Elevated Inflammatory Response in Caveolin-1-deficient Mice with *Pseudomonas aeruginosa* Infection Is Mediated by STAT3 Protein and Nuclear Factor κB (NF-κB)*

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Caveolin-1 (Cav-1), an important composition protein within the flask-shaped membrane invaginations termed caveolae, may play a role in host defense against infections. However, the phenotype in *Pseudomonas aeruginosa*-infected cav1 knock-out (KO) mice is still unresolved, and the mechanism involved is almost entirely unknown. Using a respiratory infection model, we confirmed a crucial role played by Cav-1 in host defense against this pathogen because Cav-1 KO mice showed increased mortality, severe lung injury, and systemic dissemination as compared with wild-type (WT) littermates. In addition, cav1 KO mice exhibited elevated inflammatory cytokines (IL-6, TNF-α, and IL-12a), decreased phagocytic ability of macrophages, and increased superoxide release in the lung, liver, and kidney. We further studied relevant cellular signaling processes and found that STAT3 and NF-κB are markedly activated. Our data revealed that the Cav-1/STAT3/NF-κB axis is responsible for a dysregulated cytokine response, which contributes to increased mortality and disease progression. Moreover, down-regulating Cav-1 in cell culture with a dominant negative strategy demonstrated that STAT3 activation was essential for the translocation of NF-κB into the nucleus, confirming the observations from cav1 KO mice. Collectively, our studies indicate that Cav-1 is critical for inflammatory responses regulating the STAT3/NF-κB pathway and thereby impacting *P. aeruginosa* infection.

*Pseudomonas aeruginosa* accounts for 25% of Gram-negative bacteria isolated from hospitals and is associated with high morbidity and mortality (1). *P. aeruginosa* frequently infects immunocompromised individuals, such as those affected by ventilator-associated infection, severe burns, and cancer (2). More than 80% of cystic fibrosis patients suffer severe *P. aeruginosa* infection (3). This bacterium becomes increasingly resistant to various antibiotics. Further understanding the mechanism of the host-pathogen interaction may result in effective approaches to preventing this infection. Thus, *P. aeruginosa* has been a focus of airway infectious diseases (1, 4–6).

Recent studies suggest that *P. aeruginosa* also invades the lung epithelial cells through lipid raft-mediated endocytosis (7–9), which is a possible reason why this bacterium develops such formidable resistance to antibiotics (10). The invasion process of *P. aeruginosa* may involve certain lipid raft-associated proteins, including the caveolin family of proteins (9). Using caveolin-1 (cav1) KO mice, two recent studies investigated the role of Cav-1 during *P. aeruginosa* infection (11, 12); however, their observations were very different. Thus, the role of Cav-1 in this infection needs to be clearly characterized.

Caveolins are a family of integral membrane proteins involved in caveola formation and receptor-dependent endocytosis (13–15). Cav-1 and -2 are co-expressed in various cells, such as endothelial cells, airway epithelial cells, and type I pneumocytes. Cav-1 is the major component of caveolae, flask-shaped plasma membrane invaginations. In the absence of Cav-1, Cav-2 cannot reach the plasma membrane and is degraded (16). Through the hetero-oligomeric complex formed between Cav-1 and Cav-2, Cav-2 can translocate to lipid rafts of the plasma membrane. The lipid rafts, also known as membrane microdomains, contain glycosphingolipids, cholesterol, and signaling/receptor proteins (17–19) and play a crucial role in many cellular activities, including airway bacterial invasion.

Signal transducers and activators of transcription (STATs) are SH2 domain-containing transcription factors involved in the inflammatory response in carcinogenesis and host defense (20–23). STATs are activated through receptors for cytokines and hormones, and these receptors do not contain any intrinsic enzymatic activity; thus, they depend on tyrosine kinases that can interact with the intracellular domain of the receptor. STAT signaling is initiated by phosphorylating and activating Janus kinases (JAKs), which in turn lead to phosphorylation of tyrosine residues on receptors (24). These receptors serve as docking sites for STATs. Suppressors of cytokine signaling...
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(SOCs) function as negative regulators of JAKs and operate by binding and inhibiting JAKs or competing with JAKs for phosphotyrosine binding sites on cytokine receptors (25). Recently, STAT3 was found to interact with Cav-1 and heat shock protein 90 in plasma membrane rafts during Escherichia coli infection (26). Cav-1 is also related to a JAK2/STAT5 pathway because Cav-1 is homologous to the pseudosubstrate for SOCS. Thus, Cav-1 may down-regulate JAK/STAT pathways, modulating the proinflammatory response. Because Cav-1 was found to be associated with STAT3 in lipid rafts by a co-localization study, it is also possible that a Cav-1 cascade may impact the PI3K/Akt pathway through molecular interactions, which may serve as a regulator for STAT3 (27). Thus, Cav-1 may help maintain the balance between host response and tissue damage that may be caused by overproduction of inflammatory cytokines.

To better characterize the role of Cav-1 in P. aeruginosa infection, we sought to determine the pathogenic mechanism in cav1 KO mice. We found that cav1 KO mice manifested more severe infection, including increased bacterial burdens, inflammation, oxidative stress, and susceptibility to infection. Our studies also showed that the STAT3/NF-κB pathway is responsible for the dysregulated response during P. aeruginosa infection.

EXPERIMENTAL PROCEDURES

Mouse cav1—KO and wild-type (WT) control mice (B6129SF2/J) were obtained from The Jackson Laboratory (Bar Harbor, ME) (28). Mice were housed and bred in the animal facility at the University of North Dakota, and the animal experiments were performed in accordance with the institutional animal care and use committee guidelines. We anesthetized mice with 45 mg/kg ketamine and intranasally instilled 0.5 ml of PBS supplemented with 10% formalin.

Cells—Mouse AM cells were isolated by BAL as described (30). AM cells were grown in RPMI 1640 medium supplemented with 10% newborn calf serum and penicillin/streptomycin antibiotics in a 5% CO2 incubator. MLE-12 cells were obtained from ATCC and maintained following the manufacturer’s instructions.

Bacterial Strains—P. aeruginosa strain PAO1 WT was a gift from S. Lory (Harvard Medical School, Boston, MA). PAK and PAO1-GFP were obtained from G. Pier (Channing Laboratory, Harvard Medical School, Boston, MA) (31).

Infection Experiments—Bacteria were grown overnight in Luria-Bertani (LB) broth at 37 °C with vigorous shaking. The next day, the bacteria were pelleted by centrifugation at 5,000 × g, resuspended in 10 ml of fresh LB broth, and allowed to grow until the midlogarithmic phase. Optical density (OD) at 600 nm was measured, and density was adjusted to 653,740.25 OD (0.1 OD = 1 × 106 cells/ml). Cells were washed once with PBS after overnight culture in serum-containing medium and changed to serum-free and antibiotic-free medium immediately before infection (29). Except for dose determination assays, cells were infected by P. aeruginosa in a multiplicity of infection (m.o.i.) of 10:1 bacteria–cell ratio for the indicated time points.

Cell Transfection—MLE-12 cells were transfected with yellow fluorescent protein (YFP)-Cav-1 and YFP-Cav-1Δ51–169 dominant negative (DN) plasmids using Lipofectamine 2000 reagent (Invitrogen) in serum-free RPMI 1640 medium following the manufacturer’s instructions. The cells were lysed after 24 h of transient expression (32).

Inflammatory Cytokine Profiling—Cytokine concentrations were measured by an ELISA kit (ebiScience Co., San Diego, CA) in samples of cell culture medium, BAL fluid, and lung homogenates collected at the indicated times after infection. The MLE-12 cells were treated as described above. Culture medium was collected after infection. For BAL fluid, the trachea was surgically exposed and cannulated, lungs were lavaged five times with 1.0-ml volume of lavage fluid, the lavageates were pooled, and cells were removed by centrifugation. For lung homogenates, excised lungs were ground in 500 μl of PBS. 96-well plates (Corning Costar 9018) were coated with 100 μl/well capture antibody in coating buffer and incubated overnight at 4 °C (33). 100-μl aliquots of serum samples were added to the coated microtiter wells. The cytokine concentrations were determined with corresponding detection HRP-conjugated antibodies. The values were read at 450 nm and analyzed.

Western Blotting—Mouse monoclonal Abs against Cav-1, phospho-STAT3, IL-6, and NF-κB; rabbit polyclonal Ab against phospho-NF-κB; and goat polyclonal Abs against TNF-α and SOCS3 were from Santa Cruz Biotechnology, Inc. Rabbit monoclonal Ab against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Cell Signaling Technology. The samples derived from cells and lung homogenates were lysed and quantitated. The lysates were boiled for 5 min, and protease inhibitor mixture was added. The supernatants were collected, and an equal amount of each sample was loaded onto 10% SDS-polyacrylamide minigels and electrophoresed to resolve proteins. The proteins were then transferred to polyvinylidene difluoride membranes (Pierce) and blocked for 2 h at room temperature using 5% nonfat milk blocking buffer. Membranes were incubated overnight at 4 °C with appropriate first antibodies diluted at 1:1,000 in 5% bovine serum albumin (BSA) Western antibody buffer. After washing three times with washing solution, the antigen-antibody complexes were incubated for 45 min at room temperature with horseradish peroxidase-conjugated secondary antibody (Rockland Immunochemicals, Gilbertsville, PA) diluted 1:2,000 (34). Signals were visualized using an enhanced chemiluminescence detection kit (SuperSignal West Pico, Pierce).

Reverse Transcription (RT)-PCR Analysis—RNA was extracted from lung homogenates and cells with TRIzol (Invitrogen) according to the manufacturer’s instructions. For detected genes, RT was performed using 1.5 μg of RNA, RNase ribonuclease inhibitor, oligo(dT), and cloned avian myeloblastosis virus reverse transcriptase (Invitrogen). PCR products were separated by 1.0% agarose gel electrophoresis containing ethidium bromide and visualized under UV light. The results for each gene were normalized in comparison with GAPDH expression (35).
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Confocal Microscopy and Indirect Immunofluorescence Staining—Cells were grown either on coverslips in a 24-well plate or in glass bottom dishes (MatTek, Ashland, MA). For immunostaining, the cells were fixed in 3.7% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS, and the non-specific binding site was blocked with blocking buffer for 30 min. Cells were incubated with primary Abs at a 1:500 dilution and permeabilized with 0.2% Triton X-100 in PBS, and the non-immunostaining, the cells were fixed in 3.7% paraformaldehyde with VECTASHIELD mounting medium (36). DAPI (Sigma-Aldrich) was used to stain the nucleus. The images were captured by an LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY) and processed using the software provided by the manufacturer.

Luciferase Assay—Transient transfections were performed with 2 × 10^5 cells plated in 6-well plates by using 2 μg of DNA and 3 μl of Lipofectamine 2000 reagent (Invitrogen) in serum-free RPMI 1640 medium following the manufacturer’s instruction. 24 h after transfection, the cells were infected with PAK at 10:1 m.o.i. for 1 h (37). Cell lysates were subjected to luciferase activity analysis by using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

3-(4,5-Dimethylthiazol-2-yl)-2,5-dimethyltetrazolium Bromide Assay—This assay measures the color change of 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide upon reduction by enzymes to assess the viability of cells. BAL fluid was centrifuged, and cells were cultured in a 96-well plate. After incubation with P. aeruginosa, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide dye was added at a final concentration of 1 μg/ml per well. The cells were incubated at 37 °C for 60 min or until the color change occurred. The dye is yellow in color and upon reduction by enzymes forms a blue formazan product. The reaction was stopped by adding 100 μl of stop solution (10% DMSO, 10% SDS in 50 mM HEPES buffer). The plate was left at room temperature overnight for complete dissolution of formazan crystals. The next day, the plate was read at 560-nm absorbance using a plate reader to quantify the dye conversion (38). Duplicates were done for each sample and control. Background correction was done with blanks containing dye alone.

NBT Assay—This assay is based on the color change of NBT dye upon reduction by released superoxide. Cells were treated as above, and same amount of dye as used above was added. The dye is yellow in color and upon reduction by superoxide forms a blue formazan product (39).

Dihydridichlorofluorescein Diacetate Assay—Dihydridichlorofluorescein diacetate dye (Molecular Probes, Carlsbad, CA) does not normally fluoresce but emits green fluorescence upon reaction with superoxide inside cells. Cells were treated as above, and an equal amount(s) of dye was added. After a 10-min incubation, fluorescence was measured using a fluorometer (BioTek, Winooski, VT) (39, 40).

Lipid Peroxidation Assay—Malondialdehyde is an end product of the lipid peroxidation process and was measured in a colorimetric assay (Calbiochem) according to the manufacturer’s instructions. Homogenized lung tissue in 62.5 mM Tris-HCl (pH = 6.8) supplemented with Complete Mini protease inhib-
杀了，器官被无菌性移除用于各种实验。肺部组织提取物被用来测量细菌负担。Cav-1 KO小鼠显著地增加菌落形成单位数的肺部组织和AM细胞与WT小鼠相比，表明严重肺损伤和肺炎。为了定量地确定中性粒细胞浸润，BALF液和血清被分析以确定百分比的PMN细胞。PMN穿透到肺部，与BALF液和血清中的cav1 KO小鼠相比，WT小鼠显示氧化应激显著增加（Fig. 2，A–C）。*P. aeruginosa*感染被先前展示通过显示中性粒细胞氧气物种（ROS）在肺部，可能累积并最终导致肺损伤（43）。为了测量这种氧化应激，cav1 KO和WT小鼠被PAK感染，AM细胞被获得。与WT小鼠相比，AM细胞中的cav1 KO小鼠显示了氧化应激18 h后被NBT染色。这些结果进一步被使用二氢氯氯化报纸中酸脱色剂染色（Fig. 2E），一种敏感的荧光染色法来量化超氧化物。此外，肺部细胞色素c膜电位也观察到在cav1 KO小鼠中，表明氧化应激导致细胞死亡。这些数据共同表明氧化应激增加在细胞凋亡过程中。这些数据集表明增加的ROS可能因为增加的凋亡细胞导致肺损伤，可能显著地影响肺和其他器官。

**Infection Dissemination in cav1 KO Mice—** A logical question is whether or not the intranasal inoculation only caused a local infection within the lung. Using tissue homogenates, we assessed bacterial burdens in several other organs. Colony-forming units of PAK were also increased in the spleen, liver, and kidneys, indicating that bacteria were able to spread from the original inoculation site (lungs) (Fig. 3, A–C). The spleen, which is the most sensitive indicator for bacterial spread, displayed a marked increase in bacterial burdens (Fig. 3A). Consistent with the PMN penetration into the serum (Fig. 2C), these results also suggest that the severe lung injury and bacterial spread into other organs are the possible cause of mortality in these mice.

To confirm the dissemination results, we next determined MPO activity in the lung and other organs to again probe neutrophil penetration because MPO is a recognized influx for oxidation in tissue. As expected, similar MPO activity was observed in the lung, liver, and kidney (Fig. 3, D–F). Increased MPO in organs other than the lung (e.g., liver and kidney) suggests that the superoxide release may result from systemic spread of the invading PAK bacteria.

As increased oxidation can also cause tissue injury by oxidative degradation of lipids, we used a thiobarbituric acid–reactive substance assay to detect lipid peroxidation in the lung, liver, and kidney tissue. Our results show that lipid peroxidation was significantly increased in all of the PAK-infected organs of cav1 KO mice as compared with WT mice (Fig. 3, G–I). The lungs showed a marked increase in lipid peroxidation compared with the liver and kidney, suggesting that the lung was the main target. These data were consistent with the severity of lung injury determined using the MPO assay and superoxide detection, suggesting that the increased lipid peroxidation in KO mice is relevant to the progression of lung injury by oxidation.

**Altered AM Function in cav1 KO Mice—** AM cells play a crucial role in bacterial clearance by phagocytosis (43). To explore...
In this relationship, we also measured the ingested PAK bacteria in AM cells. Increased bacterial burdens were found in the AM cells of cav1 KO mice compared with those of WT mice as assessed by colony-forming units (Fig. 4A). However, this result still cannot address whether the bacteria were dealt with through active phagocytosis or passive uptake. To determine the function of AM cells, equal amounts of PAO1-GFP, a strain of P. aeruginosa with green fluorescence (31, 39), were added into cultured AM cells isolated from cav1 KO mice or WT mice for a 2-h incubation. This allowed us to conveniently quantify phagocytosis by measuring fluorescence intensity using a fluorescence plate reader (BioTek) (29, 32). Phagocytosis of cav1 KO AM cells was significantly lower than that of WT AM cells, indicating that Cav-1 may play a role in phagocytosis by AM cells (Fig. 4B). These results also indicate that increased pre-ingestion of the pathogen resulted in reduced phagocytic capacity in AM cells following infection.

Survival of AM cells postinfection is also an essential factor for maintaining their bacterial clearance function. Thus, to determine the AM viability, AM cells were infected with PAK for 1 h at m.o.i. 10:1. After adding polymyxin B (to remove the surface bacteria; Sigma-Aldrich), survival levels of AM cells were measured, which again showed a decrease in the survival of AM cells in cav1 KO mice after PAK infection (Fig. 4C). These results indicate that Cav-1 may play a critical role in AM-mediated host defense against PAK infection.

**Altered Inflammatory Response in cav1 KO Mice**—Cav-1 has been implicated in bacterially induced inflammatory responses.
Cytokine concentrations of BAL fluid were measured 18 h post-PAK infection to probe various cytokines for reflecting the inflammatory response. The BAL fluid of cav1 KO mice showed no significant difference in inflammatory response compared with that of WT mice. However, in cav1 mouse lungs, IL-2 and IL-10 levels were significantly elevated compared with WT mice as assayed by ELISA (p < 0.05) (Fig. 5, A–D). This indicates that the lung epithelium may be the main source of increased cytokines. To validate the above data, mRNA levels of the above quantified cytokines were also measured by semiquantitative RT-PCR. mRNA expressions of TNF-α, IL-6, IFN-γ, and IL-12a were all up-regulated by ~2–4-fold as determined by densitometry (Fig. 5E). Similarly, levels of TNF-α, IFN-γ, IL-1β, IL-12a, and IL-6 (particularly IL-12a and IL-6) were increased in cav1 KO lung tissues as assessed by Western blotting with densitometrical analysis (Fig. 5F). Collectively, these data indicate that cav1 KO mice showed a more intense proinflammatory response (TNF-α, IL-1β, and IL-6) following PAK infection as compared with WT mice.

Activation of NF-κB Pathway and STAT3 by PAK Infection in cav1 KO Mice—To illustrate the mechanism that causes the dysregulated response to PAK infection in cav1 KO mice, we assessed several signaling proteins in lung tissues by Western blotting (Fig. 5G). We found that NF-κB was highly activated in protein expression (2-fold) and phosphorylation (1.4-fold). To
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Further dissect the pathway of cytokine response, we also analyzed STAT activation. STAT3, a transcription factor associated with NF-κB activation in various cellular processes, showed significant expression in cav1 KO mouse lungs (4-fold), and phospho-STAT3 was drastically increased (24-fold; Fig. 5G). Because STAT5 was not significantly increased (data not shown), our data suggest that STAT3 might play a specific regulatory role in this infection (20). We then evaluated the immediate upstream regulator of STAT3 and found that phospho-JAK2 was dramatically increased in cav1 KO mice (34-fold) (Fig. 5G). We also analyzed the roles of SOCS3, a negative regulator of STAT3. A significant increase in SOCS3 was seen in cav1 KO mice as compared with WT mice by Western blotting analysis (data not shown), suggesting that SOCS3 was responsive to the infection-induced inflammatory response. These results strongly indicate the activation of STAT3 in cav1 KO mice as compared with WT mice.

**PAO1 Infection Displays Similar Phenotypes in cav1 KO Mice—** To determine whether the infection response and its mechanism are similar across different bacterial strains, we studied the immune response to another well-studied P. aeruginosa strain, PAO1, in cav1 KO mice. A similar infection condition was used except for a higher cfu count because PAO1 is less cytotoxic than PAK. Consistent with observations in PAK infection, cav1 KO mice also showed elevated bacterial burdens in lungs and in other major organs (the liver, spleen, and kidney) after PAO1 infection (Fig. 6, A–D). Our findings demonstrate that lung tissues were severely injured in cav1 KO mice as compared with WT mice. In addition, sepsis was also observed in cav1 KO mice versus WT mice by PAO1 infection. Further-more, the inflammatory response indicated by cytokines and superoxide was also increased in cav1 KO mice following PAO1 infection (data not shown). We then investigated the NF-κB pathway and STAT3 activation using Western blotting analysis. Similar to the PAK strain, the PAO1 strain also induced significant inflammatory responses (phospho-NF-κB, 6.4-fold; phospho-STAT3, 5.4-fold; and phospho-JAK2, 5.8-fold) in cav1 KO mice as compared with WT mice. These results indicate that PAO1 infection also activated the STAT3 pathway along with NF-κB activation (Fig. 6E). In addition, levels of inflammatory cytokines IL-6 (7.3-fold) and TNF-α (5.6-fold) were also increased in cav1 KO mice as compared with WT mice. Interestingly, the SOCS3 level was much higher in cav1 KO mice than WT mice (data not shown) and was also more significantly activated than in the PAK-infected mice (Fig. 5G). These data indicate that PAO1 infection in cav1 KO mice exhibited a phenotype similar to that of the PAK strain and that a more rigorous inflammatory response was induced by the lower cytotoxicity strain PAO1 as compared with PAK.

**NF-κB Pathway Is Altered by Mutating Cav-1 in MLE-12 Cells and by STAT3 Inhibitor—** To better understand the role of STAT3 in the NF-κB pathway, we transfected murine alveolar epithelial MLE-12 cells with either WT cav1- or a DN cav1-expressing plasmid (45). MLE-12 cells have been widely used as a model for analyzing murine lung epithelial function (39, 41, 46). Transfected cells were then infected with PAK for 1 h at m.o.i. 10:1 and lysed for the cfu assay. As expected, increased bacterial burdens were observed in cav1 DN MLE-12 cells (Fig. 7A). Next, we assessed the mRNA levels for NF-κB, JAK2, and STAT3, which were not significantly activated at an early phase
(1 h postinfection). However, mRNA levels of IL-6 and IL-12a significantly increased. To ascertain the role of STAT3, WP1066 (a STAT3 inhibitor; Sigma-Aldrich) was used to pre-treat the cav1 DN cells. WP1066 has been demonstrated to inhibit the phosphorylation of STAT3 at the low μM range, thereby effectively blocking STAT3 signaling (47). After treat-
ment with the STAT3 inhibitor, activation of IL-6 and TNF-α was abolished, whereas the total mRNA levels were not altered (Fig. 7B). These data support the view that STAT3 plays a crucial role in cytokine production. Furthermore, we determined the protein levels of the aforementioned cytokines and their regulators. Consistent with the mRNA expression, the protein levels of IL-6 and TNF-α were increased in cav1 DN-transfected cells when compared with WT controls by semiquantitative RT-PCR (Fig. 7C), which showed that cav1 DN transfection resulted in a profile similar to that observed in cav1 KO mice. By contrast, protein levels of IL-6 and TNF-α were suppressed by WP1066 pretreatment. Importantly, phosphorylation of NF-κB, JAK2, and STAT3 in cav1 DN cells (Fig. 7C). These results confirm our findings observed in cav1 KO mice, indicating that the typical phenotypes may be attributable to a dysregulated proinflammatory cytokine response through the Cav-1/STAT3/NF-κB axis.

Inactive (resting) NF-κB and STAT3 are normally retained in the cytosol of MLE-12 cells (48). We hypothesized that these factors can be activated by infection, which may induce their translocation to the nucleus (49). We examined the localization of NF-κB by confocal microscopy and found that both NF-κB and STAT3 were activated and translocated into the nucleus in cav1 DN MLE-12 cells but not in vector-treated MLE-12 cells (Fig. 8, A and B). To further probe the activation of NF-κB, we transfected MLE-12 cells and determined luciferase expression. Consistent with the imaging data, the luciferase promoter assay

**FIGURE 8. Elevated nuclear translocation of NF-κB and STAT3 induced in cav1 DN MLE-12 cells.** A and B, WT and cav1 DN MLE-12 cells were infected with PAK for 1 h. The localization of NF-κB and STAT3 was visualized by indirect immunofluorescence staining. Nuclear translocation of NF-κB and STAT3 was detected in both WT and cav1 DN MLE-12 cells (arrows show the nuclear translocation). WP1066 (2 μM) was used to pretreat the cells for 0.5 h before infection. C, activation of NF-κB was detected in both WT and cav1 DN MLE-12 cells using a luciferase promoter assay. Transient transfections were performed with 2 × 10⁵ cells plated in 6-well plates using 2 μg of DNA and 3 μl of Lipofectamine 2000 reagent for 24 h. After 1 h of PAK infection at 10:1 m.o.i., cell lysates were subjected to luciferase activity analysis using the Dual-Luciferase Reporter Assay System. The data are representative of three experiments in triplicate (one-way ANOVA (Tukey’s post hoc); *, p < 0.05). D, the diagram delineates a pathway in Cav-1 KO cells against P. aeruginosa (PA) infection. Cav-1 deficiency impacts the phosphorylation of both NF-κB and STAT3 in the cytoplasm during P. aeruginosa infection. The translocation of both factors to the nucleus continuously induces cytokine production and aggravates disease progression. DIC, differential interference contrast.
defined the activation of NF-κB (Fig. 8C). Importantly, we reaffirmed that the activation of NF-κB was also associated with the STAT3 pathway because the elevation in luciferase activity was significantly inhibited by WP1066. A simplified model represents the pathway implicated in cav1 KO mice and cav1 DN cells (Fig. 8D).

**DISCUSSION**

In this study, we investigated the phenotype of *P. aeruginosa* infection in cav1 KO mice. Severe disease phenotypes in cav1 KO mice were observed in our experiments, including decreased survival, increased inflammatory response, and more severe lung injury compared with WT mice. We reasoned that dysregulated cytokine response and increased superoxide release contribute to this exaggerated pathogenesis. Importantly, we have identified a novel mechanism involved in the phenotype in which the STAT3 pathway is responsible for the dysregulated inflammatory response. Our results are consistent with the hypothesis that Cav-1 plays an essential role in the clearance of *P. aeruginosa* because both phagocytosis of the bacteria by alveolar macrophages and cytokine production by alveolar epithelial cells were Cav-1-dependent (50). Similar phenotypes (increased susceptibility, increased production of cytokines, and elevated bacterial burdens) have been observed in cav1 KO mice infected with other pathogens, such as *Salmonella typhimurium* (51). Thus, Cav-1 may render critical immune defense against a wide range of microorganisms.

Previous studies have demonstrated that caveolins are widely expressed in lung epithelial cells (52, 53). Because caveolins are associated with lipid rafts (54), they are thought to be an important part of the host response to *P. aeruginosa* infection in lung epithelial cells (7). The majority of previous studies have focused on illustrating the role of caveolins in regulating the cell responses to pathogen virulence factors like LPS (28, 55–57). However, limited reports have actually investigated the role of Cav-1 in the innate immunity of the lung against *P. aeruginosa* infection. Two previous reports showed different phenotypes in cav1 KO mice with a single pathogen, *P. aeruginosa* (11, 12). This discrepancy must be addressed to understand the real role of this critical protein. Our timely studies reveal that Cav-1 is indispensable in host defense against *P. aeruginosa* infection, consistent with the observation made by Gadjeva et al. (12). Furthermore, our studies reveal mechanistically that Cav-1 is a powerful negative regulator of proinflammatory cytokines during this infection.

We have demonstrated that ROS levels were significantly increased and that mitochondrial potential was decreased in AM cells of cav1 KO mice as compared with those in WT mice. These data showed an elevated oxidative stress in cav1 KO mice that might cause lung injury and systemic bacterial infection. Overproduction of superoxide can impair innate immunity, including the phagocytosis function of AM cells, which form the front line of innate defense in the lung. Indeed, AM cells have been shown to be dysfunctional in cav1 KO mice, which exhibit reduced phagocytosis and increased susceptibility to cell death after *P. aeruginosa* infection. Although similar methods were utilized in a cross-pathogen comparison study by Lisanti and co-workers (51), no difference in bacterial ingestion was found between cav1 KO mice and WT mice with *S. typhimurium* infection. This discrepancy may be due to inherent differences in the two pathways or the distinct experimental settings. In addition, PMN cells, another important cell type responsible for bacterial clearance, show lowered phagocytosis against *P. aeruginosa* under Cav-1 deficiency (12). Together, these observations demonstrate that the bacterial clearance mechanism is impaired in Cav-1-deficient mice at least in the case of *P. aeruginosa* infection. Furthermore, the sustained recruitment of PMN cells into the lung necessary for bacterial clearance may release excess superoxide and proteases, resulting in more severe lung injury and systemic bacterial infection.

Another major contributing factor to lung injury and mortality is the inflammatory response. We found that the lungs of cav1 KO mice exhibit increases in proinflammatory cytokines, but a similar increase was not found in the BAL fluid of these mice. Thus, lung epithelial cells might also contribute to the production of cytokines (41, 58, 59), whereas macrophages and neutrophils are traditionally regarded as the main players in inflammatory responses (60). To further explore the dysregulation of inflammatory response in KO mice, modifications of the cell signaling pathway in the lungs were examined. In particular, we found NF-κB, a major transcription factor for cytokine production in alveolar epithelial cells, to be highly activated (61).

To investigate the molecular events associated with this activation, we studied the JAK/STAT pathway. Interestingly, STAT3, but not STAT5, was found to be highly activated in cav1 KO mice following infection. The interaction between NF-κB and STAT3 has been discussed extensively in various experimental models, particularly in cancer research, whereas the STAT3/NF-κB axis is still relatively undefined in Gram-negative infections. Among the cytokines whose expression was altered, TNF-α and IL-6 were the most significantly upregulated. Considering that TNF-α and IL-6 are able to induce the activation of NF-κB and STAT3, respectively, these significant changes might be partially attributable to positive feedback (22, 62). Interestingly, we also observed significantly increased expression and phosphorylation of Akt and GSK3β (data not shown), which may be the other pathways responding to or resulting from the dysregulated cytokine production and may interact with the JAK2/STAT3 pathway in Cav-1-deficient animals. Consistent with our observations, previous reports have shown the relevance of the PI3K/Akt axis (27) and GSK3β to a JAK/STAT pathway (63). These indicate that in cav1 KO mice infection by various bacteria may activate several different pathways to compensate for the loss of this critical protein. Indeed, there is little doubt that Cav-1 is important for regulating lipid raft-mediated endocytosis and various other cellular events (32, 64, 65). Despite such activation in various pathways, the loss of Cav-1 cannot be completely rescued to combat infections and usually leads to lethal consequences because crucial cellular signaling systems may have been dysregulated. For example, Cav-1 has been reported previously to interact with STAT3 directly through molecular interactions (51). Deficiency in Cav-1 may impact STAT5 activity (44), whereas the ultimate effect on STAT5 activity is cell-dependent (51). Furthermore, no previous studies have linked Cav-1 with the...
STAT3 pathway in response to a respiratory pathogen. Thus, linking Cav-1 with STAT3/NF-κB is a novel finding for this host response. Finally, the role of the STAT3 pathway in AM cells is currently unclear and is worth studying.

Thus, we hypothesized that STAT3 might play a vital role in regulating NF-κB activation. We used MLE-12 cells to establish a Cav-1-deficient lung epithelial culture model. Our data indicate that cav1 DN plasmid-transfected MLE-12 cells showed a similar inflammatory response and cell signaling activation as seen in cav1 KO mice. STAT3 inhibitor WP1066 was then used to confirm the role of STAT3 in MLE-12 cells. As expected, cav1 KO mice, and our data indicate that Cav-1 initiates a negative regulation in the STAT3/NF-κB pathway. Although SOCS3, a chief negative regulator of STAT3, was also increased in cav1 KO mice (data not shown), the overactivation of STAT3 and the associated inflammatory response was not constrained. Thus, our study suggests that Cav-1 may potentiate the negative regulatory effects of SOCS3 on the inflammatory response, whereas loss of Cav-1 significantly dampens this role, making this an interesting question for future studies.

In conclusion, we have demonstrated a typical phenotype of P. aeruginosa infection in cav1 KO mice, and our data indicate an important role for Cav-1 in innate immunity in mice. Cav-1 deficiency impaired the phagocytic ability and other immune defense mechanisms, resulting in sustained infiltration of PMN cells into the lung and an intense inflammatory response. Along with the bacterial infection-induced oxidative stress, the accumulation of PMN cells and failure of bacterial clearance may cause severe lung injury and systemic bacterial dissemination, which finally results in host mortality. In addition, we revealed a novel Cav-1/STAT3/NF-κB axis directly contributing to a dysregulated cytokine profile in cav1 KO mice. More importantly, we confirmed this Cav-1/STAT3/NF-κB pathway in cav1 knockout MLE-12 cells during P. aeruginosa infection. Taken together, these observations have provided an insight into the role of Cav-1 in innate immunity against P. aeruginosa and might indicate novel targets for clinical therapy.

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REFERENCES
1. Driscoll, J. A., Brody, S. L., and Kollef, M. H. (2007) Drugs 67, 351–368
2. Lyczak, J. B., Cannon, C. L., and Pier, G. B. (2000) Microbes Infect. 2, 1051–1060
3. Heijerman, H. (2005) J. Cyst. Fibros. 4, Suppl. 2, 3–5
4. Fagon, J. Y., Chastre, J., Domart, Y., Trouillot, J., and Gilibert, C. (1996) Clin. Infect. Dis. 23, 538–542
5. Dunn, M., and Wunderink, R. G. (1995) Clin. Chest Med. 16, 95–109
6. Crouch Brewer, S., Wunderink, R. G., Jones, C. B., and Lerner, K. V., Jr. (1996) Chest 109, 1019–1029
7. Zaas, D. W., Duncan, M. J., Li, G., Wright, J. R., and Abraham, S. N. (2005) J. Biol. Chem. 280, 4864–4872
8. Grasmé, H., Jendrossek, V., Riehle, A., von Kürthy, G., Berger, J., Schwarz, H., Weller, M., Kolesnick, R., and Gubins, E. (2003) Nat. Med. 9, 322–330
9. Garcia-Medina, R., Dunne, W. M., Singh, P. K., and Brody, S. L. (2005) Infect. Immun. 73, 8298–8305
10. Chastre, J., and Fagon, J. Y. (2002) Am. J. Respir. Crit. Care Med. 165, 867–903
11. Zaas, D. W., Swan, Z. D., Brown, B. I., Li, G., Randell, S. H., Degan, S., Sunday, M. E., Wright, J. R., and Abraham, S. N. (2009) J. Biol. Chem. 284, 9955–9964
12. Gadjeva, M., Paradis-Blieu, C., Priebe, G. P., Fichorova, R., and Pier, G. B. (2010) J. Immunol. 184, 296–302
13. Scherer, P. E., Okamoto, T., Chun, M., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 131–135
14. Tang, Z., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 2255–2261
15. Williams, T. M., and Lisanti, M. P. (2004) Genome Biol. 5, 214
16. Mora, R., Bonilha, V. L., Marmorstein, A., Scherer, P. E., Brown, D., Lisanti, M. P., and Rodriguez-Bouland, E. (1999) J. Biol. Chem. 274, 25708–25712
17. Thomas, S., Preda-Pais, A., Casares, S., and Bruneau, T. D. (2004) Mol. Immunol. 41, 399–409
18. Thomas, S., Kumar, R. S., and Bruneau, T. D. (2004) Arch. Immunol. Ther. Exp. 52, 215–224
19. Korade, Z., and Kenworthy, A. K. (2008) Neuropharmacology 55, 1265–1273
20. Grivennikov, S. I., and Karin, M. A. (2010) Cell 141, 21824
21. Ivashkiv, L. B. (2010) Cell Host Microbe 8, 132–135
22. Takeda, K., Kaisho, T., Yoshida, N., Takeda, J., Kishimoto, T., and Akira, S. (1998) J. Immunol. 161, 4652–4660
23. Jones, M. R., Quinton, L. J., Simms, B. T., Lupa, M. M., Kogan, M. S., and Mizgerd, J. P. (2006) J. Infect. Dis. 193, 360–369
24. Wang, L., Kurotsuki, T., and Corey, S. J. (2007) Oncogene 26, 2851–2859
25. Norkin, O., Dolganiuc, A., Catalano, D., Kody, M., Madrekar, P., Syed, A., Efros, M., and Szabo, G. (2008) Alcohol Clin. Exp. Res. 32, 1565–1573
26. Maruvada, R., Argon, Y., and Prasadarao, N. V. (2008) Cell. Microbiol. 10, 2326–2338
27. Shen-Tu, G., Schauer, D. B., Jones, N. L., and Sherman, P. M. (2010) Lab. Invest. 90, 265–281
28. Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F. C., Schedd, A., Haller, H., and Kurzchalia, T. V. (2001) Science 293, 2449–2452
29. Kannan, S., Audet, A., Huang, H., Chen, L. J., and Wu, M. (2008) J. Immunol. 180, 2396–2408
30. Wisniewski, P. E., Spech, R. W., Wu, M., Doyle, N. A., Pasula, R., and Martin, W. J., 2nd (2000) Am. J. Respir. Crit. Care Med. 162, 733–739
31. Priebe, G. P., Brinig, M. M., Hatano, K., Grout, M., Coleman, F. T., Pier, G. B., and Goldberg, J. B. (2002) Infect. Immun. 70, 1507–1517
32. Kannan, S., Audet, A., Knittel, J., Mullegama, S., Gao, G. F., and Wu, M. (2006) Eur. J. Immunol. 36, 1739–1752
33. Kannan, S., Huang, H., Seeger, D., Audet, A., Chen, Y., Huang, C., Gao, H., Li, S., and Wu, M. (2009) PLoS One 4, e4891
34. Wu, M., Stockley, P. G., and Martin, W. J., 2nd (2002) Electrophoresis 23, 2573–2576
35. Wu, M., Harvey, K. A., Ruzmetov, N., Welch, Z. R., Sech, L., Jackson, K., Stillwell, W., Zaloga, G. P., and Siddiqui, R. A. (2005) Int. J. Cancer 117, 340–348
36. Kannan, S., Pang, H., Foster, D. C., Rao, Z., and Wu, M. (2006) Cell Death Differ. 13, 311–323
37. Yan, C., Cao, J., Wu, M., Zhang, W., Jiang, T., Yoshimura, A., and Gao, H. (2010) J. Biol. Chem. 285, 37227–37239
38. Wu, M., Brown, W. L., and Stockley, P. G. (1995) Bioconjug. Chem. 6, 587–595
39. Wu, M., Huang, H., Zhang, W., Kannan, S., Weaver, A., McKibben, M., Herington, D., Zeng, H., and Gao, H. (2011) Infect. Immun. 79, 75–87
40. Wu, M., Audet, A., Cusick, J., Seeger, D., Cochran, R., and Ghribi, O. (2009) J. Neurochem. 111, 1011–1021
41. Kannan, S., Huang, H., Seeger, D., Audet, A., Chen, Y., Huang, C., Gao, H., Li, S., and Wu, M. (2009) PLoS One 4, e4891
42. Wu, M., Hussain, S., He, Y. H., Pasula, R., Smith, P. A., and Martin, W. J., 2nd (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 14589–14594
43. Suntres, Z. E., Omri, A., and Shek, P. N. (2002) Microb. Pathog. 32, 27–34
44. Park, D. S., Lee, H., Frank, P. G., Razani, B., Nguyen, A. V., Parlow, A. F., Russell, R. G., Hult, J., Pestell, R. G., and Lisanti, M. P. (2002) Mol. Biol. Cell 13, 3416–3430
45. Brazer, S. C., Singh, B. B., Liu, X., Swaim, W., and Ambudkar, I. S. (2003) J. Biol. Chem. 278, 27208–27215
46. Renier, M., Tamanini, A., Nicolis, E., Rolfini, R., Imler, J. L., Pavirani, A., and Cabrini, G. (1995) Hum. Gene Ther. 6, 1275–1283
47. Kenneth, R. F., Charles, A. H., and Timothy, L. C. (2005) Clin. Cancer Res. 11, A277
48. Sugahara, K., Mizutani, A. and Yamamoto, H. (2010) Am. J. Respir. Crit. Care Med. 181, A3613
49. Arita, Y., Ito, T., Oono, T., Kawabe, K., Hisano, T., and Takayanagi, R. (2008) World J. Gastroenterol. 14, 4473–4479
50. Kowalski, M. P., Dubouix-Bourandy, A., Bajmoczi, M., Golan, D. E., Zaidi, T., Coutinho-Sledge, Y. S., Gygi, M. P., Gygi, S. P., Wiemer, E. A., and Pier, G. B. (2007) Science 317, 130–132
51. Medina, F. A., de Almeida, C. J., Dew, E., Li, I., Bonuccelli, G., Williams, T. M., Cohen, A. W., Pestell, R. G., Frank, P. G., Tanowitz, H. B., and Lisanti, M. P. (2006) Infect. Immun. 74, 6665–6674
52. Barth, K., Weinhold, K., Guenther, A., Young, M. T., Schnittler, H., and Kasper, M. (2007) FEBS J. 274, 3021–3033
53. Barar, J., Campbell, L., Hollins, A. J., Thomas, N. P., Smith, M. W., Morris, C. J., and Gumbleton, M. (2007) Biochem. Biophys. Res. Commun. 359, 360–366
54. Jacobson, K., Mouritsen, O. G., and Anderson, R. G. (2007) Nat. Cell Biol. 9, 7–14
55. Shaykhiev, R., Sierigk, J., Herr, C., Krasteva, G., Kummer, W., and Bals, R. (2010) FASEB J. 24, 4756–4766
56. Garrean, S., Gao, X. P., Brovkovych, V., Shimizu, J., Zhao, Y. Y., Vogel, S. M., and Malik, A. B. (2006) J. Immunol. 177, 4853–4860
57. Cohen, A. W., Hnasoko, R., Schubert, W., and Lisanti, M. P. (2004) Physiol. Rev. 84, 1341–1379
58. Amano, H., Morimoto, K., Senba, M., Wang, H., Ishida, Y., Kumatori, A., Yoshimine, H., Oishi, K., Mukaida, N., and Nagatake, T. (2004) J. Immunol. 172, 398–409
59. Thorley, A. J., Goldstraw, P., Young, A., and Tetley, T. D. (2005) Am. J. Respir. Cell Mol. Biol. 32, 262–267
60. Ozaki, T., Maeda, M., Hayashi, H., Nakamura, Y., Moriguchi, H., Kamei, T., Yasuoka, S., and Ogura, T. (1989) Am. Rev. Respir. Dis. 140, 1595–1601
61. Moodie, F. M., Marwick, J. A., Anderson, C. S., Szulakowski, P., Biswas, S. K., Bauter, M. R., Kilty, L., and Rahman, I. (2004) FASEB J. 18, 1897–1899
62. Wang, C. Y., Mayo, M. W., and Baldwin, A. S., Jr. (1996) Science 274, 784–787
63. Nakayama, M., Hisatsune, J., Yamasaki, E., Isomoto, H., Kurazono, H., Hatakeyama, M., Azuma, T., Yamaoka, Y., Yahiro, K., Moss, J., and Hiroyama, T. (2009) J. Biol. Chem. 284, 1612–1619
64. Anderson, R. G. (1998) Annu. Rev. Biochem. 67, 199–225
65. Anderson, R. G., and Jacobson, K. (2002) Science 296, 1821–1825