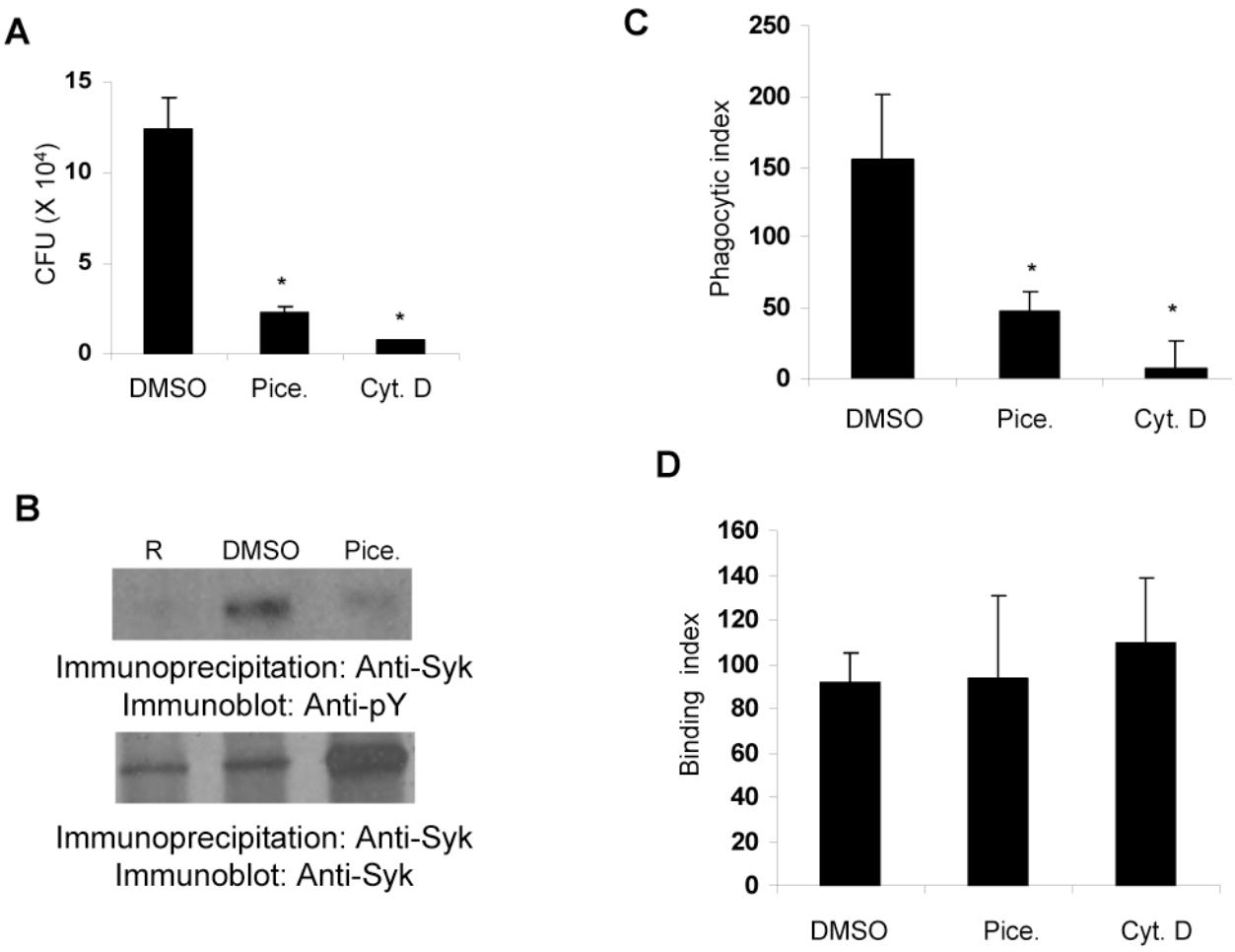

Figure 2. Syk inhibition suppresses the phagocytosis of *Francisella*

A. RAW 264.7 cells were treated with vehicle control (0.1% DMSO) or 25 μg/mL piceatannol (Pice.) or 5 μg/mL of cytochalasin-D (Cyt. D) for 30 minutes; cells were infected with 100 MOI of *F. novicida* for 1 hour and phagocytosis was assessed by CFU assays. **B.** RAW 264.7 cells were treated with vehicle control (0.1% DMSO) or 25 μg/mL piceatannol (Pice.) for 30 minutes; cells were infected with 100 MOI of *F. novicida* for 1 minute and phosphorylation of Syk was examined as described in the legend of Figure 1. **C&D.** RAW 264.7 cells were treated with 0.1 % DMSO or 25 μg/mL piceatannol (Pice.) or 5 μg/mL cytochalasin-D (Cyt. D) for 30 minutes, cells were infected with 100 MOI of *F. novicida* for 1 hour, phagocytosis (**C**) and binding (**D**) of the bacteria was analyzed by immunofluorescence. Phagocytic and binding indices are defined as the number of bacteria phagocytosed or adherent to 100 macrophages, respectively. Data represent mean and standard deviation of 3 independent experiments. *, $p<0.05$ compared to DMSO value.

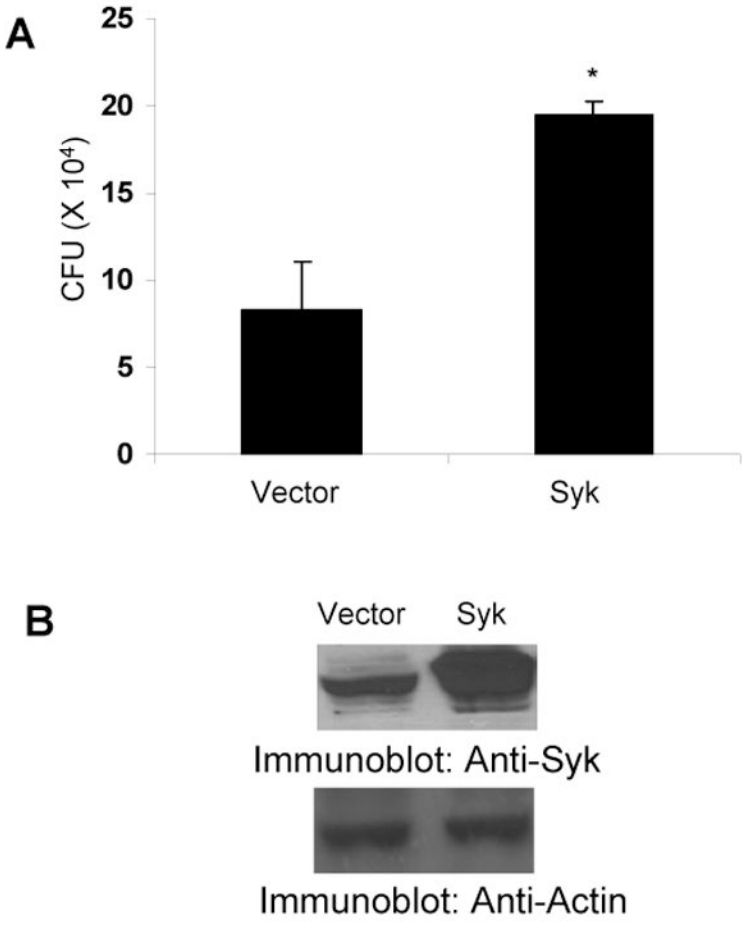

Figure 3. Syk overexpression enhances the phagocytosis of *Francisella*

A. RAW 264.7 cells were transfected with vector alone or a Syk-encoding plasmid. 16 hours post-transfection the cells were infected with 100 MOI of *F. novicida* for 1 hour and phagocytosis was assessed by CFU assays. The graph represents mean and standard deviation of values obtained from 3 independent experiments (a.u., arbitrary units). *, $p<0.05$ compared to vector value. **B.** Protein-matched lysates from the transfectants were resolved by SDS/PAGE and the expression of Syk was analyzed by Western blotting with Syk antibody (upper panel). The same membrane was reprobed with Actin antibody (lower panel). These data are representative of at least three independent experiments.

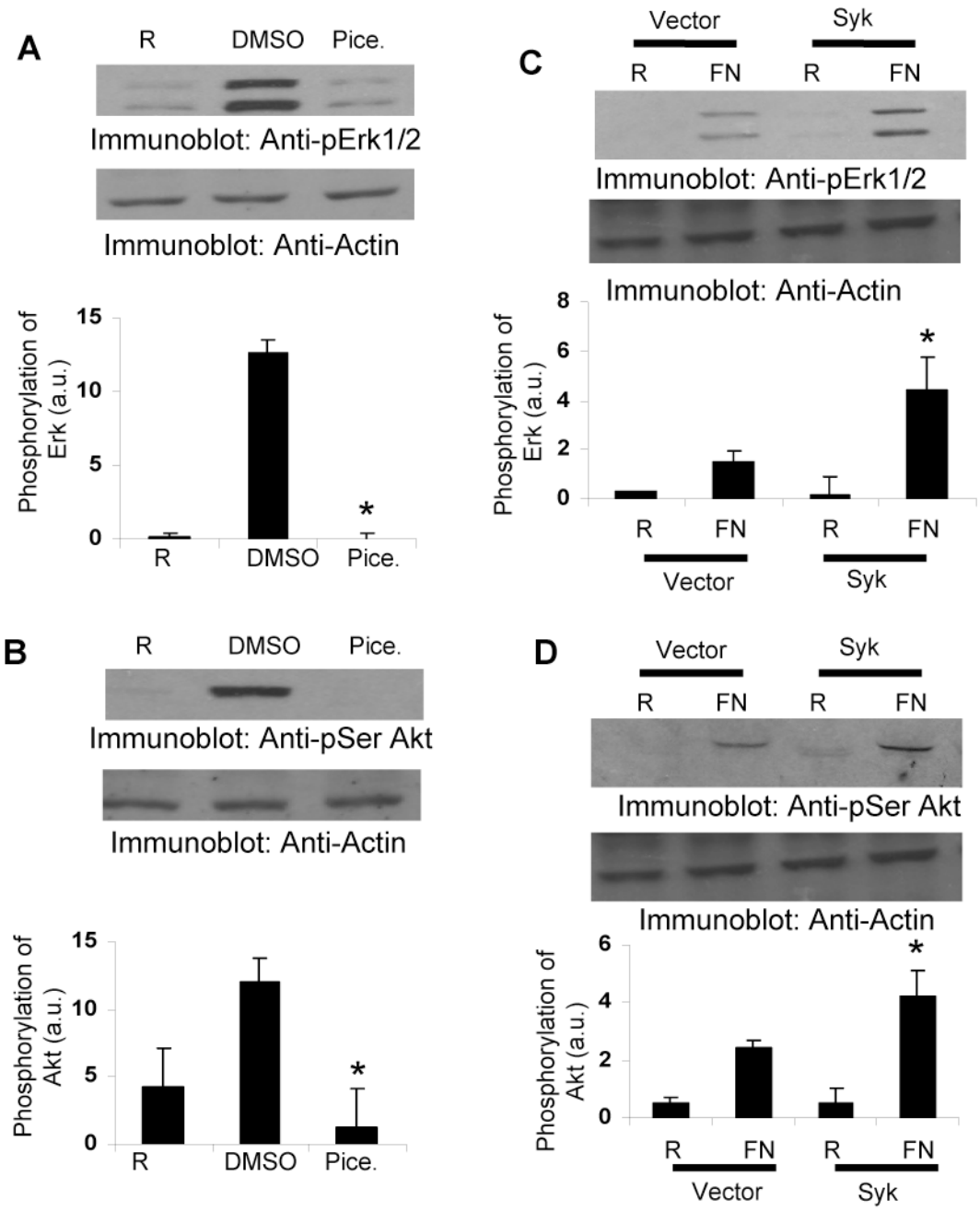

Figure 4. Syk is required for the activation of Erk and Akt

A&B. RAW 264.7 cells were treated with vehicle control (0.1% DMSO) or 25 μg/mL piceatannol (Pice.) for 30 minutes; cells were infected with 100 MOI of *F. novicida* for 15 minutes and protein-matched lysates were resolved by SDS/PAGE and analyzed by Western blotting with indicated phospho-specific antibodies (upper panels). The membranes were reprobed with Actin antibody (middle panels). The phosphorylation signals were quantitated, normalized to actin in each lane and graphed (lower panels). **C&D.** Vector and Syk transfectants were infected with 100 MOI of *F. novicida* and phosphorylation levels of Erk and Akt in the transfectants were analyzed by Western blotting with phospho-specific antibodies (upper panels). The membranes were reprobed with Actin antibody (middle panels).

Phosphorylation signals were normalized to actin content (lower panels). The graphs represent mean and standard deviation of values obtained from three independent experiments. R, resting, uninfected cells. *, $p<0.05$.

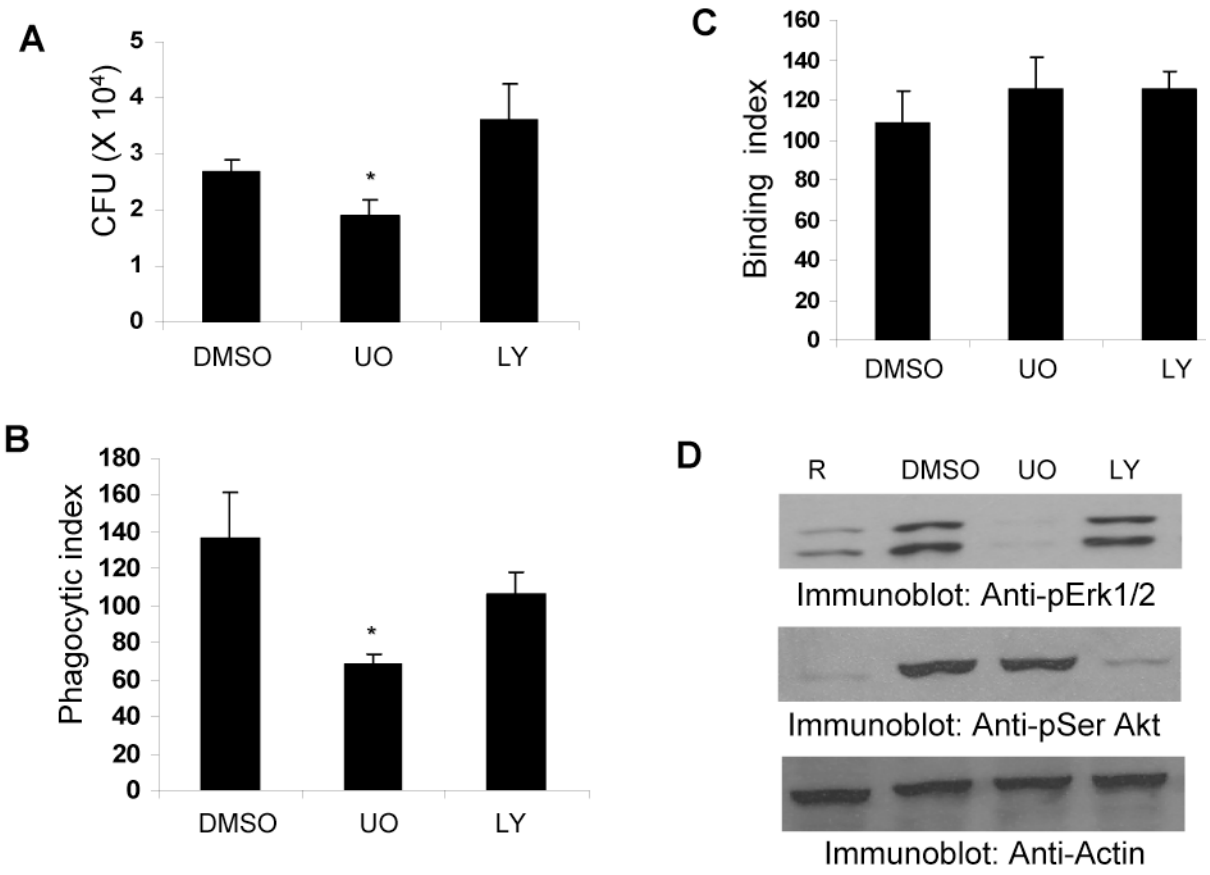

Figure 5. Erk but not PI3K/Akt is required for phagocytosis of *Francisella*

A-C. RAW 264.7 cells were treated with vehicle control (0.2% DMSO) or 2.5 μM UO126 (UO) or 20 μM of LY294002 (LY) for 30 minutes; cells were infected with 100 MOI of *F. novicida* for 1 hour and the phagocytosis (**A&B**) or adherence (**C**) of *Francisella* was examined by CFU (**A**) and microscopy assays (**B&C**). The graphs represent mean and standard deviation of values obtained from 3 independent experiments. *, $p<0.05$ compared to DMSO value. **D.** RAW 264.7 cells were treated with vehicle control or inhibitors (as described above), infected for 1 hour and protein-matched lysates were analyzed by Western blotting with phospho-specific antibodies (upper and middle panels). The same membrane was reprobed with Actin antibody to ensure equal loading (lower panel). R, resting, uninfected cells.

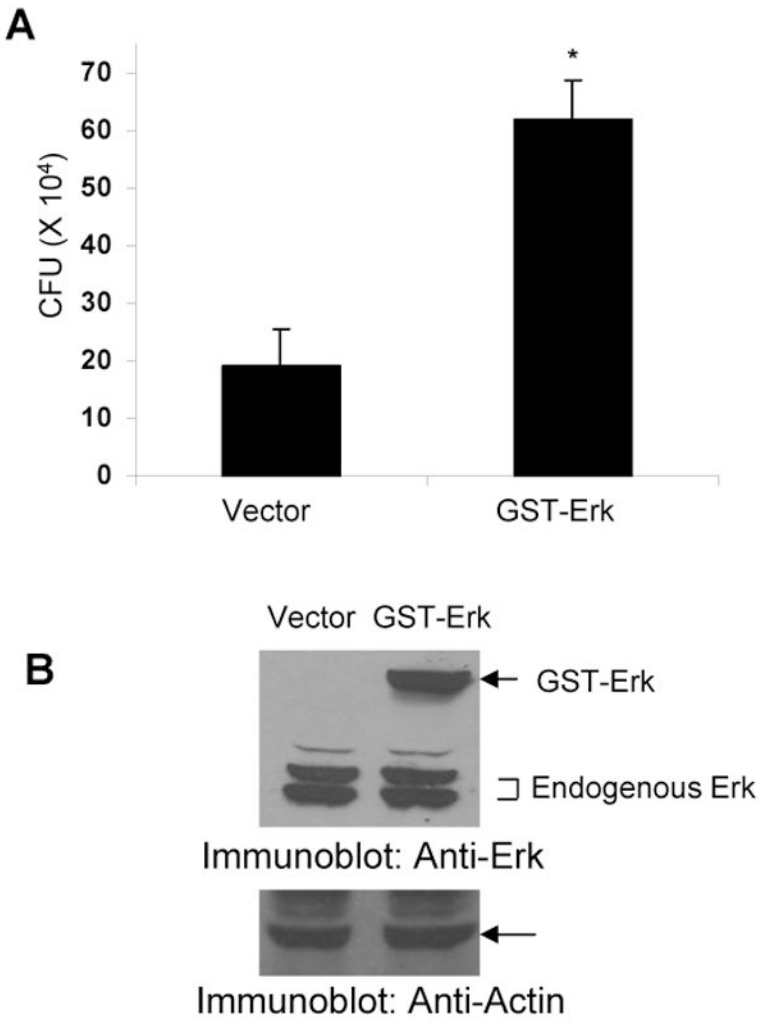

Figure 6. Erk overexpression enhances the phagocytosis of *Francisella*

RAW 264.7 cells were transfected with vector or GST-Erk2 encoding construct. 16 hours post-tranfection the cells were infected with 100 MOI of *F. novicida* for 1 hour and phagocytosis was assessed by CFU assays. The graph represents mean and standard deviation of values obtained from 3 independent experiments. *, $p<0.05$ compared to vector value. **B.** Vector and GST-Erk2 transfectants were lysed, protein-matched lysates were resolved by SDS/PAGE and the expression of Erk was analyzed by Western blotting with Erk antibody (upper panel). The same membrane was reprobed with Actin antibody (lower panel). These data are representative of three independent experiments. NS, non-specific band.

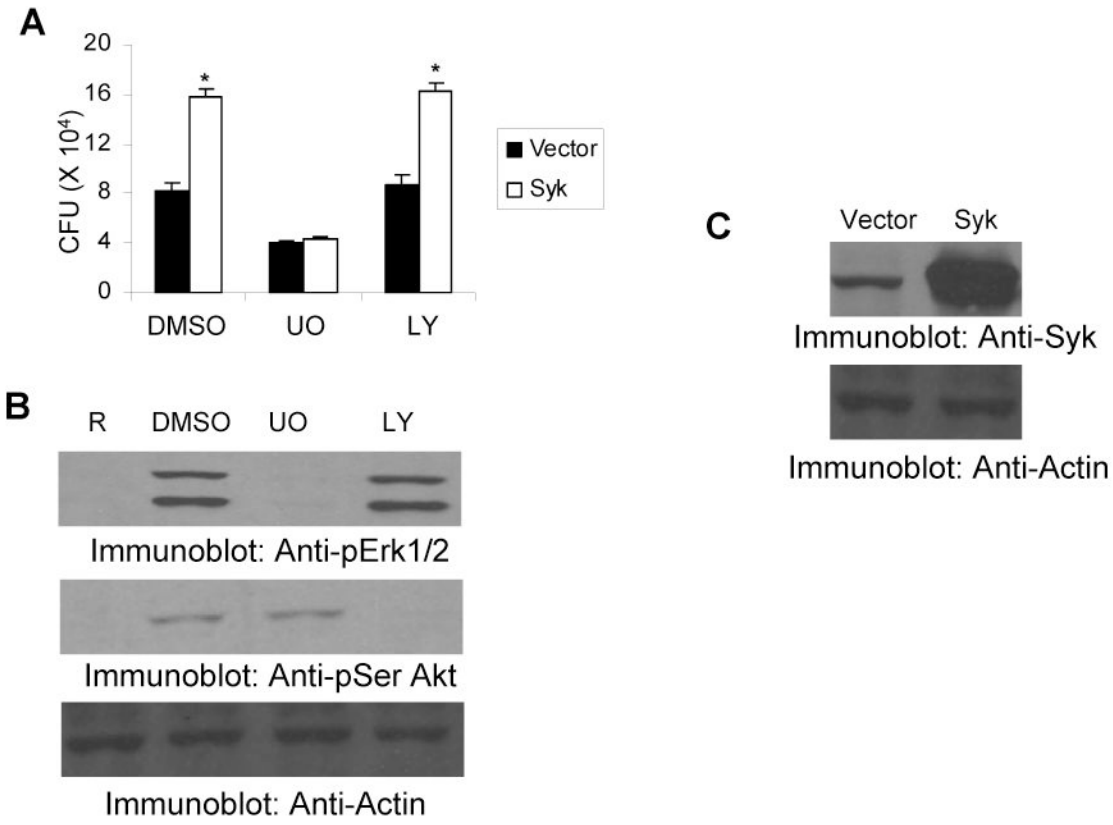

Figure 7. Syk-dependent increase in the phagocytosis of *Francisella* is abrogated by Erk inhibition

A. Vector and Syk transfectants were treated with 0.2% DMSO or 2.5 μM UO126 (UO) or 20 μM of LY294002 (LY) for 30 minutes; cells were infected with 100 MOI of *F. novicida* for 1 hour and phagocytosis was examined by CFU assays. The graph represents mean and standard deviation of values obtained from three independent experiments. *, $p<0.05$, compared to corresponding vector transfectants. **B.** RAW 264.7 cells were treated with vehicle control or with inhibitors (as described above), infected for 1 hour and protein-matched lysates were analyzed by Western blotting with phospho-specific antibodies (upper and middle panels). The same membrane was reprobed with Actin antibody to ensure equal loading (lower panel). R, resting, uninfected cells. **C.** Vector and Syk transfectants were lysed, protein-matched lysates were resolved by SDS/PAGE and the expression of Syk was analyzed by Western blotting with Syk antibody (upper panel). The same membrane was reprobed with Actin antibody (lower panel). These data are representative of three independent experiments.

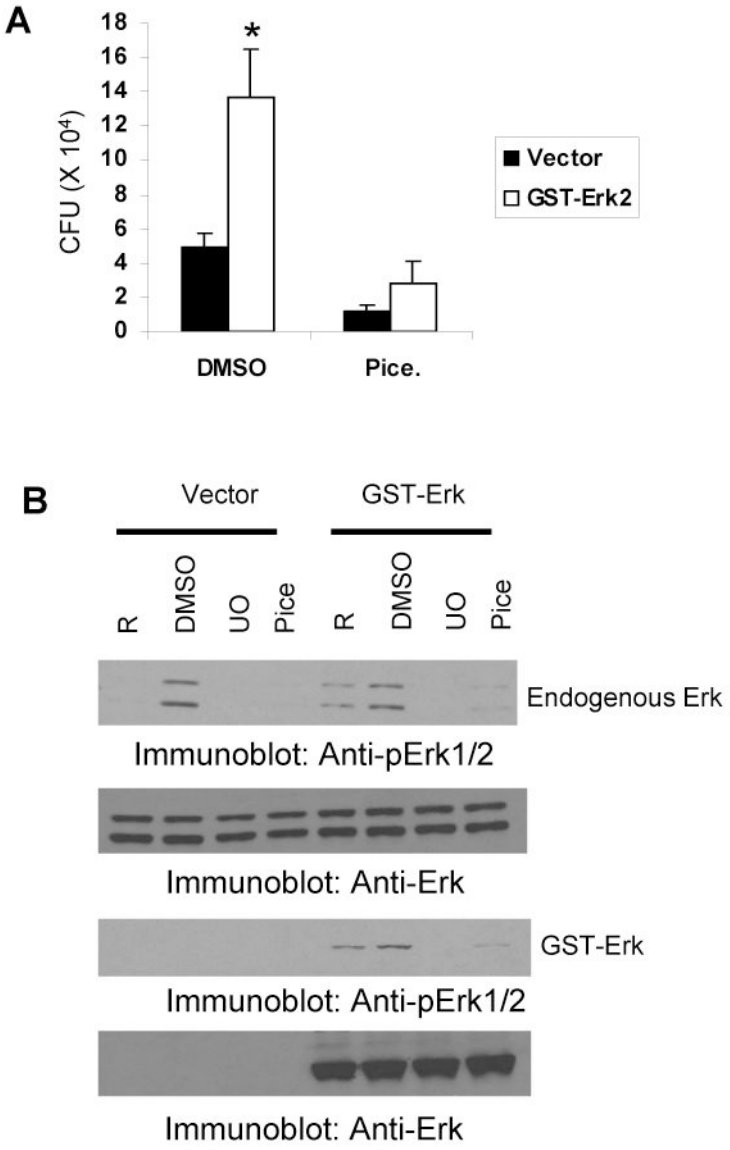

Figure 8. Erk-dependent increase in the phagocytosis of *Francisella* is abrogated by Syk inhibition

A. Vector and GST-Erk2 transfectants were treated with 0.1% DMSO or 25 μg/mL piceatannol for 30 minutes; cells were infected with 100 MOI of *F. novicida* for 1 hour and phagocytosis was examined by CFU assays. The graph represents mean and standard deviation of values obtained from three independent experiments. *, $p<0.05$, compared to corresponding vector transfectants. **B.** RAW 264.7 cells were treated with DMSO or piceatannol (as described above), infected for 15 minutes and protein-matched lysates were analyzed by Western blotting with phospho-Erk antibody (upper panel). The same membrane was reprobed with Erk2 antibody to test the over-expression of Erk2 (lower panel). R, resting, uninfected cells. These data are representative of 2 independent experiments.