The Early Dendritic Cell Signaling Induced by Virulent *Francisella tularensis* Strain Occurs in Phases and Involves the Activation of Extracellular Signal-Regulated Kinases (ERKs) and p38 In the Later Stage*

Ivo Fabrik‡, Marek Link‡, Daniela Putzova‡, Lenka Plzakova‡, Zuzana Lubovska§, Vlada Philimonenko§¶, Ivona Pavkova‡, Pavel Rehulka‡, Zuzana Krocova‡, Pavel Hozak§¶, Marina Santic¶, and Jiri Stulik‡**

Dendritic cells (DCs) infected by *Francisella tularensis* are poorly activated and do not undergo classical maturation process. Although reasons of such unresponsiveness are not fully understood, their impact on the priming of immunity is well appreciated. Previous attempts to explain the behavior of *Francisella*-infected DCs were hypothesis-driven and focused on events at later stages of infection. Here, we took an alternative unbiased approach by applying methods of global phosphoproteomics to analyze the dynamics of cell signaling in primary DCs during the first hour of infection by *Francisella tularensis*. Presented results show that the early response of DCs to *Francisella* occurs in phases and that ERK and p38 signaling modules induced at the later stage are differentially regulated by virulent and attenuated ΔΔΔΔ strain. These findings imply that the temporal orchestration of host proinflammatory pathways represents the integral part of *Francisella* life-cycle inside hijacked DCs. *Molecular & Cellular Proteomics* 17: 10.1074/mcp.RA117.000160, 81–94, 2018.

*Francisella tularensis* is a Gram-negative bacterium and intracellular pathogen that is responsible for tularemia disease (1). Although humans are not the primary hosts, *Francisella* capacity to cause respiratory infections with the relatively high mortality rates prompted the classification of the bacterium as a potential biological weapon (2). The disease itself is characterized by the delayed onset of the adaptive immunity which is then followed by the hypercytokinemia (3). The suboptimal host response comes as a consequence of *Francisella* ability to avoid the activation of phagocytes in which the bacterium replicates (4). *Francisella* initiates its intracellular life cycle by the entry into the host cell where it transiently resides within the phagosome. Following 30–60 min post infection (p.i.)¹, *Francisella* escapes from the vacuole into the host cell cytosol and it replicates there (5). Dendritic cells (DCs), as professional phagocytes, are also susceptible to *Francisella* infection. Like the situation in other host cells, *Francisella*-infected DCs are not sufficiently stimulated, do not produce proinflammatory cytokines and do not undergo the classical process of maturation (6). Consequently, these DCs have only a limited capacity to prime the adaptive response and they serve rather as migrating bacterial reservoirs. The weak immunostimulatory phenotype of infected DCs correlates with *Francisella* tendency to evade the host proinflammatory signaling. *Francisella*-mediated activation of nuclear factor

---

¹ The abbreviations used are: p.i., post infection; AMPK, AMP-activated protein kinase; AP-1, activator protein 1; BMDC, bone marrow-derived dendritic cell; CREB, cAMP-responsive element-binding protein; DC, dendritic cell; ERK, extracellular signal-regulated kinase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GSK, glycogen synthase kinase; HILIC, hydrophilic interaction chromatography; IFN-β, interferon-β; IKK, inhibitor of NF-κB kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPKAPK, MAPK-activated protein kinase; MOI, multiplicity of infection; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor κ-light-chain- enhancer of activated B cells; p70S6K, p70 ribosomal S6 kinase; PAK, p21-activated kinase; PI3K, phosphoinositide 3-kinase; RSK, p90 ribosomal protein S6 kinase; SILAC, stable isotope labeling by amino acids in cell culture; TLR, toll-like receptor.
Dendritic Cell Signaling Induced by *F. tularensis*

κ-light-chain-enhancer of activated B cells (NF-κB) is dependent on toll-like receptor 2 (TLR2)/myeloid differentiation primary response protein 88 (MyD88) stimulation (7). However, the bacterium can reduce the NF-κB-driven gene expression either through the modulation of phosphoinositide 3-kinase (PI3K)/Akt pathway or by the rapid escape from the phagosome (7, 8). In cytosol, the sensing of *Francisella* DNA triggers stimulator of interferon genes (STING)-dependent type I interferon (IFN) response which helps to orchestrate the inflammation assembly and caspase 1 activation (9, 10). Nevertheless, although virulent *Francisella* stimulates in DCs the expression of IFN-β, the pyroptosis is suppressed (11). *Francisella* manipulation of DC response therefore evolves in time and follows the bacterial needs. From this perspective, the early *Francisella*-DC interactions represent the crucial phase which directs the future events of DC activation and potentially shapes the adaptive immune response.

To better understand these early processes, we analyzed the cell signaling dynamics of primary DC during the first hour of *Francisella* infection by SILAC (stable isotope labeling by amino acids in cell culture) based phosphoproteomic approach. Our results reveal the existence of distinct phases of protein phosphorylation in infected DCs. Although the initial stage seems to relate to the general process of the bacterial entry, the induction of extracellular signal-regulated kinase (ERK) and p38 signaling during the later phase is regulated differently by the used virulent *Francisella* strains.

**EXPERIMENTAL PROCEDURES**

**Cultivation of Bacteria**—All *Francisella tularensis* strains were cultured on McLeod agar enriched for bovine hemoglobin and IsoVitalex (both Becton Dickinson, Franklin Lakes, NJ) at 37 °C.

**Generation/SILAC Labeling of Bone Marrow-Derived DCs (BMDCs) and J774.2 Cultivation**—BMDCs were generated from bone marrow progenitors isolated from femurs and tibias of 6- to 8-week-old female C57BL/6 mice. Approximately 1 × 10^7 bone marrow cells were seeded on 10 cm tissue plastic Petri dish into 10 ml of RPMI 1640 media containing 10% (v/v) fetal bovine serum (FBS; Sigma Aldrich, St. Louis, MO) and penicillin/streptomycin and these were left at 37 °C in a humidified atmosphere of 5% CO₂. After overnight depletion of adherent cells, suspension cells were seeded on a new dish in RPMI 1640, 10% FBS, and 5% (v/v) supernatant from Ag8653 cells transfected by cDNA of murine granulocyte-macrophage colony-stimulating factor (GM-CSF). Cells were passaged every 2–3 days.

**Infections and Treatments**—Bacterial suspension was mixed with the suspension at 4 °C. For 24 h infection intervals, BMDCs were seeded into fresh RPMI 1640 medium containing 10% FBS (or SILAC medium with 10% dialyzed FBS in the case of proteomics experiments) at 2.5 × 10^6 cells/ml. Infection was initiated by the addition of bacteria suspended in the medium of the same composition followed by thorough mixing. Multiplicity of infection (MOI) was 50. Infected cells were kept at 37 °C/5% CO₂ and the infection was stopped by the addition of excess of ice-cold PBS followed by centrifugation of suspension at 4 °C. For 24 h infection intervals, BMDCs were seeded at 2 × 10^6 cells/ml and MOI was 10. Cells treated by *E. coli* 055:B5 lipopolysaccharide (LPS; 500 ng/ml) were used as a positive control.

In experiments with killed *Francisella*, bacteria were first fixed by 3.7% (v/v) parafomaldehyde (PFA) at 4 °C overnight and then used as an infection agent at apparent MOI 100. For synchronization of BMDC infection, bacterial suspension was mixed with the suspension of 7.5 × 10^6 cells (MOI 50) in flat-bottom falcon tube and immediately copelleted in centrifuge (400 × g, 5 min, RT). Following the centrifugation (t = 0), cells were kept at 37 °C/5%/5% CO₂ for the indicated time p.i. Mock-infected BMDCs were centrifuged without bacteria. When indicated, seeded BMDCs were pretreated either by DMSO or by bafilomycin A1 or SB203580 (both Sigma Aldrich) for 1 h at final concentrations of 100 nM or 10 μM, respectively, followed by the addition of bacteria. DMSO/inhibitors were present in the medium throughout the infection. Adherent J774.2 cells were infected in 6-well plates (1 × 10^6 cells/well) by cocentrifugation (400 × g, 5 min, RT) with bacteria (MOI 50) and left for 60 min at 37 °C/5% CO₂.

**Cell Lysis and Protein Digestion**—BMDC pellets were lysed by sodium deoxycholate (SDC)-containing buffer as previously described (13). Protein concentrations were measured using the Micro BCA kit (Thermo Pierce, Waltham, MA) and the corresponding light and heavy isotope-labeled lysates were mixed in a 1:1 ratio based on protein content. Proteins were reduced by the addition of DTT (final concentration 10 mM) for 1 h at 37 °C, followed by the alkylation with iodoacetamide (IAA; final concentration 20 mM) for 30 min at room temperature in the dark. The excess of IAA was quenched by the addition of DTT to a final concentration of 20 mM and the reaction was left to proceed for 15 min at room temperature. Proteins were digested by trypsin (Promega, Madison, WI) at a ratio 50:1 (w/w) at 37 °C overnight. Digestion was stopped by the addition of TFA to a final concentration of 1% (v/v) to precipitate SDC. Suspension was then mixed with an equal volume of ethyl acetate, vortexed and centrifuged. Upper organic phase was removed and the extraction process was repeated four times to completely extract SDC (14). Water phase containing peptides was then desalted on Discovery DSC-18 SPE cartridges (500 mg/3 ml; Sigma Aldrich) and the eluate in 80% ACN/0.1% TFA was vacuum-dried.

**HPLC Fractionation**—Fractionation of peptides was performed using Alliance 2695 liquid chromatograph (Waters, Milford, MA). For phosphoproteome analysis, BMDC digests were fractionated by hydrophilic interaction chromatography (HLIC) (15). Peptide material of 3 to 5 mg was injected onto TSKgel Amide-80 HR column (5 μm, 4.6 × 250 mm) with guard column (5 μm, 4.6 × 10 mm; both Tosoh Bioscience, Tokyo, Japan) under conditions of 20% mobile phase A (2% ACN/0.1% TFA) and 80% mobile phase B (98% ACN/0.1% TFA) at flow rate of 0.5 ml/min. Peptide separation was performed by a linear gradient formed by mobile phase A and mobile phase B, from 80 to 60% of mobile phase B in 40 min and from 60 to 0% of mobile phase B in 5 min. Through the gradient elution window, 20 fractions were manually collected into microcentrifuge tubes. For analysis of BMDC proteome at 60 min p.i., 200–300 μg of peptides were fractionated by high-pH reversed phase liquid chromatography as previously described (16) and 10 fractions were manually collected through the gradient elution window. Prior LC-MS analysis, fractions 1 and 2 were pooled with fractions 9 and 10, respectively.

**Phosphopeptide Enrichment**—Collected HLIC fractions were first acclified by 2% (v/v) TFA solution containing 100 μM glutamic acid as an eluoder, followed by the addition of 1.5–2.5 mg of TiO₂ particles (10 μM, GL Sciences, Tokyo, Japan). Suspensions were then vor-
texted for 20 min at room temperature and centrifuged. Pellets of TiO$_2$ were sequentially washed in buffers A (65% ACN/2% TFA/100 mM glutamic acid), B (65% ACN/0.5% TFA), and C (65% ACN/0.1% TFA). TiO$_2$-bonded phosphopeptides were eluted by vortexing particles for 10 min at room temperature in ammonia solution ($pH \sim 11$) followed by the acidification of supernatants by TFA to reach $pH$ 2. Each HILIC fraction was subjected to two identical cycles of the enrichment and TiO$_2$-eluates from the same fraction were pooled, desalted on 3M Empore SPE cartridges (Sigma Aldrich) and the eluates in 65% MeOH/0.1% TFA were vacuum-dried.

**Liquid Chromatography-Mass Spectrometry**—The Ultimate 3000 RSLCnano system connected through Nanospray Flex ion source with Q Exactive mass spectrometer (Thermo Scientific, Waltham, MA) were used for instrumental analysis (17). For phosphoproteome analysis, approximately one third of sample material from each phosphopeptide fraction was introduced onto trap column (PepMap100 C18, 3 $\mu$m, 0.075 $\times$ 20 mm) and then separated by running a linear gradient (0.1% FA in water as phase A; 80% ACN, 20% water and 0.1% FA as phase B) from 4 to 34% B in 48 min and from 34 to 55% B in 10 min, at a flow rate of 300 nL/min, on analytical column (PepMap C18, 2 $\mu$m, 0.075 $\times$ 150 mm). The full MS/Top10 setup was used for mass spectra acquisition. The positive ion MS spectra from 350–1750 $m/z$ range were obtained in the Orbitrap at a resolution of 70,000 (at $m/z$ 200). Multiply charged precursors ions with minimal threshold intensity of $5 \times 10^3$ counts and not fragmented during previous 30 s were admitted for higher energy collisional dissociation (HCD). Tandem mass spectra were acquired with following settings: resolution at 17,500, AGC target value at $1 \times 10^5$, maximum ion injection time at 100 ms, and normalized collision energy set to 27. Data acquisition was under control of Xcalibur software v3.0. Proteome samples fractions were analyzed by the same instrumentation using a linear gradient from 4 to 30% B in 88 min and from 30 to 55% B in 25 min for separation. Mass spectrometer operated in Top12 setup and collected MS spectra were in range of 350–1650 $m/z$. Intensity threshold for triggering MS/MS was $6 \times 10^3$ counts. The rest of instrumental parameters were identical to those of phosphoproteome analysis.

**Phosphosite and Protein Identification and Quantification**—Phosphoproteome and proteome data sets were processed by MaxQuant ver. 1.5.2.8 coupled with Andromeda search engine (18). Data were searched against FASTA database consisting of reference proteome for *Mus musculus* searched against FASTA database consisting of reference proteome and proteome data sets were processed by MaxQuant. Molecular & Cellular Proteomics 17.1

$\text{RSLCnano system connected through Nanospray Flex ion source}$

**Experimental Design and Statistical Rationale**—SILAC experiments were performed in biological triplicate for each time point and bacterial strain used and the respective mock-treated BMDCs served as a control. In two replicates, light BMDCs were infected and heavy cells were mock-treated. In one replicate, SILAC groups were swapped (supplemental Fig. S1). In total, 18 digests (three time points/two bacterial strains/triplicate) were fractionated by HILIC. Aliquots of 6 digests (60 min p.i./two bacterial strains/triplicate) were also subjected to proteome analysis. Significantly regulated phosphoproteins for each triplicate (time point/bacterial strain) were found by global mean rank test (GMRT) (19) using R package MeanRankTest (https://www.evotive.com/MeanRankTest) with parametric FDR level set to 0.05. Only those phosphoproteins quantified in all three replicates of the given experimental condition were allowed for testing. With respect to the obtained phosphoproteomic data, GMRT was chosen for two reasons: (1) rank tests in general are advantageous for global methods when fold changes are relatively low (20) and (2) GMRT was shown to reliably control FDR even for small number of replicates (19).

**Phosphosite Fuzzy c-means Clustering**—Fuzzy c-means clustering (21) of phosphosites with localization probability $>0.75$ (22, 23), which were quantified in all three time points for WT-infected BMDC samples with the relative standard deviation (RSD) $<30\%$, was performed by $M fuzz$ R package (24). For a given phosphosite, normalized log$_2$-ratios from replicates were first averaged and multiplied by −1 for each time point and then normalized by Z-score. Fuzzifier was set to 3.65 (25) and the number of clusters was 4. Human protein reference database (HPRD) (26) kinase motifs enriched in phosphosite clusters were found by Fisher exact test (Benjamini-Hochberg (BH) FDR level set to 0.05) using Perseus software ver. 1.5.4.1 (27).

**InnateDB Terms Enrichment and the Construction of Protein-Protein Interaction Network**—For the comparison of BMDC response induced by WT and $\Delta dsbA$ *Francisella* strains, normalized log$_2$-ratios of phosphosites quantified in all three replicates of WT-infected BMDC samples were tested by unpaired two sample Student’s $t$ test against those quantified in all three replicates of $\Delta dsbA$-infected BMDCs samples of the same time interval. Phosphosites (and corresponding phosphoproteins) having $p < 0.05$ were further considered as differentially regulated between BMDCs infected by WT and $\Delta dsbA$. For InnateDB term analysis, phosphoproteins were first annotated by Pathway analysis web-based tool from InnateDB database (http://www.innatedb.com/) (28) and Fisher exact test was then used to find enriched InnateDB Pathway name terms in the group of differentially regulated phosphoproteins (BH FDR level set to 0.05). Only terms containing at least 3 regulated phosphosites are reported. In case in which the same group of phosphosites was annotated by several similar enriched InnateDB terms, one representative term was selected. Using the described approach, none InnateDB terms were found enriched for 10 and 30 min p.i. For the construction of protein-protein interaction network, only proteins bearing phosphosites differentially regulated between WT- and $\Delta dsbA$-infected BMDCs at 60 min p.i. were considered. Sequences were mapped to murine protein sequences on STRING (v10) (29) using BLAST to obtain database identifiers and best matches with at least 80% identity were used. STRING interaction network containing interactions with $>700$ score
was then loaded into Cytoscape ver 3.2.1 and only differentially regulated phosphoproteins or proteins having at least two differentially regulated neighbors (“connecting” nodes) were kept. The emerging network was then reduced to contain smallest possible number of “connecting” nodes while keeping all regulated phosphoproteins in one network (i.e. removing “connecting” nodes with relatively low number of neighbors). In cases where it could not be decided, all equivalent nodes were kept.

**Immunofluorescence Microscopy**—Bacteria were incubated for 1 h with 5 μM 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) prior the infection. BMDCs were then infected as described under Experimental Procedures, transferred to glass slide using cytosin and fixed by 3.7% PFA. The excess of PFA was quenched by 50 mM NH4Cl and cells were permeabilized by 50 μg/ml digitonin for 1 min. BMDCs were then stained for 20 min by Alexa Fluor 594 Phalloidin (6.6 μM) and for 5 min by 4',6-diamidino-2-phenylindole (DAPI, 300 nM) and mounted by Mowiol. Microscope slides were viewed by fluorescence microscopy on Nikon Eclipse Ti (Nikon, Tokyo, Japan). Percentage of infected cells was calculated as an average of 500 cross-sectional images per time point.

**Transmission Electron Microscopy**—Infected or uninfected cells were quickly washed with Sørensen buffer (0.1 M sodium/potassium phosphate buffer, pH 7.3; SB) at 37 °C, fixed with 2.5% glutaraldehyde in SB for 2 h, washed with SB, embedded in blocks of 1% low-melting point agarose (type VII, Sigma Aldrich), and postfixed with 1% OsO4, solution in SB for 2 h. The cells were dehydrated in series of ethanol with increasing concentration, subsequently in propyleneoxide, and embedded in Epon-Durcupan resin. Polymerized blocks were cut into 80 nm ultrathin sections, collected on 200 mesh formvar/ficoll-coated nickel grids, stained with saturated aqueous solution of uranyl acetate for 4 min. The sections were examined in FEI Morgagni 268 transmission electron microscope operated at 80 kV. The images were captured using Mega View III CCD camera (Olympus Soft Imaging Solutions, Münster, Germany).

**Flow Cytometry**—Following 24 h of infection, suspension BMDCs were harvested and stained by following antibodies: anti-CD11c-phycocerythrin (PE)-Cy7 (BD Pharmingen, San Jose, CA), anti-CD80-PE (Beckman Coulter, Brea, CA), anti-CD86-PE (Beckman Coulter), and anti-I-A/I-E major histocompatibility complex II (MHCIi) conjugated with biotin (Novus Biologicals, Littleton, CO), respectively. Anti-MHC II antibodies were then stained by streptavidin-FITC (Invitrogen, Carlsbad, CA). Cells were fixed and before analysis on CyAn ADP flow cytometer (Beckman Coulter) stained by propidium iodide. Data acquisition and interpretation were performed using Summit 4.3 software (Beckman Coulter).

**ELISA**—Quantification of cytokines in cell culture supernatants was performed using DuoSet ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer instructions.

**Quantitative Real-time PCR**—RNA was isolated from cells using RNeasy kit from Qiagen. 1 μg of total RNA was reverse transcribed using oligo (dT) primers (New England Biolabs, Ipswich, MA). Quantitative real-time PCR analysis was performed and analyzed using ABI Prism 7500 Fast RT-PCR System (Applied Biosystems, Foster City, CA). Data were normalized to the housekeeping gene 18S rRNA (Rn18S1) and expressed as fold change relative to RNA samples from mock-treated cells using the comparative Ct method (∆∆Ct). The following TaqMan Gene Expression Assays were used (Applied Biosystems): Il10 (Mm01288386_m1), Il12b (Mm01288989_m1), Il6 (Mm00439552_s1), and Il6 (Mm03392990_g1).

**Western Blot**—Cell pellets were lysed in RIPA buffer containing protease (Roche, Basel, Switzerland) and phosphatase (cocktail set II, Merck, Darmstadt, Germany) inhibitors. Denatured and reduced proteins were separated by SDS-PAGE and transferred to PVDF membranes. Blots were blocked by milk and incubated with primary antibodies overnight followed by secondary antibody conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). Bands were visualized by ECL (Amersham Biosciences, Little Chalfont, United Kingdom). Anti-β-actin and anti-tubulin antibodies were purchased from Sigma Aldrich and Abcam (Cambridge, United Kingdom), respectively. The rest of primary antibodies were obtained from Cell Signaling (Danvers, MA).

**RESULTS**

**Francisella Induces Two Waves of Protein Phosphorylation in BMDCs During the First Hour of Infection**—SILAC-labeled primary murine bone marrow-derived DCs (BMDCs) (12) were infected by fully virulent Francisella tularensis subsp. holarctica FSC200 strain (WT) in suspension without the synchronization by centrifugation to avoid TLR2-MyD88-dependent NF-κB activation caused by a mechanical force (30). BMDCs were lysed at 10, 30, and 60 min p.i. and the lysates were processed and analyzed as described under Experimental Procedures (supplemental Fig. S1). In total, 17,535 phosphoproteins from Francisella-infected BMDC proteome were identified. From these, more than 5000 were quantified in all replicates of each experimental condition (time/bacterial strain; supplemental Fig. S2 and supplemental Table S1). In general, Francisella-induced phosphorylation events in BMDCs were most apparent at 10 min p.i. (Fig. 1A). Consistently, the highest number of phosphosites was classified as significantly regulated at 10 min p.i. (Fig. 1B and supplemental Fig. S3). Surprisingly, following the drop at 30 min p.i., there was a new increase in BMDC protein phosphorylation at 60 min p.i. This observation was not caused artificially by the variability of data. Phosphoproteome samples were grouped together according to the time p.i. in PCA (supplemental Fig. S4A). Although the relatively small phosphorylation changes and sub-optimal SILAC labeling efficiency of primary BMDCs (12) led to the low correlation of label swap replicates, there was no difference between 30 and 60 min p.i. data sets in terms of the distribution of RSDs for phosphosites SILAC ratios (supplemental Fig. S4B). The increase in BMDC signaling at 60 min p.i. was also not affected by the changes in protein expression or degradation (supplemental Fig. S5). To further analyze phosphorylation dynamics in infected BMDCs, fuzzy c-means clustering was used to group reproducibly quantified phosphosites according to their time profiles (Fig. 1C and 1D). In line with results shown in Fig. 1B, cluster peaking at 10 min p.i. (cluster A) contained the highest number of phosphosites (Fig. 1C). To identify potentially involved kinases, clustered phosphosites were searched for Human Protein Reference Database (HPRD) kinase motifs (Table I). Activities of several kinases seemed to follow the profile of the largest phosphosite cluster A; e.g. p70 ribosomal S6 kinase (p70S6K), Akt or p21-activated kinase 2 (PAK2) (Table I). Importantly, activities of these kinases were confirmed by Western blot (Fig. 1E). The large part of phosphoproteins regulated at 10 min p.i. was functionally linked to regulation of cytoskeleton and vesicular transport (supplemental Table S1). Among them, GTPase-activating proteins (GAPs) and guanine nucleotide exchange
factors (GEFs) of Rac and Cdc42 GTPases (e.g. Arhgap1, Arhgefs or Docks) represented the most notable examples. GEFs activate Rac/Cdc42 and promote their binding to PAKs, which in turn stimulates PAK autophosphorylation and activation. Such autophosphorylation of PAK1 (S204) and PAK2 (S55 and S197) was indeed detected at 10 min p.i., therefore indirectly confirming previous kinase motif analysis (Fig. 1D, 1E and Table I) and supporting the active role of Rho GTPase-PAK signaling in Francisella internalization. In contrast to 10 min p.i., only few potential kinases could be associated with the second signaling maximum occurring 60 min p.i. (clusters B and D in Fig. 1D and Table I, respectively). Although the activity of AMP-activated protein kinase (AMPK) gradually increased and peaked at 60 min p.i. (cluster D in Fig. 1D and Fig. 1E), ERKs displayed more complex behavior as their kinase motif was enriched in phosphosite cluster displaying maxima at 10 and 60 min p.i. (cluster B in Fig. 1D).

Francisella Mutant Lacking dsbA Gene May Serve As An Avirulent Control to WT Strain in BMDCs—The identification of DC signaling pathways regulated in response to Francisella

**Fig. 1.** Protein phosphorylation induced in BMDCs during the first hour of infection by *Francisella tularensis* subsp. *holarctica FSC200 (WT) strain*. SILAC-labeled BMDCs were infected with WT (MOI 50) for 10, 30, or 60 min (supplemental Fig. S1) and processed as described under Experimental Procedures. A, Histograms of normalized SILAC log2-ratios (Mock/WT) of phosphosites from infected BMDCs. Data collected from means of three biological replicates with RSD <30%. B, Count of significantly regulated phosphosites per given time p.i. (Global Mean Rank Test, FDR <0.05). C, Count of phosphosites which were assigned to fuzzy c-means clusters. D, Fuzzy c-means clustered phosphosites from infected BMDCs. E, Western blot analysis of selected kinases. Data are representative from biological duplicate.

**TABLE I**

| Cluster | HPRD motif                  | Enrichment factor | FDR      |
|---------|-----------------------------|-------------------|----------|
| A       | p70 Ribosomal S6 kinase     | 2.33              | 3.02×10⁻⁷|
| A       | Aurora-A kinase             | 1.97              | 6.82×10⁻⁴|
| A       | Phosphorylase kinase        | 1.64              | 1.50×10⁻⁵|
| A       | Akt kinase                  | 1.57              | 3.82×10⁻⁴|
| A       | MAPKAPK1 kinase             | 1.55              | 2.39×10⁻⁴|
| A       | PAK2 kinase                 | 1.53              | 2.65×10⁻³|
| A       | PKC epsilon kinase          | 1.46              | 6.21×10⁻³|
| A       | MAPKAPK2 kinase             | 1.30              | 7.85×10⁻³|
| A       | 14–3–3 domain binding motif | 1.27              | 2.05×10⁻⁴|
| A       | Calmodulin-dependent protein kinase II | 1.21              | 1.30×10⁻³|
| B       | Growth associated histone H1 kinase | 1.48              | 2.38×10⁻³|
| B       | ERK1,2 kinase               | 1.26              | 7.96×10⁻⁵|
| B       | WW domain binding motif     | 1.21              | 3.31×10⁻⁴|
| B       | GSK-3, ERK1, ERK2, CDK5     | 1.21              | 3.68×10⁻⁴|
| D       | Calmodulin-dependent protein kinase I | 2.10              | 1.14×10⁻²|
| D       | AMP-activated protein kinase | 1.70              | 1.49×10⁻²|
virulent behavior could be facilitated by the confrontation of results with experiments in which attenuated strain was used as a control. Francisella strains lacking dsbA gene (ΔdsbA) are attenuated in vivo and provide the protection against subsequent challenge by parental strain (31–33). The engagement of adaptive immunity suggests that ΔdsbA would represent suitable alternative to WT in terms of DC response. To assess in vitro ability of ΔdsbA to induce BMDC maturation, cells were infected by FSC200 WT and ΔdsbA mutant of the same background and surface expression of MHC II, CD80 and CD86 was measured 24 h p.i. by flow cytometry (Fig. 2A). For all presented maturation markers, ΔdsbA-infected BMDCs showed higher expressions than cells infected by WT. Similarly, in vitro secretion of proinflammatory cytokines IL-12p40, IL-1β, and IL-6 was higher in BMDCs infected by ΔdsbA at 24 h p.i. (Fig. 2B). The observed differences between strains were not skewed by BMDC cell death (supplemental Fig. S6A). Note however, that both WT and ΔdsbA strains were relatively poor inducers of BMDC cytokine secretion compared with Francisella Live Vaccine Strain (LVS; Fig. 2B). Moreover, the viability of cells in general (supplemental Fig. S6B) might further affect the cytokine production because the levels of IL-12p40 secreted by ΔdsbA-infected BMDCs did not exceed those of uninfected cells and the production in WT-infected BMDCs was even lower (Fig. 2B). Consistently, the levels of IL-12p70 and IL-23 secreted by BMDCs infected with either WT or ΔdsbA were below the level of detection. To explore the intracellular fate of bacterial strains, WT- and ΔdsbA-infected BMDCs were subjected to transmission electron microscopy at 60 min p.i. Similarly to what was previously reported (34), WT bacteria were located primarily in the cytosol at this time p.i. (Fig. 2C). In contrast, the majority of ΔdsbA

Fig. 2. Francisella ΔdsbA mutant stimulates in vitro BMDC activation and maturation. BMDCs were infected by FSC200 WT, FSC200 ΔdsbA or LVS at MOI 10 or treated by E. coli LPS (500 ng/ml) and left for 24 h. A, Flow cytometric analysis of cell surface expression of MHC II, CD80 and CD86. Histograms are representative from biological triplicate. B, Concentrations of IL-12p40, IL-1β and IL-6 in culture supernatants were determined by ELISA. Data are presented as means ± S.E. of independent replicates; n = 3; *p < 0.05 (Student’s t test). C, Electron micrographs of BMDCs infected by WT and ΔdsbA at 60 min p.i. (MOI 50). Black arrow shows free bacteria in the cytosol. White arrow indicates the damaged phagosomal membrane. Lengths of scale bars are 0.2 μm and 0.1 μm for micrographs showing WT and ΔdsbA, respectively. Data are representative from biological triplicate.
bacteria (~60%) were surrounded by the damaged vacuolar membrane (Fig. 2C) which suggested the intracellular trafficking of ΔdsbA in BMDCs differed from that of virulent strain. Importantly, although the unsynchronized infection reduced BMDC infectivity, the dynamics of WT and ΔdsbA host entry did not differ significantly (supplemental Fig. S6B). Taken together, the results confirmed the attenuated nature of ΔdsbA in BMDCs in vitro and encouraged its use as an avirulent control for phosphoproteomics experiments where WT was an infection agent (supplemental Fig. S1).

**Fig. 3.** BMDCs infected by WT maintain mTOR/p70S6K signaling at 60 min p.i. A, Time-dependent changes in phosphorylation of known Akt targets in WT- and ΔdsbA-infected BMDCs. B, Time-dependent site phosphorylation in proteins connected to mTOR localization to lysosome - all sites were significantly upregulated at 60 min p.i. only in WT-infected BMDCs (Global Mean Rank Test, FDR <0.05). Bar graphs show the situation at 60 min p.i. for sites quantified only at this time point. Headings of graphs in (A) and (B) contain gene name/protein name, Uniprot accession number and phosphosite position. SILAC-based phosphorylation changes are expressed as a mean ± S.E. (n = 3) of normalized fold change (FC, infected/mock) at respective time p.i.; *p < 0.05 (Student’s t test applied on normalized SILAC log2-ratios). In graphs, “C” designates no change (FC = 1) and up- and downregulated phosphosites are represented by positive and negative FC values, respectively. C, Western blot analysis of phosphosites connected to mTOR activity in infected BMDCs. Data are representative from biological duplicate.

Signaling of mTOR/p70S6K is Sustained 60 min p.i. in WT-infected BMDCs—BMDCs infected by ΔdsbA mutant were processed and analyzed for phosphoproteome changes exactly as for WT-treated cells. Dominant feature of WT-infected BMDCs was an early Akt activation (see above) (8). To examine whether WT and ΔdsbA mutant differed in their ability to trigger Akt signaling, time profiles of several identified Akt targets phosphorylated in both WT- and ΔdsbA-infected BMDCs at 10 min p.i. were compared (Fig. 3A, supplemental Table S1). Phosphorylation trends of Tuberin (Tsc2; T1465), proline-rich AKT1 substrate 1 (PRAS40; T247), glycogen synthase kinase-3 β (GSK-3β; S9) or AMPK α1 (S496) followed the predicted Akt activity (cluster A in Fig. 1D) similarly in both WT- and ΔdsbA-infected BMDCs. Importantly, these proteins are responsible for the suppression of mammalian target of rapamycin complex 1 (mTORC1) activity and Akt-mediated phosphorylation release mTORC1 from their inhibition (35–38). This suggested that mTOR activity should follow that of Akt in both WT- and ΔdsbA-infected BMDCs (Fig. 1D). However, several phosphosites associated with mTOR signaling were found to be upregulated at 60 min p.i. in BMDCs infected by WT (Fig. 3B, supplemental Table S1). Although phosphorylation of mTOR on S1261 was shown to be crucial for the induction of mTORC1 activity (39), functional roles of phosphosites S278 on SH3 domain-binding protein 4 (SH3BP4) and S381 on RagC are unknown. Interestingly, SH3BP4 negatively regulates mTORC1 activity on lysosome lumen through binding of Rag complex via SH3BP4 region containing S278 (40). Differential regulation of mTOR between WT- and ΔdsbA-infected BMDCs at 60 min p.i. was confirmed by Western blot (Fig. 3C). Independently of the bacterial strain used, PRAS40 (T247) phosphorylation (compare with Fig. 3A) and mTOR/p70S6K signaling were induced 10 min p.i. However, mTOR/p70S6K crosstalk at 60 min p.i. was preserved...
only in WT-infected BMDCs. These results suggest that in contrast to BMDCs infected by ΔdsbA, mTOR/p70S6K signaling is maintained in WT-infected BMDCs at 60 min p.i. and that this activity is Akt-independent.

Cell Signaling of BMDCs Infected by WT and ΔdsbA Starts to Diverge at 60 min p.i.—Observed differences in mTOR activity prompted further inspection of phosphoproteomic data to search for system-wide variations in WT- and ΔdsbA-induced BMDC signaling. To determine correlations in phosphoproteomes, phosphosites quantified in the given time point p.i. in both WT- and ΔdsbA-infected BMDCs were plotted against each other in scatter plots (Fig. 4A). Phosphorylation changes induced by WT and ΔdsbA invasion at 10 min p.i. correlated well. However, WT-infected BMDCs showed more prominent protein phosphorylation with increasing time p.i. (Fig. 4A). This behavior affected also counts of regulated phosphosites (supplemental Fig. S3 and supplemental Table S1). Although at 10 min p.i. the numbers of significantly regulated phosphosites in BMDCs infected by WT and ΔdsbA were comparable, almost three times more phosphosites from WT-infected cells passed the significance test when compared with ΔdsbA at 60 min p.i. (supplemental Fig. S3). Similarly, the numbers of phosphosites, which were considered as differentially regulated between WT- and ΔdsbA-infected BMDCs (see Experimental Procedures), were almost two times higher at 60 min p.i. (125 sites) than those at 10 or 30 min p.i. (70 and 56, respectively; supplemental Fig. S7).

Interestingly, the weak correlation of strain-specific signaling temporally corresponded to the second wave of protein phosphorylation at 60 min p.i. in WT-infected BMDCs (Fig. 1B). BMDC signaling pathways engaged differentially by WT and ΔdsbA at 60 min p.i. were found by InnateDB terms enrichment analysis (Fig. 4B). Taken together, WT and ΔdsbA induce similar patterns of protein phosphorylation in BMDCs during the entry. BMDC cell signaling starts to diverge in later time points p.i. and this is largely because of activation of TLR- and mitogen-activated protein kinase (MAPK)-related pathways in WT-infected cells.

ERKs and p38 Modules are Major Components of WT-induced BMDC Signaling at 60 min p.i.—To explore phosphoproteins differentially regulated at 60 min p.i. to a greater depth, protein-protein interaction network was constructed (supplemental Fig. S8). The assembly of the network was subjected to two requirements: (1) the network should contain at least 3 differentially regulated phosphosites are presented.
unidentified proteins should also be included to keep the network together (see Experimental Procedures). Notably, the center of the network was formed by a cluster of closely interacting nodes corresponding to MAPKs and MAPK-activated protein kinases (MAPKAPKs) (Fig. 5A). The kinetics of phosphorylation for sites with available quantitative data is shown in Fig. 5B. The phosphorylation profiles of ERK2 and p38 in their activation loops (Y185 and T180, respectively) suggested that these MAPKs are activated at 60 min p.i. only in WT-infected BMDCs. Interestingly, although the activation of p38 in WT-infected BMDCs peaked at 60 min p.i., the phosphorylation of ERK2 was upregulated also at 10 min p.i. in both WT- and ΔΔsbA-infected BMDCs. Of note, the time profile of ERK2-activation phosphosite in WT-infected BMDCs resembled the V-like shaped phosphosite cluster B (Fig. 5D) for which ERKs kinase motif was enriched (Table I). Although not passing the significance level for differential regulation in WT- and ΔΔsbA-infected BMDCs at 60 min p.i., phosphorylation kinetics paralleled the time profiles of phosphosites in activation loops of the respective upstream MAPK (Fig. 5B). Although there was no direct proof of c-Jun N-terminal kinases (JNKs) involvement in WT-induced BMDC signaling at 60 min p.i., the phosphorylation of JIP3 was differentially regulated in WT- and ΔΔsbA-infected BMDCs at this time p.i. (Fig. 5C). To confirm SILAC-based results, the activation states of ERKs, p38 and RSK1 were assessed by Western blot (Fig. 5D). In line with phosphoproteomics data, ERKs and RSK1 were activated in both WT- and ΔΔsbA-infected BMDCs at 10 min p.i. However, in contrast to WT-infected BMDCs, both kinases returned to their near-basal states in ΔΔsbA-infected cells at 60 min p.i. As expected, the induction of p38 at 60 min p.i. was observed only in BMDCs infected by WT strain (Fig. 5D). The described MAPK activation profiles were not biased by the efficiency or by the synchronization of the infection (supplemental Fig. S9). WT-specific induction of ERKs and p38 at 60 min p.i. raised a question whether the altered host signaling could be related to the increased presence of WT in the cytosol (Fig. 2C). Bafilomycin A1 treatment is employed to block

**Fig. 5.** ERK and p38 modules are induced in WT-infected BMDCs at 60 min p.i. A, Protein–protein interaction network constructed on STRING background containing 80 proteins differentially phosphorylated in WT- and ΔΔsbA-infected BMDCs at 60 min p.i. (large circles). Magnified part shows MAPK interaction cluster. The color of large circles corresponds to the absolute value of the difference between means of phosphosite normalized SILAC log2–ratios in WT- and ΔΔsbA-infected BMDCs at 60 min p.i. The whole network is in supplemental Fig. S8. For details see Experimental procedures. B, Time-dependent and (C) 60 min p.i. changes in phosphorylation of MAPK-connected phosphosites in WT- and ΔΔsbA-infected BMDCs. See Fig. 3 legend for the description of the graphs. D, Western blot analysis of phosphosites connected to MAPKs activity in infected BMDCs. Data are representative from biological duplicate.
phagosomal escape in host cells (46, 47). However, bafilomycin A1 alone was a potent inducer of p38 in BMDCs (supplemental Fig. S10) which ruled out its use in this case. Nevertheless, BMDCs treated by escape-negative paraformaldehyde (PFA)-killed bacteria showed a similar level of ERK activation as in WT-infected cells and only p38 activity was dependent on the viability of bacteria (supplemental Fig. S10B). Altogether, the results indicate that ERKs and p38 and their downstream effectors RSK1, RSK2, Msk2 and MK2 represent major signaling modules specifically induced in WT-infected cells at 60 min p.i. and that the induction of p38 branch requires viable bacteria.

WT-induced p38 Signaling Regulates the Early Expression of Pro- and Anti-inflammatory Cytokines in Infected BMDCs—The induction of MAPK/MAPKAPK cascades in WT-infected BMDCs suggested that these cells might also mobilize their transcriptional machinery. Indeed, the observation of WT-driven phosphorylation of proteins associated with inhibitor of NF-κB kinase (IKK) signaling indicated the upregulation of gene expression. Tab2 protein, needed for IKK/CREB activation, was phosphorylated (S450) in WT-infected BMDCs at 60 min p.i. (supplemental Table S1). Similarly, a known substrate of both canonical IKKs and IKK-related kinases (48) - Tank - was phosphorylated on two residues (S107 and S258; Fig. 6A and supplemental Table S1, respectively). Finally, IKK targets NF-κB inhibitor-α (IκB-α; S18) (Fig. 6A), IκB-β (Fig. 6B) and Abin2 (S147) (supplemental Table S1) were found to be phosphorylated in WT-infected BMDCs at 30 min or 60 min p.i., respectively, and these events are known to have a positive impact on NF-κB-dependent gene expression (49, 50). In addition, the transcriptional activity of activator protein 1 (AP-1) was also upregulated in BMDCs infected by WT as inferred from the phosphorylation of Jun (S73) (Fig. 6A). The described situation favored the expression of Il12b in WT-infected BMDCs (Fig. 6D) as the gene transcription is NF-κB- and AP-1-dependent (S1, 52). Notably, although the phospho-

Fig. 6. p38-regulates the gene expression in WT-infected BMDCs. A, Time-dependent changes in phosphorylation of selected phosphosites in WT- and ΔdsbA-infected BMDCs. B, Phosphorylation of IκB-α and CREB in WT- and ΔdsbA-infected BMDCs (MOI 50) during the first hour of infection. C, Phosphorylation of Jun, CREB and p70S6K in WT-infected BMDCs at 60 min p.i. Cells were pretreated either by DMSO or p38-inhibitor SB203580 for 60 min before infection. D, Stable expression of Il12b and Il10 mRNA in WT- and ΔdsbA-infected BMDCs (MOI 50) and in mock-treated cells during 4 h p.i. E, Stable expression of Il12b and Il10 mRNA at 2 h p.i. in WT-infected BMDCs. Cells were pretreated either by DMSO or p38-inhibitor SB203580 for 60 min before infection. F, A model of phosphorylation-mediated early DC signaling in response to infection by Francisella tularensis subsp. holarctica FSC200. For the description of graphs in (A), see Fig. 3 legend. The changes in mRNA transcription in (D) and (E) are expressed as a mean ± S.E. (n = 3) FC (infected over mock left for 1 h); *p < 0.05 (Student’s t test applied on ΔΔCt values from WT- and ΔdsbA-infected BMDCs). In graphs, “C” designates no change (FC = 1). Western blots are representative from biological duplicates.
ylation of Jun was p38-independent (Fig. 6C), the expression of Il12b relied on p38 activity (Fig. 6E) which suggests that WT-induced p38 might support NF-κB-dependent transcription (53). The early Il12b expression, followed by IL-12p40 secretion (1–6 h p.i.; Fig. 6D and supplemental Fig. S11), contrasted with the situation at 24 h p.i., in which the levels of IL-12p40 produced by WT-infected BMDCs were below those produced by ΔdsbA-infected cells (Fig. 2B and supplemental Fig. S11). The nature of the attenuation of IL-12p40 production in WT-infected BMDCs could not be explained based on the presented phosphoproteomic data as these describe the very early moments of the infection. Nevertheless, several lines of evidence suggested that triggering of MAPK/MAPKAKP signaling in WT-infected BMDCs at 60 min p.i. leads to the activation of cAMP-responsive element-binding protein (CREB) which is responsible for the transcription of genes with immunosuppressive functions (54). First, InnateDB terms connected to events in the nucleus (including “CREB phosphorylation”) were found enriched in the group of proteins differentially phosphorylated in WT- and ΔdsbA-infected BMDCs at 60 min p.i. (Fig. 4B). Second, although none of CREB phosphosites were identified as regulated in phosphoproteomic screen, CREB represented an interaction hub in MAPK signaling cluster in the constructed protein-protein interaction network (Fig. 5A). CREB-regulated transcription coactivator 2 (TORC2) was phosphorylated in WT-infected BMDCs at 60 min p.i. (supplemental Table S1) at ~2 position (S612) to calcineurin binding motif (55). Finally, WT-induced MK2 and Msks MAPKAPKs (see above) can phosphorylate the regulatory S133 CREB site (56, 57). In line with the latter, CREB was phosphorylated in WT-infected BMDCs at 60 min p.i. (Fig. 6B) in p38-dependent manner (Fig. 6C). Notably, CREB was also phosphorylated at 10 min p.i. in both WT- and ΔdsbA-infected BMDCs and the biphasic activation profile in WT-infected cells implied the participation of either ERKs (Fig. 1D and 5B) and/or Akt (see Discussion) in the process. The activation of CREB in infected macrophages and DCs leads to the expression of anti-inflammatory genes such as Il10 (54). Consistently with WT-induced CREB phosphorylation, the increase in Il10 mRNA was more prominent in WT-infected BMDCs (Fig. 6D) and the stable expression relied on p38 activity (Fig. 6E). The dominant position of MAPK-regulated transcription factors in BMDC transcriptional response to WT might seem surprising considering the cytosolic localization of bacteria at 60 min p.i. (Fig. 2C). Indeed, cytosolic Francisella is known to induce expression of type I IFN-related genes through the activation of interferon-regulatory factor 3 (IRF3) by TANK-binding kinase 1 (TBK1) in STING-dependent manner (9, 10, 58, 59). The interaction network in supplemental Fig. S8 contained group of proteins potentially involved in cytosolic sensing of bacteria (supplemental Fig. S12A). However, the cluster was relatively small and both participants identified in phosphoproteomic screen, Tank (Fig. 6A) and Traf1 (supplemental Fig. S12B), were also shown to participate in the regulation of NF-κB activity (Fig. 6B) (48, 60, 61). In line with the inconclusive findings, the expression of Ifnb1 was low in both WT- and ΔdsbA-infected BMDCs when compared with cells infected with LVS (supplemental Fig. S12C). Taken together, these results show that signaling cascades responsible for the activation of NF-κB and CREB in BMDCs are stimulated during the first hour of WT infection and the induced early expression of both Il12b and Il10 genes depends on WT-activated p38 signaling.

**DISCUSSION**

In this work, we explored phosphorylation signaling of DCs during their early (< 1 h) interactions with either virulent Francisella tularensis subsp. holarctica FSC200 (WT) or its avirulent ΔdsbA mutant. Such events are known to be differentially regulated in host cells infected by virulent and attenuated strains (7, 62–64) and they represent the initial point of divergence in the activation and the immunogenic development of DCs (6). We demonstrate here that virulent Francisella triggers two distinct waves of DC protein phosphorylation within the first hour of infection (Fig. 6F). The initial phagocytosis-induced DC response (10 min p.i.) involves Akt-mediated stimulation of mTOR, the activation of ERK-RSK module, and the regulation of Rac/Cdc42 GAPs and GEFs and PAK autophosphorylation. The active role of ERKs and mTOR in Francisella internalization was reported before (65, 66), but this is the first study to show the involvement of PAKs in the process. Their function however remains unclear because PAK activation is associated with macrophagic uptake (67) which is not the primary mechanism of Francisella entry into phagocytes (68). The initial DC response induced by the bacterial engulfment rapidly declines at 30 min p.i. The notable exception represents the upregulation of IKK signaling (e.g. phosphorylation of IκBs and Tank) which occurs more prominently in DCs infected by WT bacterium. The difference between strains becomes even more evident at 60 min p.i. when WT induces the new wave of host proteome phosphorylation. The most active components of this second signaling peak are ERK1/2-RSK1/2 and p38-MK2 kinase modules. The lower potency of ΔdsbA to stimulate such response represents an interesting feature of the invasion. DsbA protein is TLR2 ligand (69) but it is unlikely that its loss affected TLR-dependent MAPK activation because there is no quantitative difference in the initial signaling response of DCs to WT and ΔdsbA. Rather, the existence of two temporally separated host phosphorylation waves suggests that infected DCs react to two distinct events. Considering the bacterial uptake as the origin of the initial DC response, we speculate that the second signaling wave detected at 60 min p.i. might be functionally linked to the rapid escape of WT bacteria into the cytosol. Two findings support this notion: (1) only viable WT bacteria are able to trigger p38 activation and (2) although the majority of ΔdsbA-containing phagosomes lose their integrity at 60 min p.i., bacteria are still surrounded by vacular membranes and therefore not fully released. It is however impossible to con-
clude whether the activation of p38/ERKs at 60 min p.i. results from the enhanced cytosolic sensing of bacterial products, WT-specific rapid disruption of phagosome, or the activity of unidentified bacterial effector. In this light, it is interesting that components of cytosolic DNA-sensing pathways are not among phosphoproteins differentially regulated between WT- and ΔdsbA-infected DCs. It was previously shown that ΔdsbA of LVS background stimulates the higher production of IFN-β than its WT counterpart (70). In contrast, the expression of Il10β in DCs infected by FSC200 WT and ΔdsbA is lower than in LVS-infected DCs and virtually indistinguishable from uninfected cells. This suggests that the observed upregulation of MAPK signaling in WT-infected BMDCs is unrelated to STING-dependent cytosolic sensing of Francisella DNA (9, 10, 58, 59). The activation of MAPKs is a part of the host proinflammatory response and it is usually associated rather with attenuated Francisella strains (62, 63). For example, previous analysis of host phosphoproteome showed that p38-target tristetraproline (TPP) was phosphorylated in response to F. novicida ΔpcoC mutant and that this positively affected the stability of proinflammatory transcripts (63). In contrast, we observe p38-mediated TPP phosphorylation (SS2: supplemental Table S1) and p38-dependent Il12b expression rather in DCs infected by virulent strain. These contradictory findings might be explained if we consider that the extent of MAPK activity induced by virulent Francisella is assessed only relatively by the comparison with attenuated bacteria. For illustration, mutants defective in phagosomal escape are known to prolong the stimulation of TLRs from within the phagosome (7) and the comparison of these strains with WTs may lead to the conclusion that virulent Francisella avoids MAPK activation. Here, we employ ΔdsbA as a reference. The mutant is attenuated and provides protection in vivo (33). However, the production of IL-1β in infected DCs suggests that ΔdsbA probably reaches the host cytosol. Such behavior would explain the relatively low ability of ΔdsbA to induce TLR-dependent early MAPKs signaling when compared with phagosome-residing mutants (7). On the other hand, different kinetics of ΔdsbA-driven phagosomal disruption and of the cellular stress connected with the process might be responsible for the apparently high MAPKs activity in WT-infected DCs at 60 min p.i. Collectively, the presented comparative study reveals that the early MAPKs signaling in Francisella-infected DCs is selectively engaged rather than suppressed. The preservation of mTOR activity observed at 60 min p.i. in WT-activated DCs probably helps to stimulate gene transcription and to re-configure the cell metabolism (71). Interestingly, although Akt mediated the suppression of mTOR inhibitors during the bacterial entry, mTOR activity at 60 min p.i. was partially supported by p38 (Fig. 6C) and possibly also by ERKs (66). The initial proinflammatory directing of WT-infected DCs is however lost during Francisella-DC interaction and, in contrast to ΔdsbA-infected cells, DCs infected by virulent bacteria are not activated or matured 24 h p.i. Although the transient early activation of p38 and IκB-α phosphorylation was in Francisella-infected cells observed before (64), implications for bacterial virulence remain unclear. It is known that the activation of p38 helps Francisella to suppress the early cell death of the host (72, 73) and to downregulate MHC II (74). We propose that the positive regulation of CREB transcription factor is another side effect of p38 activation which favors Francisella-mediated host survival and immunity bypass (54). It was previously shown that GSK-3β inhibition led to CREB activation and IL-10 production in LVS-infected macrophages (75). Akt-mediated inhibition of GSK-3β could be indeed responsible for CREB induction at 10 min p.i. observed in both WT- and ΔdsbA-infected DCs. However, phosphorylation of CREB at 60 min p.i. and the early expression of Il10 in DCs infected by WT was p38-dependent. Although the levels of secreted IL-10 were below the limit of detection, we speculate that p38-dependently produced IL-10 may through autocrine/paracrine stimulation instruct DCs to suppress the initial inflammation and to alter the maturation (76). Taken together, our phosphoproteomic data show that the very early DC response to Francisella is divided into temporally separate phases which correspond to different stages of bacterial infection. We report that cytosolic virulent Francisella induces MAPKs and early gene expression and that these processes are the earliest events regulated differentially in DCs infected by WT and attenuated ΔdsbA strains of fully virulent FSC200 bacterial background. The temporal orchestration of host proinflammatory pathways therefore represents the integral part of Francisella life-cycle inside hijacked DCs. In this regard, the detailed analysis of early-intermediate gene expression in cells exposed to the bacterium may provide new insights into how DCs acquire their unproductive phenotype.

Acknowledgments—We thank Prof. Yousef Abu Kwaik for critical reading of the manuscript and Jitka Zakova and Lenka Lukšíková (University of Defense) for excellent technical assistance.

DATA AVAILABILITY

Proteomics data were deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data set identifiers PXD005747 (phosphoproteome) and PXD006759 (proteome) and MS/MS spectra can be viewed on MS-Viewer (http://msviewer.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msviewer) using the search keys uomuhcoiy9 (phosphoproteome) and oqiga3hmr5 (proteome).

* This work was supported by the Czech Science Foundation (15-02584S). Marina Santic was supported by the Croatian Science Foundation (HRZZ-9003). The Microscopy Centre - Electron Microscopy CF, IMG AS CR is supported by the Czech-BioImaging large RI project (LM2015062 funded by MEYS CR) and by OP RDE (CZ.02.1.01/0.0/0.0/16_013/0001775).

[S] This article contains supplemental material.
Dendritic Cell Signaling Induced by *F. tularensis*

"To whom correspondence should be addressed: Department of Molecular Pathology and Biology, Faculty of Military Health Sciences, University of Defence, 500 01 Hradec Kralove, Czech Republic. Tel.: 00420-973-253-220; Fax: 00420-495-513-018; E-mail: jiri.stulik@unob.cz.

REFERENCES

1. Kubelkova, K., and Macela, A. (2015) Putting the Jigsaw Together - A Brief Insight Into the Tularaemia. *Open Life Sci.* 10,

2. Oyston, P. C. F., SJostedt, A., and Titzball, R. W. (2004) Tularaemia: bioterrorism defence reviews interest in Francisella tularensis. *Nat. Rev. Microbiol.* 2, 967–978

3. Sharma, J., Mares, C. A., Li, Q., Morris, E. G., and Teale, J. M. (2011) Features of sepsis caused by pulmonary infection with *Francisella tularensis* Type A strain. *Microb. Pathog.* 51, 39–47

4. Asare, R., and Kwai, Y. A. (2010) Exploitation of host cell biology and evasion of immunity by *Francisella tularensis*. *Front. Microbiol.* 1, 145

5. Santic, M., Al-Khodor, S., and Abu Kwaik, Y. (2010) Cell biology and molecular ecology of *Francisella tularensis*. *Cell. Microbiol.* 12, 129–139

6. Fabrik, I., Hårtlova, A., Rehulka, P., and Stulik, J. (2013) Serving the new masters - dendritic cells as hosts for stealth intracellular bacteria. *Cell. Microbiol.* 15, 1473–1483

7. Cole, L. E., Santiago, A., Barry, E., Kang, T. J., Shirey, K. A., Roberts, Z. J., Sobol, M., Philimonenko, A., Hozak, P., Krocova, Z., Gekara, N., Filipp, D., Kashyap, M. K., Mohmood, R., Ramachandra, Y. L., Krishna, V., Rahi, B. A., Mohan, S., Ranganathan, P., Ramabhadran, S., Chaerkady, R., and Pandey, A. (2009) Human Protein Reference Database-2009 updates and continuing curation. *Nucleic Acids Res.* 37, D767–D772

8. Tyyskari, T., Sintcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., and Cox, J. (2016) The Perseus computational platform for comprehensive analysis of (proteomics) data. *Nat. Methods* 13, 731–740

9. Breuer, K., Foroushani, A. K., Laird, M. R., Chen, C., Srinabina, A., Lo, R., Winsor, G. L., Hancock, R. E. W., Brinkman, F. S. L., and Lynn, D. J. (2013) InnateDB: systems biology of innate immunity and beyond–recent updates and continuing curation. *Nucleic Acids Res.* 41, D1228–D1233

10. Straskova, A., Spidlova, P., Putzova, D., Pavkova, I., Sobol, M., Philimonenko, A., Hozak, P., Kroova, Z., Gekara, N., Filipp, D., Kashyap, M. K., Mohmood, R., Ramachandra, Y. L., Krishna, V., Rahi, B. A., Mohan, S., Ranganathan, P., Ramabhadran, S., Chaerkady, R., and Pandey, A. (2009) Human Protein Reference Database-2009 updates and continuing curation. *Nucleic Acids Res.* 37, D767–D772

**Molecular & Cellular Proteomics 17.1**
phosphorylation promotes mTORC1-mediated signaling and cell growth. Mol. Cell. Biol. 29, 4308–4324

50. Kim, Y.-M., Stone, M., Hwang, T. H., Kim, Y.-G., Dunley, J. R., Griffin, T. J., and Kim, D.-H. (2012) SH3BP4 is a negative regulator of amino acid-Rag GTPase-mTORC1 signaling. Mol. Cell 46, 833–846

51. Dalby, K. N., Monrice, N., Caudwell, F. B., Avruch, J., and Cohen, P. (1998) Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. J. Biol. Chem. 273, 1496–1505

52. Kang, S., Dong, S., Gu, T.-L., Guo, A., Cohen, M. S., Lonial, S., Khoury, H. J., Fabbro, D., Gilland, D. G., Bergsagel, P. L., Taunton, J., Polakiewicz, R. D., and Chen, J. (2007) FGFR3 activates RSK2 to mediate hematopoietic transformation through tyrosine phosphorylation of RSK2 and activation of the MEK/ERK pathway. Cancer Cell 12, 201–214

53. Engel, K., Schultz, H., Martin, F., Kotylarow, A., Plath, K., Hahn, M., Heinemann, U., and Gaestel, M. (1995) Constitutive activation of mitogen-activated protein kinase-activated protein kinase-2 by mutation of phoshoxydase sites and an A-helix motif. J. Biol. Chem. 270, 27213–27221

54. Tomas-Zuber, M., Mary, J. L., Lamour, F., Bur, D., and Lesslauer, W. (2001) C-terminal elements control location, activation threshold, and p38 docking of ribosomal S6 kinase B (Rskk). J. Biol. Chem. 276, 5892–5899

55. Matsuura, H., Nishitoh, H., Takeda, K., Magasawa, A., Tito, M., Yoshiohka, K., and Ichijo, H. (2002) Phosphorylation-dependent scaffolding role of JASAP1/JIP3 in the ASK1-JNK signaling pathway. A new mode of regulation of the MAP kinase cascade. J. Biol. Chem. 277, 40703–40709

56. Santic, M., Asare, R., Skrobonja, I., Jones, S., and Abu Kwaik, Y. (2008) Acquisition of the vacular ATPase proton pump and phagosomal acidification are essential for escape of Francisella tularensis into the macrophage cytosol. Infect. Immun. 76, 2671–2677

57. Chong, A., Wehrly, T. D., Nair, V., Fischer, E. R., Barker, J. R., Klose, K. E., Santic, M., Asare, R., Skrobonja, I., Jones, S., and Abu Kwaik, Y. (2008) Francisella tularensis LVS initially activates but subsequently down-regulates intracellular signalling and cytokine secretion in mouse mononuclear and human peripheral blood mononuclear cells. Microb. Pathog. 38, 239–247

58. Sanajada, S., Takaseu, G., Mshima, R., Yoshida, R., Kobayashi, T., and Yoshimura, A. (2008) FLN29 deficiency reveals its negative regulatory role in the Toll-like receptor (TLR) and retinoic acid-inducible gene I (RIG-I)-like helicase signaling pathway. J. Biol. Chem. 283, 33858–33864

59. Huang, M. T.-H., Mortensen, B. L., Taxman, D. J., Craven, R. J., Taft-Benzen, S., Kijek, T. M., Fuller, J. R., Davis, B. K., Allen, I. C., Brickey, W. J., Gris, D., Hen, W., Kavula, T. H., and Ting, J. P.-Y. (2010) Deletion of Ripa alleviates suppression of the inflammasome and MAPK by Francisella tularensis. J. Immunol. 185, 5476–5485

60. Nakayasu, E. S., Tempel, R., Cambronne, X. A., Petyuk, V. A., Jones, M. B., Gritsenko, M. A., Monroe, M. E., Yang, F., Smith, R. D., Adkins, J. N., and Yates, J. R., Takemori, H., Okamoto, M., and Heffron, R. (2013) Comparative phosphoproteomics reveals components of host cell invasion and post-transcriptional regulation during Francisella infection. Mol. Cell. Proteomics 12, 3297–3309

61. Telepnev, M., Goloviov, I., and Sijslott, A. (2005) Francisella tularensis LVS initially activates but subsequently down-regulates intracellular signalling and cytokine secretion in mouse mononuclear and human peripheral blood mononuclear cells. Microb. Pathog. 38, 239–247

62. Para, V. L., Butchar, J. P., Rajaram, M. V. S., Cremer, T. J., and Trindadapandi, S. (2008) The tyrosine kinase Syk promotes phagocytosis of Francisella through the activation of Erk. Mol. Immunol. 45, 3012–3021

63. Edwards, M. W., Aultman, J. A., Harber, G., Bhattacharya, J. M., Sztul, E., Xu, Q., Zhang, P., Michalek, S. M., and Katz, J. (2013) Role of mTOR downstream effector signaling molecules in Francisella tularensis internalization by murine macrophages. PLoS One 8, e83226

64. Van den Broeke, C., Radu, M., Chernoff, J., and Favoreel, H. W. (2010) An emerging role for p21-activated kinases (Paks) in viral infections. Trends Cell. Biol. 20, 160–169

65. Moreau, G. B., and Mann, B. J. (2013) Adherence and uptake of Francisella into host cells. Virulence 4, 826–832

66. Thakran, S., Li, L., Lavine, C. L., Miller, M. A., Bina, J. E., Bina, X. R., and Re, F. (2008) Identification of Francisella tularensis lipoproteins that stimulate the toll-like receptor (TLR) 2/TLR1 heterodimer. J. Biol. Chem. 283, 3751–3760

67. Petkova, D., Panda, S., Hardtova, A., Stulik, J., and Gekara, N. O. (2017) Subversion of innate immune responses by Francisella involves the disruption of TRAF3 and TRAF6 signalling complexes. Cell. Microbiol. doi: 10.1111/cmi.12769

68. Sukhbaatar, N., Hengstschläger, M., and Weichhart, T. (2016) mTOR-Mediated Regulation of Dendritic Cell Differentiation and Function. Trends Immunol. 37, 778–789

69. Hrstka, R., Stulik, J., and Vojtesek, B. (2005) The role of MAPK signal pathways during Francisella tularensis LVS infection-induced apoptosis in murine macrophages. Microbes Infect. 7, 619–625

70. Santic, M., Pavokovic, G., Jones, S., Asare, R., and Kwaik, Y. A. (2010) Regulation of apoptosis and anti-apoptosis signalling by Francisella tularensis. Microbes Infect. 12, 126–134

71. Brummett, A. M., Navratil, A. R., Bryan, J. D., and Woolard, M. D. (2014) Janus kinase 3 activity is necessary for phosphorylation of cytosolic phospholipase A2 and prostaglandin E2 synthesis by macrophages infected with Francisella tularensis live vaccine strain. Infect. Immun. 82, 970–982

72. Zhang, P., Katz, J., and Michalek, S. M. (2009) Glycogen synthase kinase-3β (GSK3β) inhibition suppresses the inflammatory response to Francisella infection and protects against tularemia in mice. Mol. Immunol. 46, 677–687

73. Corinti, S., Albanesi, C., La Sala, A., Pastore, S., and Girolomoni, G. (2001) Regulatory activity of autocrine IL-10 on dendritic cell functions. J. Immunol. Baltim. Md 1950 166, 4312–4318