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Caveolin-1 Modifies the Immunity to \textit{Pseudomonas aeruginosa}

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The inflammatory response to \textit{Pseudomonas aeruginosa} is not properly regulated in the lungs of patients with cystic fibrosis (CF). In the lung epithelium of individuals with wild-type CF transmembrane conductance regulator, lipid rafts containing CF transmembrane conductance regulator are rapidly formed in response to \textit{P. aeruginosa} infection, and this response is closely linked to resistance to infection and disease. We found these rafts also contained high levels of caveolin-1 and thus examined the sensitivity of cav1 knockout (KO) mice to \textit{P. aeruginosa} challenge in both acute and chronic \textit{P. aeruginosa} infection models. We found that cav1 KO mice had increased sensitivity to \textit{P. aeruginosa} infection, as represented by an increased mortality rate, elevated bacterial burdens recovered from lungs and spleens, and elevated inflammatory responses. These findings correlated with the decreased ability of cav1-deficient neutrophils to phagocytose \textit{P. aeruginosa}. In addition, \textit{P. aeruginosa} colonized cav1 KO mice much better compared with the wild-type controls in a model of chronic infection, indicting an important contribution of Cav-1 to innate host immunity to \textit{P. aeruginosa} infection in the setting of both acute pneumonia and chronic infection typical of CF. \textit{The Journal of Immunology}, 2010, 184: 000–000.

Although \textit{Pseudomonas aeruginosa} is a frequent cause of acute nosocomial pneumonia in patients undergoing mechanical ventilation, burn victims, patients with corneal trauma, and patients with healing surgical wounds (1) and cystic fibrosis (CF) (2, 3), healthy individuals are generally highly resistant to serious infection. There are clearly multiple, redundant innate immune mechanisms contributing to this resistance. However, it is also clear a key factor is the CF transmembrane conductance regulator; CI, confidence interval; IL-1, interleukin-1; IN, intranasally; KO, knockout; PMN, polymorphonuclear cell; WT, wild-type.

Abbreviations used in this paper: AP, alkaline phosphatase; BAL, bronchoalveolar lavage; Cav, caveolin; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CI, confidence interval; IL-1β, β form of pro-IL-1; IN, intranasally; KO, knockout; PMN, polymorphonuclear cell; WT, wild-type.

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shown that during bacterial entry into cells, *P. aeruginosa* colocalizes with Cav-1 and CFTR in variety of human cell lines (18) in association with the CFTR receptor for *P. aeruginosa* (19, 20). Cav-1 was also identified as a prominent protein found in lipid rafts of cells with WT CFTR after only 15 min of infection with *P. aeruginosa* (20). In this study we examined the role of Cav-1 in both an acute and chronic *P. aeruginosa* infection model. We found that cav-1 KO mice have increased sensitivity to *P. aeruginosa* infection, as represented by an increased mortality rate, elevated bacterial burdens recovered from lungs and spleens, and elevated inflammatory responses. These findings correlated with the decreased ability of neutrophils to phagocytose *P. aeruginosa*. In addition, *P. aeruginosa* colonized cav-1 KO mice much better compared with the WT controls in a model of chronic infection, indicating an important contribution of Cav-1 to innate host immunity to *P. aeruginosa* infection in the setting of CF.

Materials and Methods

**Mice**

Breeding pairs of cav-1 KO and control mice (B6129SF2/J) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed and bred in the Channing Laboratory Animal Facilities (Boston, MA). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Harvard Medical Area Office for Research Subject Protection.

**Acute *P. aeruginosa* pneumonia model**

Gender-matched 6- to 9-wk-old cav-1 KO and control mice were sedated with ketamine hydrochloride (65 mg/kg) and xylazine (13 mg/kg), and then infected intranasally (IN) with doses of *P. aeruginosa* from 2 × 10^5 to 2 × 10^6 CFU, using strains PAO1 or PAK as described (20). Mice were sacrificed at 6 h or 16 h postinfection by i.v. injection of phenobarbital. Lungs were inflated by instillation of PBS, removed, weighed, and homogenized in 10% FBS in DMEM, and aliquots were plated on *P. aeruginosa*-selective cetrimide plates to enumerate bacterial levels.

**Flow cytometry analysis of cells in bronchoalveolar lavage**

Following 20 h of infection of cav-1 KO and control animals with *P. aeruginosa* strain PAO1, mice were euthanized with pentobarbital, and bronchoalveolar lavage (BAL) fluid was obtained by infusion, via a tracheal needle, of 1 ml of PBS that was then recovered via the same needle. This process was repeated two more times. Cells were recovered from the BAL by centrifugation, the concentration determined in a hemocytometer, and then 1 × 10^5 cells were stained with Ab to the polymorphonuclear cell (PMN) markers CD11b (M1/70) and Gr-1 (RB6.8C5 clone) using PE-conjugated or FITC-conjugated Abs, respectively, (BD Biosciences) and examined for differences in morphology postinfection by a routine histologic procedure. Four-micrometer sections were cut and stained with hematoxylin and eosin (H&E).

**Histopathology analysis**

Lung tissues were fixed in 1% formaldehyde then embedded in paraffin and examined for differences in morphology postinfection by the Histopathology Core Facility personnel.

**Inflammatory cytokine profiling**

Levels of mouse cytokines in BAL were simultaneously measured using a Meso Scale Discovery multiplex 7-spot electrochemiluminescence assay read by an ultra low noise charge-coupled device Imager 2400 (Meso Scale Discovery, Gaithersburg, MD). The cytokines included the β form of pro-IL-1 (IL-1β), IL-6, IL-12p70, IL-10, IFN-γ and the α chemokine neutrophil attractant and activator CXCL1/GRO (also known as KC). The Meso Scale Discovery electrochemiluminescence platform has been previously validated against cytokine standards recommended by the World Health Organization and U.K. National Institute for Biological Standards and Control and by comparison with traditional ELISA (23).

**Phagocytosis assay**

One milliliter of mouse blood was collected into heparinized tubes using 20- or 22-gauge needles to obtain blood from the heart of euthanized animals. The blood from three to four mice was pooled for these experiments. The blood was washed once with 100 × g at 22°C for 10 min to collect the buffy coat layer. The cellular suspension was overlaid on Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) and Histopaque 1119 (Sigma-Aldrich) gradients and centrifuged for 30 min at 700 × g. The neutrophil fraction was collected at the interface of Histopaque 1077 and Histopaque 1119 layers. A total of 1 × 10^6 purified PMN were resuspended in RPMI 1640 and 10% FBS and mixed with *P. aeruginosa* strain PO1 at the ratio of 100 PMN:1 bacteria. Aliquots of PMN/bacteria were incubated at 37°C for 180 min. Extracellular bacteria were quantified by plating an aliquot of the mixture on *P. aeruginosa*-selective cetrimide plates. The phagocytosed bacteria was quantified by lysing the cells with Bacto tryptic soy broth/0.5% (BD Biosciences, Chicago, IL) Triton and plating on cetrimide plates.

**Chronic model of *P. aeruginosa* infection**

Establishment of chronic infection of mice, first developed for transgenic CF mice, was carried out as described (24). In this model, the recovery of *P. aeruginosa* on throat swabs and development of a significant Ab response are taken as documentation that long-term infection of the respiratory tract had been established. Mice (*n* = 10 per group) were treated for 5 days with oral levofloxacin in the drinking water to clear out enteric flora that colonize the murine oropharynx and interfere with establishment of *P. aeruginosa* infection (24). Oropharyngeal infection was induced by placing the bacteria in the drinking water (10^7 CFU/ml) for 5–7 d. The infected water was replaced with acidified water (pH 4.5) to prevent bacterial growth in the water and cross-infection within a cage (24). Oropharyngeal throat swabs were taken after the infection period to verify that all mice were exposed to *P. aeruginosa* and evaluated by ELISA as described (24). At the age of 9 mo, animals were sacrificed and lungs removed for histopathology as described for chronically infected transgenic CF mice (24).

Serum samples were collected from the chronically colonized animals. Serum dilutions were analyzed by ELISA for the presence of Abs to *P. aeruginosa*. Briefly, *P. aeruginosa* strain N6 (a homoygous *cav* mutant early clinical isolate from a patient with CF) was grown overnight on tryptic soy broth plates. A bacterial suspension was prepared with inoculating 10 ml of PBS with *P. aeruginosa* to an OD<sub>490</sub> = 2.0. This bacterial stock was treated with NaOCl to kill the bacterial cells then used at 1/1000 dilution in 0.05 M carbonate/bicarbonate buffer (pH 9.6) to coat Immunolon HBX microtiter plates (Fisher Scientific, Pittsburgh, PA) overnight at 4°C. Subsequently, plates were washed with PBS/0.05% Tween 20, blocked with 5% BSA/PBS for 2 h at 37°C, and serial 2-fold dilutions of sera starting at 1:200 were added. Plates were then incubated for 90 min at 37°C, washed with PBS/0.05% Tween 20, incubated with *P. aeruginosa*-adsorbed anti-mouse IgG-alkaline phosphatase (AP) conjugate (A4656; Sigma-Aldrich) for 1 h at 37°C, washed with PBS/0.05% Tween 20, and developed with p-nitrophenyl phosphate substrate (N2765; Sigma-Aldrich, St. Louis, MO). Plates were read at 405 nm within 30 min of substrate addition. To adsorb the anti-mouse IgG-AP conjugate with *P. aeruginosa*, *P. aeruginosa* strain N6 was grown overnight at 37°C on a tryptic soy agar plate. A bacterial suspension was prepared by washing the bacteria off the tryptic soy agar plate into 50 ml of 5% BSA/PBS then 10 µl of the anti-mouse IgG-AP conjugate added. After 30 min at 4°C, the bacteria were removed by centrifugation and the supernatant filtered and used in the ELISA.

**Statistical analysis**

Determination of the significance of the differences in outcomes between cav-1 KO and WT control animals after *P. aeruginosa* infection were calculated by Kaplan-Meier survival curve comparisons and the marginal probability of colonization between mouse strains was estimated equations (25) using the R software for statistical computing (www.r-project.org). The throat culture results obtained over time were used to calculate an overall probability of colonization of a given strain of mouse with a given strain of *P. aeruginosa*. Two-sided significance tests of the differences in the probability of colonization between LPS-smooth strains were obtained by using the ratio of the estimated difference to its robust standard error (25), which follows a standard normal distribution. Cytokine levels were analyzed for significance by two-tailed t tests (Prism 4, GraphPad).
Results

Cav regulates the susceptibility to P. aeruginosa-induced acute pneumonia

To determine if Cav-1 affects susceptibility to P. aeruginosa lung infection, groups of cav1 KO and genetically related WT mice were challenged with various doses of two strains of P. aeruginosa: PAO1 or PAK. When mice were challenged with 1 × 10^8 or 4 × 10^8 CFU/animal of strain PAO1, cav1 KO mice displayed increased lethality (log-rank test, \( p = 0.02 \)) (Fig. 1). Although the differences in the overall survival were small (~20%), the calculated hazard ratio (0.041) indicated an increased hazard for early death of about 3-fold with a 95% CI from 1.26 to 16. When cav1 KO and WT control mice were challenged with a different P. aeruginosa strain—PAK (2 × 10^8 CFU/mouse)—increased mortality among cav1 KO mice was also seen. Seven out of seven animals succumbed to infection within 24 h, whereas all the WT animals survived the challenge (\( p = 0.0006 \), Fisher exact test for overall survival; odds ratio = 0.004, 95% CI 0.00008–0.26). These findings indicate that Cav-1 is needed for full resistance to P. aeruginosa infection.

Cav-1 deficiency is associated with elevated bacterial burdens in the lung

The cause of mortality from acute P. aeruginosa lung infection in mice is strongly associated with systemic bacterial spread to major organs, of which the spleen is the most sensitive indicator. To determine the bacterial burden in lungs and spleens of cav1 KO and WT mice, animals were challenged IN with 1 × 10^8 CFU/mouse of P. aeruginosa strain PAO1 and sacrificed at different time points postinfection. Interestingly, 6 h postinfection, cav1 KO mice showed a nonsignificant tendency for decreased levels of bacteria when compared with the WT mice (Fig. 2A). However, after 12 h of infection, cav1 KO mice showed 3-fold higher levels of strain PAO1 in the lungs when compared with the WT control mice (Fig. 2; \( p = 0.004 \), Mann-Whitney \( U \) test). Elevated numbers of PAO1 bacteria were also found in the spleens of cav1 KO animals when compared with WT infected animals (Fig. 2A; \( p = 0.05 \), Mann Whitney \( U \) test). A similar outcome in the lung was obtained when mice were challenged with PAK strain (Fig. 2B).

Cav deficiency modifies P. aeruginosa–induced inflammation

Because caveolae harbor a variety of signaling components and the Cav-1 protein has been implicated as an important regulator of inflammatory responses, we analyzed the cytokine profile induced in the lung of cav1 KO and WT mice 6–12 h following P. aeruginosa infection (Fig. 3) (26). The cytokine measurements demonstrated that Cav-1-deficient mice had elevated levels of IL-1β, TNF-α, IFN-γ, KC, IL-6, IL-10, and IL-12p70 when compared with WT control mice (\( p < 0.05 \)). These differences were observed as early as 6 h postinfection and remained at later time points, e.g., 12 h postinfection (Fig. 3A, 3B). KC levels were dramatically increased in cav1 KO mice at 6 h after challenge with P. aeruginosa strain PAO1 when compared with WT mice. This tendency was preserved at the later time points where Cav-1-deficient mice maintained 2- to 3-fold elevated KC and IL-6 levels when compared with WT mice. Twelve hours postchallenge with strain PAO1, IL-1β levels in the cav1-deficient mice were raised almost 5-fold over that in the BAL from the WT mice, demonstrating that the inflammatory response induced by P. aeruginosa that is characterized by production of IL-1β, TNF-α, KC, and IL-6 is enhanced in the absence of Cav-1 protein.

Analysis of phagocytosis of P. aeruginosa strain PAO1 by cav1-deficient and sufficient PMNs

To determine if cav1-deficient PMNs ingest live P. aeruginosa as efficiently as the PMNs obtained from WT mice, we performed phagocytic studies. Consistent with a previously described defect in cav1-deficient macrophages to phagocytose bacteria (27), we found that cav1-deficient PMNs also phagocytosed significantly less P. aeruginosa bacteria than did the WT control PMNs (Fig. 4).

Histopathologic analysis of lungs in cav1 KO mice

To determine how the infection with P. aeruginosa affected lung pathology, tissue sections were stained with H&E. The micrographs showed no dramatic differences in the morphology of the infected groups in either cav1 KO or WT mice. Both groups of infected mice showed signs of acute inflammation, with elevated neutrophil infiltrates (Fig. 5). Thus, the effect of the loss of Cav-1 on susceptibility to P. aeruginosa infection was not manifest as a major change in the histopathology of the lung during acute infection, indicating that the effects on phagocytosis and control of inflammatory responses were the primary driving force in the different outcomes from infection.

Cav deficiency results in increased chronic lung colonization with P. aeruginosa

Because cav1 deficiency resulted in increased mortality from acute P. aeruginosa pneumonia, it was of interest to establish if this defect would allow for chronic lung infection to develop following infection via the drinking water with a clinical isolate of P. aeruginosa, strain N6, obtained from a patient with CF early in the course of colonization. Cultures of throat swabs obtained right after levofloxacin treatment confirmed that all mice were initially free of detectable P. aeruginosa. After exposure to P. aeruginosa in the drinking water for 5 d, followed by replacement of the contaminated water with acidified water that prevents bacterial growth in this medium, all of the animals had positive throat culture swabs for P. aeruginosa, indicating both groups had initially acquired the pathogen via the water. After 7 wk, to allow for establishment of lung infection, all mice were treated for 2 wk with meropenem (1 mg/l) in their drinking water to kill bacteria residing in the upper oropharynx but not those in the lower
infection (none of the WT controls had both of these measures of chronic 
evidenced by positive throat cultures and Ab responses, whereas 
mouse of P. aeruginosa only 30% percent of the WT had positive throat cultures for 
cav confined to the upper oropharynx and had not reached tissue sites 
throat cultures, indicating that in these mice, the colonization was 
did not have higher serum IgG Ab titers than the WT with negative 
throughout the study. The WT mice with positive throat cultures 
of meropenem from the drinking water and were maintained 
or spleens of cav mice (\(n = 7\)) were infected IN with 1 \(\times 10^8\) CFU/mouse of P. aeruginosa. The colonization with P. aeruginosa was then 
followed weekly for an additional 20 wk (Fig. 6). The percent of 
P. aeruginosa colonized cav KO mice reached 100%, whereas 
only 30% percent of the WT had positive throat cultures for 
P. aeruginosa (\(p = 0.0017\); generalized estimating equation). 

To determine if chronic colonization with P. aeruginosa resulted in 
changes in lung morphology, lung tissue was obtained from 
chronically colonized WT and cav KO mice, embedded in paraffin, sectioned, and stained with H&E. No gross changes in 
morphology were observed (Fig. 6), consistent with prior results 
with CFTR-deficient mice chronically infected with nonnuclid P. aeruginosa (24) who also showed only modest changes in the 
lungs after 6–9 mo of chronic infection. 

An additional response to lung infection not seen in mice with 
only oropharyngeal colonization but not lung infection is the de-
development of Ab responses to P. aeruginosa cells. Mice were 
tested for IgG Ab to killed P. aeruginosa N6 cells. cav KO mice 
had higher titers of IgG Abs to P. aeruginosa cells than did the 
WT controls (Table I; Mann-Whitney U test, \(p = 0.01\)). These 
differences were manifested as early as 4 wk after the withdrawal of meropenem from the drinking water and were maintained 
throughout the study. The WT mice with positive throat cultures 
did not have higher serum IgG Ab titers than the WT with negative 
throat cultures, indicating that in these mice, the colonization was 
confined to the upper oropharynx and had not reached tissue sites 
such as the lung, where Ab responses are induced. Overall, all 10 
cav1 KO mice developed chronic P. aeruginosa lung infection, as 
evidenced by positive throat cultures and Ab responses, whereas 
none of the WT controls had both of these measures of chronic 
infection (\(p < 0.0001\), Fisher exact test).

Discussion 
Prior results (15, 17, 28) have associated Cavs with lung epithelial 
cell responses to P. aeruginosa, but the actual importance of these 
responses in regard to susceptibility and resistance to infection was 
not investigated. Although numerous studies have indicated Cavs 
are important components of innate immune responses to pathogen 
virulence factors like LPS (9, 10, 29), there are only a limited 
number of studies that aimed to clarify the significance of Cav-1 in 
modulating innate immune responses against live pathogens. 

In this study, we tested the hypothesis that the P. aeruginosa-
dependent recruitment of Cav-1 to lipid rafts of airway epithelial 
cells (28) represented a significant host factor involved in control-
ing infection with this pathogen. cav1 KO mice were significantly 
more likely to have a lethal outcome from P. aeruginosa lung infection, and this phenotype was associated 
with higher production of inflammatory cytokines, elevated 
bacterial burdens, and a decreased ability of neutrophils to phago-
cytose P. aeruginosa. 

Lisanti and coworkers (30) showed that cav1 KO mice displayed 
a significant decrease in survival when challenged with Salmonella enterica serovar Typhimurium. The increased sensitivity 
correlated with elevated bacterial burdens in the spleen and in-
creased production of inflammatory cytokines, chemokines, and 
NO, suggesting that cav1 KO mice were unable to control sys-
temic infection with Salmonella (30). However, it was surprising 
that the authors did not find differences in bacterial ingestion 
between cav1 KO and WT macrophages, indicating that the ele-

cavated mortality in cav1 KO mice could be due to their inability to 
control the inflammatory responses. This phenotype is comparable 
to our findings, wherein during an acute pneumonia that develops 
into a systemic bacterial infection, Cav-1 modifies inflammation. 
We found that when cav1 KO and WT control mice were infected 
with P. aeruginosa, the cav1-deficient mice had elevated in-
flammatory cytokines in their BAL, including IL-1β, TNF-α, IL-
6, IFN-γ, and KC. These results show that in a setting of an acute 
systemic infection, Cav-1 plays a key role in regulating in-
flammatory responses to P. aeruginosa infection so they are not 
overabundant.

In addition to the dramatic differences in cytokines found in cav1 
KO mice infected with P. aeruginosa compared with WT controls, 
we also found significant differences in the ability of neutrophils 
to ingest bacteria. These data are consistent with a previously
published report that describes phagocytic defects in Cav-1-deficient macrophages that are unable to maximally ingest Escherichia coli K-12 bioparticles (31). Subsequent studies demonstrated that Leishmania chagasi or Francisella tularensis use Cav-dependent phagocytosis to gain access to macrophages (27, 32). These observations signify that a common phagocytic mechanism that is shared by PMNs and macrophages is the use of Cav-rich platforms to mediate phagocytosis and/or facilitate vesicle fusion. In the setting of live bacterial infection, cav1 deficiency may contribute to inefficient

FIGURE 3. Cav-1 modifies the inflammatory responses to P. aeruginosa lung infection. cav1 KO (n = 7) and WT controls (n = 7) were infected with 1 × 10⁸ CFU/mouse of P. aeruginosa strain PAO1. BAL fluid was harvested at 6 h (A) and 12 h (B) postinfection, and the cytokine levels were quantified. Bars represent means and SD from replicate measurements from a representative experiment.

FIGURE 4. Cav-1 deficiency inhibits the ability of neutrophils to phagocytose P. aeruginosa. PMNs derived from cav1 KO and WT mice were exposed to P. aeruginosa for 180 min to allow for phagocytosis to occur. PMNs were lysed and aliquots were plated to determine the number of ingested bacteria (A). The phagocytic index represents the number of ingested bacteria per 1 × 10⁸ PMNs. The number of extracellular bacteria was determined by plating and aliquot from the phagocytic reaction on P. aeruginosa selective media without lysing the PMNs (B). Bars represent means and error bars the SD from a representative experiment. p values determined by a Student t test.

FIGURE 5. Cav-1-deficient mice present with comparable morphology after acute infection with P. aeruginosa strain PAO1 to that of WT control mice. Groups of seven cav1 KO and WT mice were infected with 1 × 10⁸ CFU of P. aeruginosa strain PAO1 and sacrificed at 12 h postinfection. Lungs were extracted and embedded in paraffin. Sections were analyzed by H&E staining. Representative images are shown out of two individual experiments.
Differences in the probability of colonization of mice are significant at a level of pharyngeal colonization of cultures after the antibiotic treatment was withdrawn. The percent of oro-strain N6 in the sterile drinking water for 1 wk, subsequently drinking water for 2 wk and subsequently reinstated on acid water. Animals weeks later, the mice were treated with the antibiotic meropenem in the corresponding titers of the WT mice by Mann-Whitney had an OD 405 reading 2-fold higher than the background. The titer was considered to be the value of the last dilution that was detected (22). The titer was assigned as the last dilution in which the Ab could be detected. The assigned titer value is indicative of the last dilution in which the Ab was detected (22). The titer was considered to be the value of the last dilution that had an OD 405 reading 2-fold higher than the background.

In conclusion, we have established that Cav-1 is an important component of the innate host immune response to the majority of noncytotoxic strains of P. aeruginosa by promoting bacterial clearance during acute pneumonia and chronic colonization. Lack of Cav-1 was found to reduce PMN recruitment and increase inflammatory cytokine production during acute pneumonia, two host factors known to affect host resistance to infection. From prior studies identifying Cav-1 as 1 of 150 proteins recruited to lipid rafts of bronchial epithelial cells (28), we have in this study validated that Cav-1 makes a major contribution to host innate immunity to P. aeruginosa. Obviously with such a large number of proteins found to rapidly respond to P. aeruginosa infection, innate immune resistance to this pathogen is quite complex and dependent on a potentially large number of interacting factors. Nonetheless, the results in this study validate that Cav-1 has a demonstrable effect in resistance to both acute and chronic P. aeruginosa infection.

**Disclosures**

The authors have no financial conflicts of interest.

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