The Journal of Infectious Diseases

# *Staphylococcus aureus* Impacts *Pseudomonas aeruginosa* Chronic Respiratory Disease in Murine Models

**Cristina Cigana,[1] Irene Bianconi,[1] Rossella Baldan,[2] Maura De Simone,[1] Camilla Riva,[1] Barbara Sipione,[1] Giacomo Rossi,[3] Daniela M. Cirillo,[2,a] and Alessandra Bragonzi[1,a]**

[1]Infections and Cystic Fibrosis Unit and [2]Emerging Bacterial Pathogens Unit, IRCCS San Raffaele Scientific Institute, Milano, Italy; [3]School of Biosciences and Veterinary Medicine, University of Camerino, Italy

**(See the editorial commentary by Kahl, on pages 854-6.)**

***Background.*** *Staphylococcus aureus* and *Pseudomonas aeruginosa* are key bacterial pathogens of the respiratory tract in patients with cystic fibrosis (CF). Although *P. aeruginosa* chronic bronchial infection is associated with a poorer prognosis, the consequences of *S. aureus* colonization on CF outcomes are controversial.

***Methods.*** In this paper, murine models of infection resembling traits of the CF human airways disease have been revisited using an infection schedule that mimics the sequence of events of pulmonary disease in CF patients. First, mice were infected with *S. aureus*, embedded in agar beads; this was followed by *P. aeruginosa* infection and analysis of bacterial load, leukocyte infiltration, and lung tissue damage.

***Results.*** We reveal that (1) *S. aureus* promotes severe lesions including abscess formation, (2) *S. aureus* increases the risk of subsequent chronic *P. aeruginosa* respiratory infection, and (3) once the chronic infection has been established, *P. aeruginosa* influences most of the inflammatory responses independent of *S. aureus*.

***Conclusions.*** Our findings established the significance of *S. aureus* colonization per se and the impact on the subsequent *P. aeruginosa* infection. This would point towards a thorough assessment for the need of treatment against *S. aureus*.

**Keywords.** cystic fibrosis; mouse model; *Pseudomonas aeruginosa*; respiratory infection; *Staphylococcus aureus*.

Cystic fibrosis (CF) is the most common life-threatening monogenic disease [1]. Over past decades, life expectancy of CF patients has increased to 37 years due to early diagnosis and improved treatments; however, the quality of life of these patients is still limited [2]. The great burden of the morbidity and mortality of the condition results from respiratory failure secondary to pulmonary infections and inflammation.

Cystic fibrosis patients are predisposed to infections caused by a number of microorganisms, the prevalence of which varies according to patient age. *Staphylococcus aureus* and *Pseudomonas aeruginosa*—the most prevalent species—are acquired in a subsequent order. Previous surveys showed that the prevalence of *S. aureus* (both methicillin-susceptible *S. aureus* [MSSA] and methicillin-resistant *S. aureus* [MRSA]) is approximately 60% in young CF patients, between the ages of 1 and 10 years, and remains stable from 30 to 40 years of age, whereas the prevalence of *P. aeruginosa* is approximately 25% in younger patients and rises up to 60% in adult CF patients older than 30 years [3].

Although *P. aeruginosa* is recognized as the leading cause of airways infection and is associated with a decline in lung function [4], the significance of *S. aureus* in the pathogenesis and course of the disease is still debated. Chronic infections by *P. aeruginosa* begin with virulent bacterial cells with swimming motility, twitching motility, and protease secretion and develop with bacterial adaptive variants that grow in biofilm structures [5]. These *P. aeruginosa* CF-adapted variants shape the innate immune response to favor their persistence [6, 7]. *S. aureus* can cause diseases mainly due to the multiplicity of its virulence factors and various strategies to escape host defense, eg, capsule polysaccharide and exoprotein Panton-Valentine leucocidin (PVL) expression, diversification into heterogeneous morphotypes, emergence of small colony variants (SCVs), biofilm formation, and internalization by various cell types including respiratory epithelial cells [8].

Although *S. aureus* is recognized as an emerging pathogen with an increased incidence and prevalence in the CF population, major questions deriving from clinical observations persist. The unsolved issues are as follows: (1) what impact does *S. aureus* infection have on the deterioration of the lung function in CF patients; and (2) whether preinfection with *S. aureus* could predispose patients to subsequent infection with *P. aeruginosa*. The answer to these questions provides crucial

Received 30 June 2017; editorial decision 20 October 2017; accepted 1 December 2017; published online December 5, 2017.

Presented in part: 30th Annual North American Cystic Fibrosis Conference, Orlando (FL), USA, October 27-29, 2016; 40th European Cystic Fibrosis Conference, Seville, Spain, June 7-10, 2017.

[a]D. M. C. and A. B. contributed equally to this work and share senior authorship.

Correspondence: Alessandra Bragonzi, PhD, Infections and Cystic Fibrosis Unit, Division of Immunology, Transplantation and Infectious Diseases IRCCS San Raffaele Scientific Institute via Olgettina 58, 20132 Milano, Italy (bragonzi.alessandra@hsr.it).

**The Journal of Infectious Diseases® 2018;217:933–42**



DOI: 10.1093/infdis/jix621

information on when and how to treat patients with positive *S. aureus* respiratory culture.

Because the predisposing role of *S. aureus* in the progression of lung disease is still being debated, no internationally accepted guidelines have been defined for treatment of MSSA or MRSA. This controversy stems from findings that treatment of *S. aureus* may lead to an earlier and enhanced acquisition of *P. aeruginosa* [9–11].

The issues relative to CF pathogenesis deserve further model-based investigation to fill the gaps in the human studies. Murine models of long-term chronic infections have been previously established to mimic *P. aeruginosa* pathogenicity [7, 12], whereas models showing the interaction between different pathogens during a respiratory infection are still in their infancy. As a result, the cascade of events, mediated by *S. aureus* and *P. aeruginosa* persistence in the pathogenesis of chronic airways infection, has not yet been addressed.

In this study, we describe further development of the model reported by Cigana et al [7] to clarify (1) whether and how *S. aureus* can be considered to be an independent contributor to CF lung disease pathogenesis, and (2) whether and how it impacts the progression of *P. aeruginosa* chronic lung infection, or not. We report that (1) in murine airway diseases, *S. aureus* promotes severe lesions—including abscess formation, (2) *S. aureus* increases the risk of subsequent chronic *P. aeruginosa* respiratory infection, and (3) once the chronic infection has been established, *P. aeruginosa* influences most of the inflammatory responses, independent of *S. aureus*.

## MATERIAL AND METHODS

### Ethics Statement

Animal studies adhered strictly to the Italian Ministry of Health guidelines for the use and care of experimental animals (protocol no. 738).

### Bacterial Strains

For *S. aureus*, human clinical isolates USA300 [13], SH1000 [14], and Newman [15] were used. For *P. aeruginosa*, reference strain PA14, and clinical isolates AA2 and AA43 from a CF patient previously described [7, 16] were used. AA43 was collected 7.5 years after the acquisition of clonal AA2 isolate and before patient's death. Bacterial strains were cultured in trypticase soy broth and plated on mannitol salt agar for *S. aureus* and Pseudomonas isolation agar for *P. aeruginosa*.

### Mouse Model

C57BL/6NCrl (B6) male mice (Charles River), 8–10 weeks old, were infected under anesthesia according to the previously described method [17, 18]. In brief, a 50-µL inoculum of an agar bead suspension containing 1–2 × $10^6$ colony-forming units (CFUs) for *S. aureus* bacteria or empty beads was implanted via a 22-gauge cannula through the trachea into the lung, with all lobes inoculated. After 7 days from *S. aureus* chronic infection, mice were inoculated with an agar bead suspension containing 1–2 × $10^6$ CFUs for *P. aeruginosa* bacteria or with empty beads for additional 14 days. Additional details are reported in the Supplementary Data.

### Bronchoalveolar Lavage Fluid and Lung Processing, Cytokine/Chemokine Quantification, and Histological Analysis

After 7, 13, or 21 days from the first challenge, the bronchoalveolar lavage (BAL) was performed as described previously [17, 18]. Total cells present in the BAL fluid (BALF) were counted, and a differential cell count was performed on cytospins stained with Diff Quick. Lungs were removed and homogenized. The BALF and lung serial dilutions were plated on selective media for CFU count. Recovery of more than 1000 CFU of bacteria from lung cultures was indicative of chronic infection [16]. Lung homogenates were centrifuged, and the supernatants were analyzed by Bioplex (Bio-Rad). Histopathology was performed according to standard procedures. Sections of murine lungs were stained by anti-CD3 monoclonal antibody (mAb) or anti-F4/80 mAb; hematoxylin and eosin, Masson's trichrome (MTS), and immunofluorescence (IF) staining were examined blindly and scored by a pathologist, as detailed in the Supplementary Data.

### Statistics

Statistics were performed with GraphPad Prism. Data analysis was performed using a non-parametric 2-tailed Mann-Whitney *U* test for single comparison or a non-parametric Kruskal-Wallis test followed by a post hoc Dunn test to correct for multiple comparisons, for CFU counts, histopathological scoring, cellular counts, and cytokine/chemokines quantification. Mantel-Cox test was used to compare survival curves. Incidences of mortality and chronic colonization were compared using Fisher exact test. Two-way analysis of variance with Bonferroni's multiple comparison test was used to compare changes in body weight. $P < .05$ was considered significant.

## RESULTS

### Chronic Persistence of Staphylococcus aureus Promotes Airway Tissue Damage in Mice

To study the long-term chronic infection and track the host response to *S. aureus* infection, we established a chronic infection (by agar bead-embedded bacteria) in C57BL/6NCrl (B6) mice with the reference clinical isolates USA300, SH1000, and Newman. All mice infected with USA300 were evidently sick (with considerable loss of body weight, scruffy coat, and inactivity), and high mortality was observed within 4 days postinfection (Figure 1A–C). Most of the mice infected with SH1000 and Newman lost less body weight, showed better clinical conditions compared with USA300, and established a nonlethal chronic colonization. At 13 days, the incidence of chronic colonization did not differ between SH1000 (80%) and Newman (87.5%) (Figure 1C and Supplementary Table 1S), and lung bacterial loads (SH1000 median: 2.9 × $10^6$ CFU/lung;

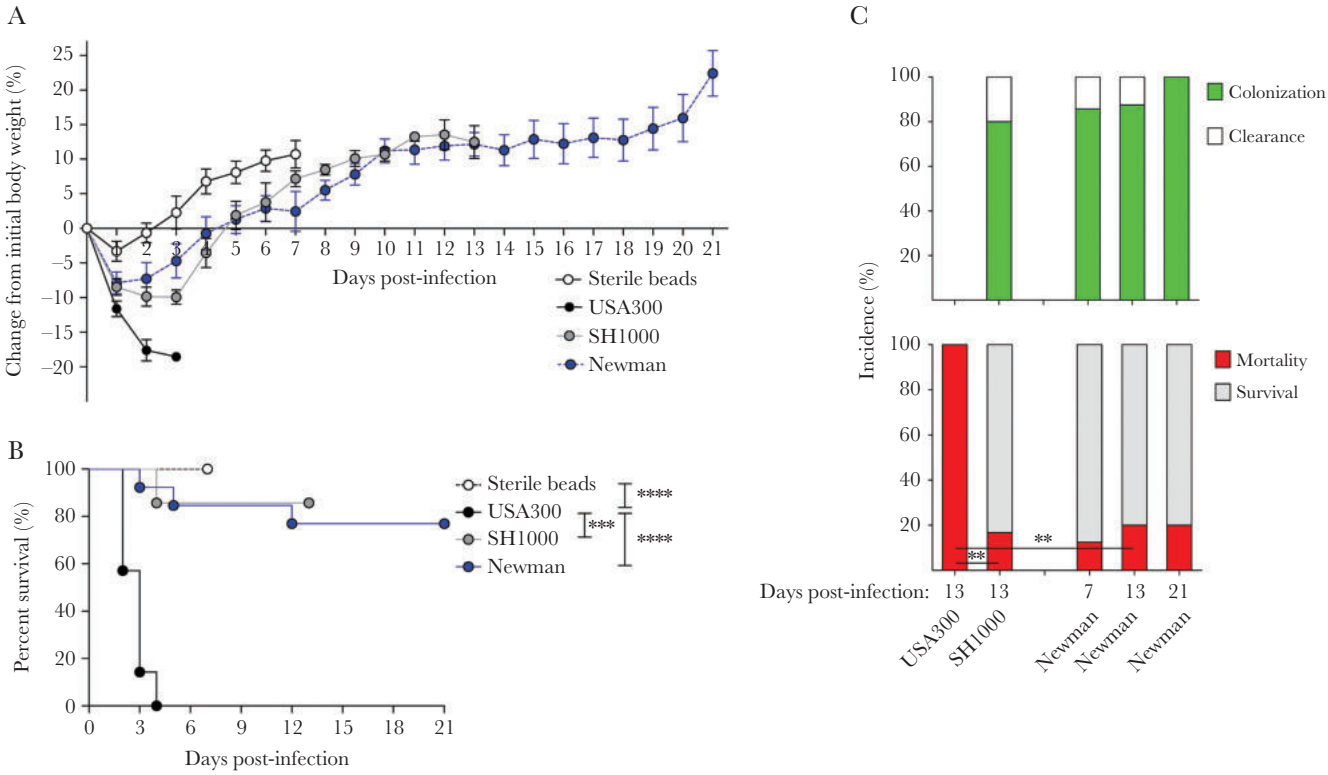


**Figure 1.** Pathogenicity of *Staphylococcus aureus* isolates in murine models of long-term chronic infection. C57BL/6NCrl (B6) mice were infected with 1–2 × $10^6$ colony-forming units/lung of USA300, SH1000, and Newman isolates embedded in agar beads for the chronic infection or inoculated with sterile agar beads and analyzed during a time course up to 21 days postinfection. (A) Body weight and (B) cumulative survival rate after infection with *S aureus* were recorded. (C) Mortality and survival were evaluated on challenged mice; clearance and capacity to establish chronic airways infection were determined on surviving mice. The data were pooled from at least 2/3 independent experiments (n = 6–20) as detailed in Supplemenary Table 1S. Statistical significance is indicated: **, $P < .01$; ***, $P < .001$; ****, $P < .0001$.

Newman median: 2.6 × $10^5$ CFU/lung) (Figure 2A) did not differ significantly.

We performed a time-course analysis where mice were sacrificed 7, 13, and 21 days following Newman infection and analyzed for bacterial loads, cell recruitment in the BALF, and cytokines/chemokines. Overall, the incidence (%) of chronic colonization remained stable (Figure 1C and Supplementary Table 1S), whereas bacterial loads decreased significantly at 13 and 21 days compared with 7 days. However, bacterial counts were stable from day 13 onwards until day 21. Leukocytes in the BALF, harvested at 7 days, decreased significantly at 21 days (Figure 2B) after bacterial loads (Figure 2A). The levels of several cytokines/chemokines were significantly higher in mice retaining infection with Newman after 7 days, but their levels decreased to basal levels over 21 days (Table 1).

Histopathological analysis 7 days postinfection with Newman revealed an initial formation of abscess-like lesions: these had increased further by 21 days (Figure 2C). A central necrotic zone, surrounded by a layer of $CD3^+$ lymphocytes and macrophages and small amounts of fibrin/collagen, was observed with most of the staphylococci sequestered within necrotic lesions. Therefore, *S. aureus* shows an ability to establish long-term chronic pulmonary infections, promoting severe lesions in our murine model.

### *Staphylococcus aureus* Preinfection Influences the Pathogenicity of Pseudomonas aeruginosa

Next, we exploited the mouse model of *S. aureus* chronic lung infection to evaluate whether and how the staphylococcal infection impacts the subsequent superinfection with *P. aeruginosa* (Supplementary Figure 1S). A reference *P. aeruginosa* strain (PA14), a clinical isolate (AA2) equipped with virulence factors, and its adapted variant (AA43) [16, 19] from a CF patient were used. Although PA14 and AA2 isolates express a wide series of virulence factors including swimming motility, twitching motility, and protease secretion [16, 20] and induce high mortality in mice, the CF-adapted AA43 isolate is mucoid and can establish long-term chronic infection [7, 16].

We observed that the *P. aeruginosa* superinfection did not affect the high incidence of chronic infection established by *S. aureus* Newman strain. Instead, preinfection with *S. aureus* Newman strain significantly decreased the incidence of mortality induced by *P. aeruginosa* PA14 and AA2 strains and significantly increased the capacity of PA14 and CF-adapted AA43 strains (Figure 3A and Supplementary Table 1S) to establish chronic infection. Although *S. aureus* impacted *P. aeruginosa* superinfection in terms of the incidence of mortality and chronic colonization, *S. aureus* and *P. aeruginosa* did not affect each other

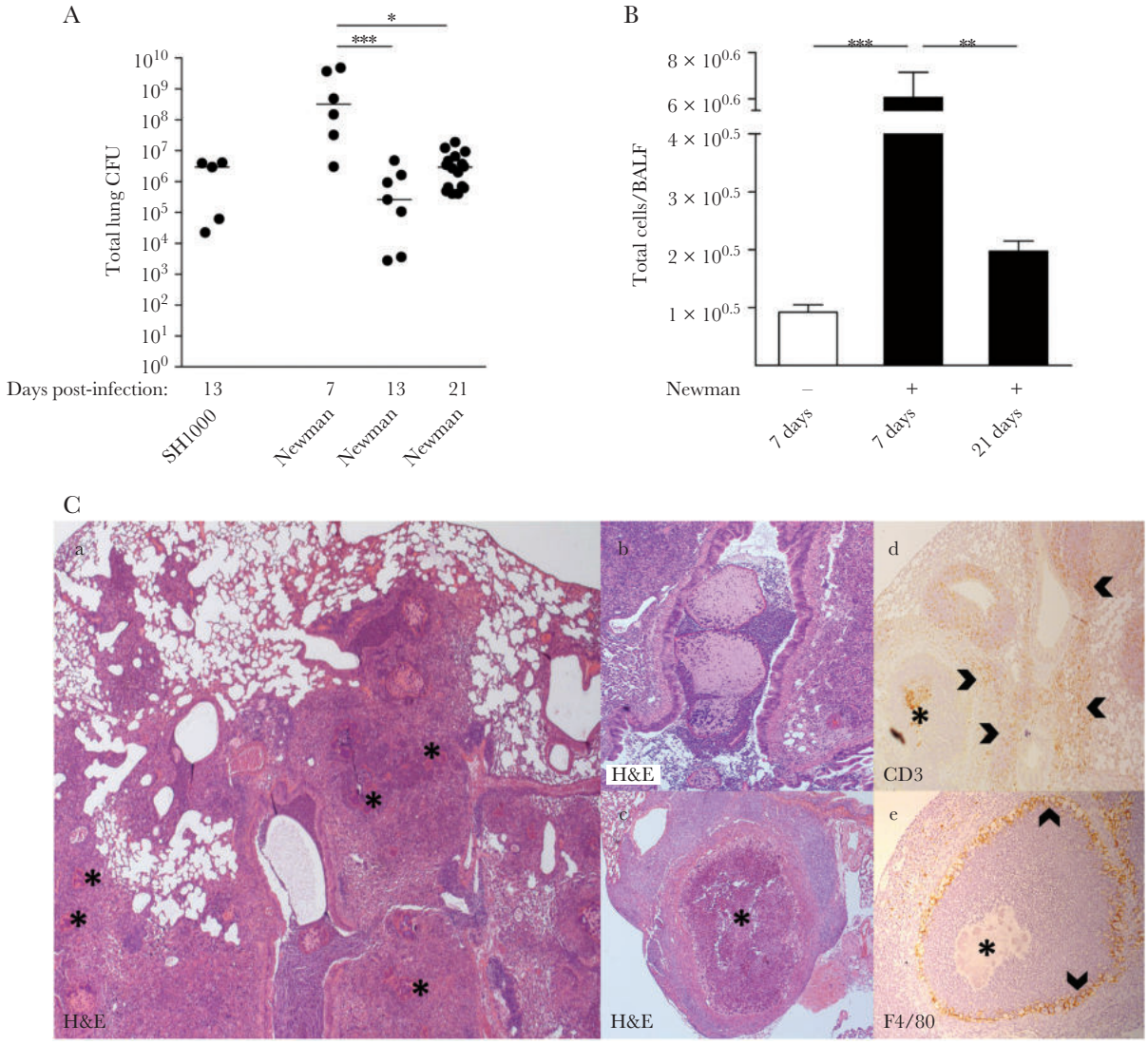


**Figure 2.** Lung infection, inflammation, and damage in murine models of long-term *Staphylococcus aureus* chronic infection. C57BL/6NCrl (B6) mice were infected with USA300, SH1000, and Newman isolates as described in Figure 1 and analyzed for bacterial load, cell recruitment in the BALF, and histopathology. (A) Dots represent colony-forming units (CFUs) in the lung of individual mice, and horizontal lines represent median values. (B) The total cells were measured in the bronchoalveolar lavage fluid (BALF). Values represent the mean ± standard error of the mean. The data were pooled from at least 2/3 independent experiments (n = 6–20) as detailed in Supplementary Table 1S. (C) The figure exemplifies the lungs of mice challenged with Newman for 7 days stained with hematoxylin and eosin (H&E) and anti-CD3 and anti-F4/80 monoclonal antibody. Magnification: (a) ×2.5, (b–d) ×5, and (e) ×10. In a–e, asterisk indicates abscess-like structure. In d and e, arrows indicate CD3- or F4/80-positive cells. Statistical significance is indicated: *, $P < .05$; **, $P < .01$; ***, $P < .001$.

in terms of bacterial burden in the lung. Overall, pulmonary *P. aeruginosa* loads were similar in mice, preinfected or not with *S. aureus* (Figure 3B). However, it should be noted that the bacterial burden of *S. aureus* was higher than that of *P. aeruginosa*, independent of the strain used. Overall, these results indicate that *S. aureus* infection influences the pathogenicity of *P. aeruginosa*.

### *Pseudomonas aeruginosa* Influences Most of the Inflammatory Responses Independent of *Staphylococcus aureus* at a Late Stage of Chronic Infection

The host response was then investigated in BALF (Supplementary Figure 2S) and total lung (Figure 4) after long-term chronic infection. We assigned histopathological scores to quantify inflammation severity, immune cells recruitment, and the formation of bronchus-associated lymphoid tissue (BALT)-like structures at 21 days (Figure 4). All conditions were scored except those with high mortality rate (PA14 and AA2 alone). The persistence of *S. aureus* resulted in a low level of inflammatory response, including infiltration of innate and adaptive immune cells both at bronchial and interstitial level (Supplementary Figure 2S and Figure 4). Subsequent infection with *P. aeruginosa* induced significantly higher inflammation and, in particular, higher infiltration of innate and adaptive immune cells, the latter organized in BALT-like structures when compared with

**Table 1. Cytokines and Chemokines Levels in Murine Lungs During *Staphylococcus aureus* Chronic Lung Infection and *Pseudomonas aeruginosa* Superinfection[a]**

| | Day 7 | | Day 21 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cytokines | Ctrl | Newman | Ctrl | Newman | Newman + PA14 | Newman + AA2 | AA43 | Newman + AA43 | Newman + PA14 vs Newman | Newman + AA2 vs Newman | Newman + AA43 vs Newman | Newman + AA43 vs AA43 |
| IL1α | 11.24 | **77.45** | 9.68 | 15.34 | 15.79 | 29.09 | 12.33 | **23.57** | ns | ns | ns | * |
| IL1β | 14.03 | **353.87** | 21.94 | 53.06 | **90.34** | 279.45 | 37.50 | **145.10** | ns | ns | ns | * |
| IL5 | 2.11 | 4.82 | 11.28 | 5.73 | 5.43 | **3.42** | 9.67 | 5.43 | ns | ns | ns | ns |
| IL6 | 1.45 | 2.27 | 4.51 | 3.69 | 3.38 | 1.83 | 3.44 | 3.11 | ns | ns | ns | ns |
| MIP1α | 0.98 | **167.55** | 1.94 | 10.57 | 13.52 | **32.78** | 3.73 | 25.60 | ns | ns | ns | ns |
| MIP1β | 24.24 | **48.53** | 26.28 | 26.64 | 23.35 | 20.38 | 21.78 | 26.57 | ns | ns | ns | ns |
| TNFα | 5.64 | 10.81 | 12.84 | 9.66 | 12.39 | 8.84 | 10.47 | 11.12 | ns | ns | ns | ns |
| KC | 5.31 | **64.68** | 10.15 | 21.39 | **41.45** | **42.02** | 17.62 | 23.60 | ns | ns | ns | ns |
| G-CSF | 2.58 | **100.37** | 10.19 | 14.75 | 26.16 | 25.87 | 5.45 | 24.33 | ns | ns | ns | ** |
| RANTES | 5.16 | 7.42 | 18.45 | 10.39 | 31.84 | 15.98 | 15.59 | 15.43 | * | ns | ns | ns |
| IL12p70 | 4.91 | **26.13** | 33.73 | 16.33 | 7.64 | 9.17 | 16.26 | 12.69 | ns | ns | ns | ns |
| IL17A | 4.33 | **31.21** | 9.26 | 22.50 | 28.67 | **32.73** | 15.49 | 21.09 | ns | ns | ns | ns |
| IL12p40 | 3.73 | **6.29** | 12.24 | 10.30 | 21.72 | 13.39 | 24.25 | 19.64 | * | ns | * | ns |
| IL13 | 60.89 | 72.48 | 104.78 | 90.97 | 88.47 | 83.62 | 75.83 | 94.64 | ns | ns | ns | ns |
| IL10 | 2.72 | **11.35** | 8.50 | 5.79 | 6.68 | 5.31 | **5.05** | **5.21** | ns | ns | ns | ns |

Significance of each value compared with its Ctrl is in bold.

Abbreviations: Ctrl, control; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; KC, keratinocyte-derived cytokine; MIP, macrophage inflammatory protein; ns, not significant; RANTES, regulated on activation, normal T-cell expressed and secreted; TNF, tumor necrosis factor.

[a]Data are expressed as mean of pg/500 µg of lung. Statistical analysis by the non-parametric Mann-Whitney *U* test and by nonparametric Kruskal-Wallis test followed by post hoc Dunn test to correct for multiple comparisons for different types of infection (*, $P < .05$; **, $P < .01$) is reported.

*S. aureus* alone. In addition, preinfection with *S. aureus* did not affect the inflammatory response by the CF-adapted variant AA43 strain, further ascribing a greater role in lung injury to *P. aeruginosa* infection rather than to *S. aureus*. When measuring pulmonary cytokines/chemokines, we found that several of them were expressed at low levels in the lungs of both *S. aureus*- or *P. aeruginosa*-infected mice (Table 1), likely reflecting the attenuation of the host response in the late phase of chronic infection as previously observed [7].

Histopathology and IF staining for *S. aureus* and *P. aeruginosa* on day 21 localized the two bacterial species in different areas of the lung (Figure 5). *P. aeruginosa* infection was localized mainly in the agar beads in bronchial lumens, but small colonies were also present in the parenchyma. *S. aureus* was localized primarily in the abscesses and rarely in the agar beads. Infection with *P. aeruginosa* yielded higher recruitment of innate immune cells, including neutrophils and macrophages, at both bronchial and parenchymal levels; the preinfection by *S. aureus* caused severe abscess-like lesions. These abscesses were characterized by large clusters of staphylococci surrounded by a massive number of immune cells, macrophages, and small amounts of fibrin/collagen.

## DISCUSSION

Whether *S. aureus* infection contributes to lung pathogenesis and has an impact on early *P. aeruginosa* infection is still an open question. Clinical data are difficult to interpret due to the large number of confounding variables. In this investigation, we adopted the agar-beads model of infection that is well established for other pathogens [7], to investigate the role of *S. aureus* in the long-term chronic lung infection. Our results indicate that *S. aureus* infection represents a risk factor for initial *P. aeruginosa* airway infection and has a role in the pathogenesis of the lung disease.

Three *S. aureus* strains of different origins and from different clinical settings were used to establish the model of long-term chronic infection. We found that USA300, a community-associated MRSA, causes acute infection, whereas the MSSA strains SH1000 and Newman can establish chronic persistent infection. *S. aureus* reference isolates (SH1000 and Newman) showed a remarkable capacity to induce chronic pneumonia, supporting our previous report with a panel of clinical CF strains [21]. Of interest, two *S. aureus* reference isolates of this paper and six clinical CF strains of our previous study [21], including those sampled at the onset of chronic colonization and after years of chronic infection, did not differ in their pathogenicity. All isolates showed significant ability to induce chronic pneumonia with high bacterial loads in the murine lung, severe lesions in bronchi, and pulmonary parenchyma. The only exception was the highly invasive and PVL-producer MRSA-USA300 [13] that induces severe sepsis, as previously observed in other models [22], rather than long-term chronic pulmonary infection. Several European studies tracked the persistence of *S. aureus* among CF patients over extended periods of time [23–25]. In most patients, long-term colonization by *S. aureus* occurred, indicating a persistence capacity similar to *P. aeruginosa*. CF patients with high *S. aureus* density in throat cultures experience a more rapid lung

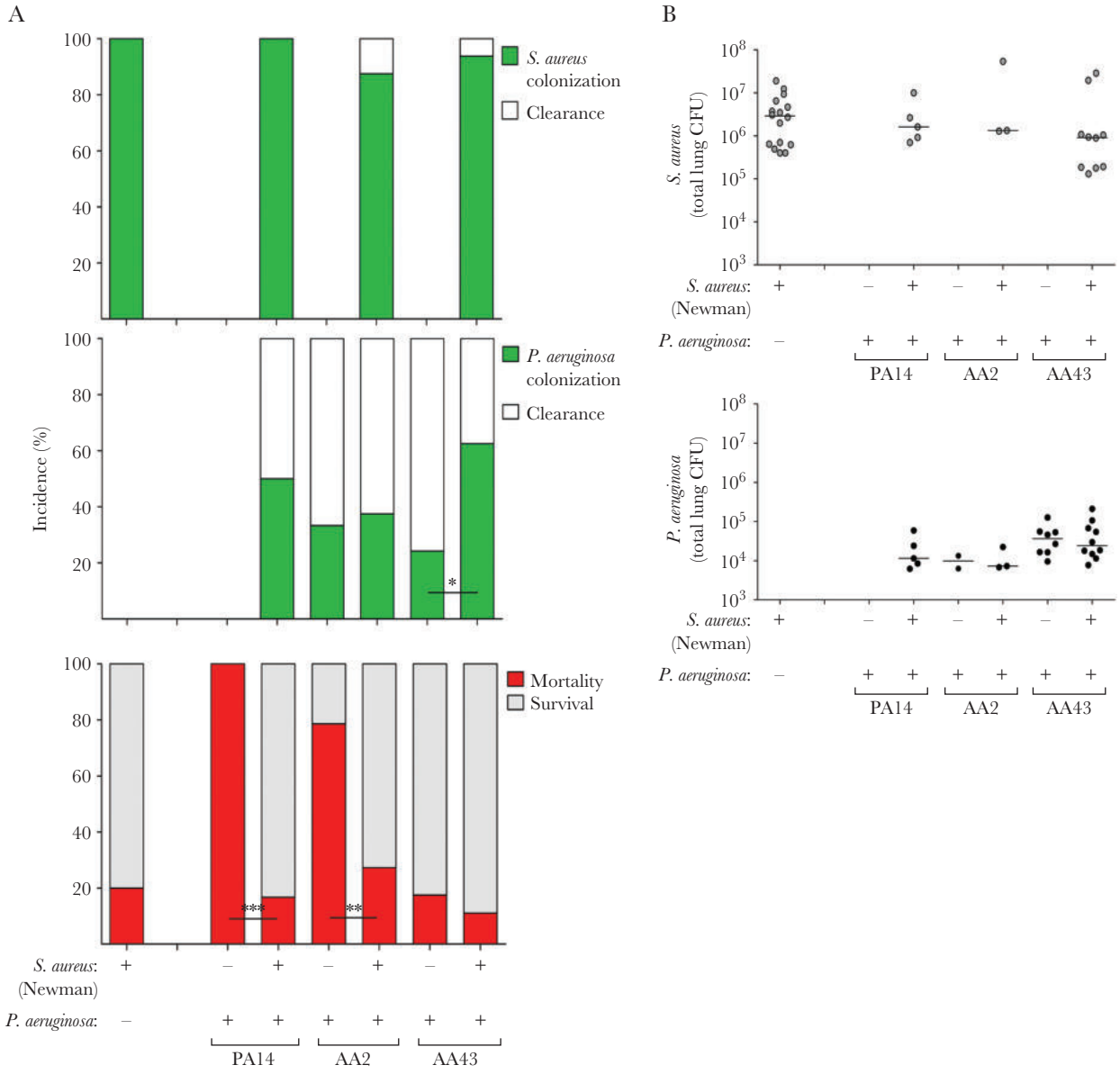


**Figure 3.** Pathogenicity of *Staphylococcus aureus* infection and *Pseudomonas aeruginosa* superinfection in a murine model of long-term chronic infection. C57BL/6NCrl (B6) mice were infected as described in Supplementary Figure 1S. (A) Mortality and survival were evaluated on challenged mice; clearance and capacity to establish chronic airways infection were determined on surviving mice. (B) *Staphylococcus aureus* and *Pseudomonas aeruginosa* colony-forming units (CFUs) were evaluated in total lung. Dots represent CFUs in individual mice, and horizontal lines represent median values. The data were pooled from at least 2/3 independent experiments (n = 7–40) as detailed in Suppementary Table 1S. Statistical significance is indicated: *, $P < .05$; **, $P < .01$; ***, $P < .001$.

function decline [25], and bacterial persistence has an impact on bacterial adaptation but with no uniform adaptive changes in isolates of the same clonal lineage in different patients and no clear impact on pathogenesis [21].

Staphylococcal respiratory infection in CF patients is rarely associated with bacteremia, and the formation of abscesses has been reported only in a few cases with PVL$^+$ MRSA infections [26]. However, in several non-CF clinical conditions, abscesses have been documented in numerous different organ tissues. In our murine model, long-term persistence of *S. aureus* in the lungs induced regional abscesses characterized by large clusters of staphylococci surrounded by massive numbers of immune cells, macrophages, and small amounts of fibrin/collagen. These severe lesions were confined to a limited region in the parenchyma. When the total lung was scored for levels of inflammation, it was observed that the infiltration of innate and adaptive immune cells was largely limited to the abscesses. Therefore, in our murine model, MSSA *S. aureus* can form the lung abscesses as described for different organs in other models of infection [27]. It remains to be established whether abscess formation in the lung is a feature of the murine model or whether further studies in patients with CF are necessary.

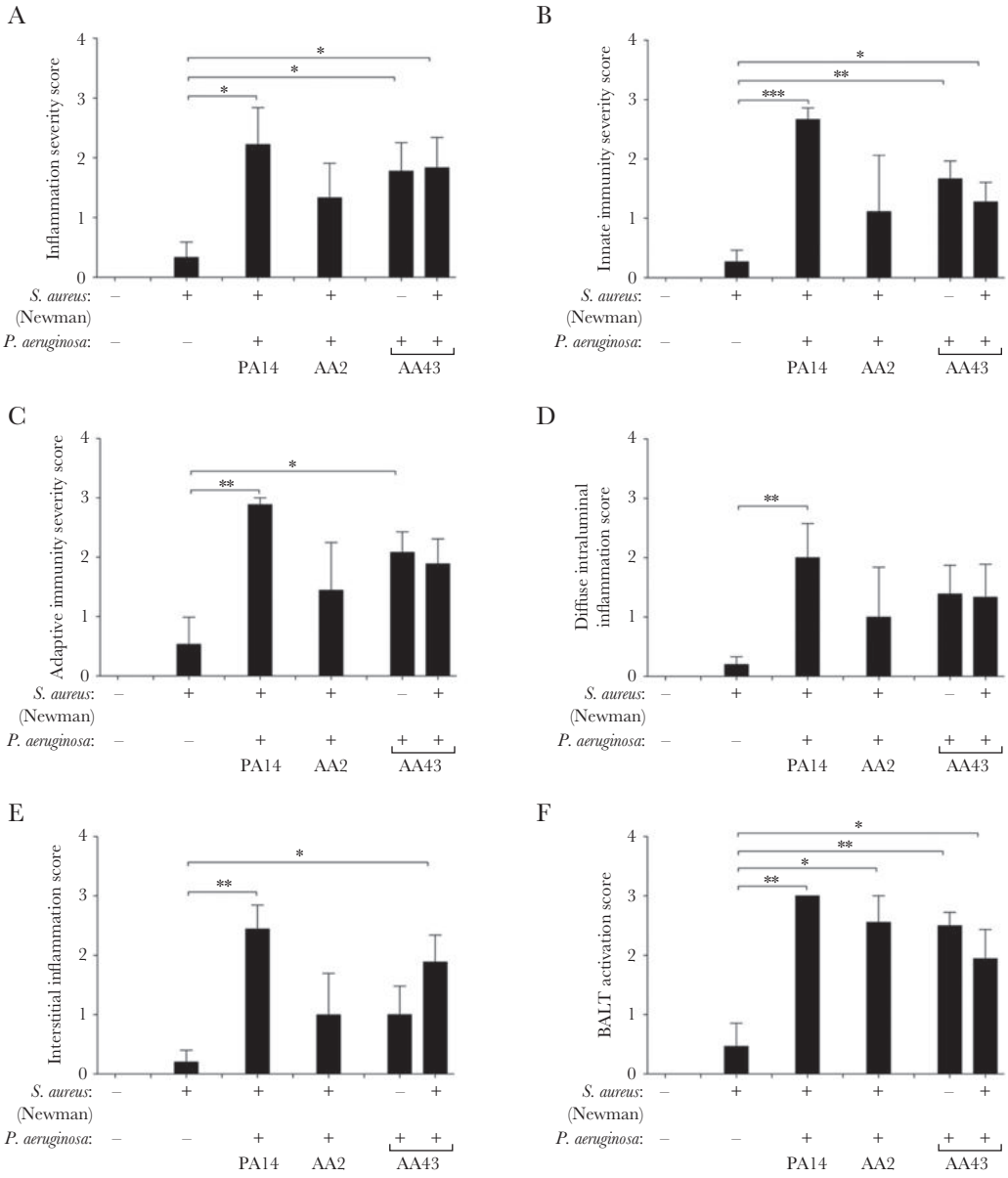


**Figure 4.** Histopathological scores on sections of murine lungs after infection with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. C57BL/6NCrl (B6) mice were infected as described in Supplementary Figure 1S. Sections of murine lungs were stained with hematoxylin and eosin, Masson's trichrome for collagen, anti-CD3 monoclonal antibody (mAb) for T cells, and anti-F4/80 mAb for macrophages, according to the standard procedures. Scorings of overall inflammation severity (A), acute innate immunity severity (B), adaptive immunity severity (C), diffuse intraluminal inflammation (D), interstitial inflammation (E), and bronchus-associated lymphoid tissue (BALT) activation (F) were performed on slices stained with H&E. The data are pooled from at least 2 independent experiments (n = 3–6). Values represent the mean ± standard error of the mean. Statistical significance is indicated: *, $P < .05$; **, $P < .01$; ***, $P < .001$.

Next, we compared these results to the mouse model of *P. aeruginosa* [7]. Direct comparison between *S. aureus* and *P. aeruginosa* in single infections showed that *S. aureus* is difficult to eradicate by the host and is more efficient in establishing a chronic infection with higher bacterial load and percentage of chronicity than *P. aeruginosa* in the murine model [16]. These findings support the hypothesis that, in some ways, *S. aureus* has higher pathogenicity compared with *P. aeruginosa*. We were surprised to find that the host immune response was limited to the areas of the abscesses compared with the diffused *P. aeruginosa* inflammation.

In humans, there is conflicting evidence for the role of *S. aureus* in lung disease in CF patients and, consequently, for the clinical impact of anti-staphylococcal therapy. Observational studies have identified relationships between *S. aureus* detection and lower lung function [28] or higher inflammation [25, 29]. On the other hand, other studies found *S. aureus* to be associated with better survival [30] and better lung function [31]. Based on our results using an animal model of chronic lung infection, we support the notion that *S. aureus* infection is asymptomatic based on specific clinical signs (eg, recovery of body weight and general health evaluation),

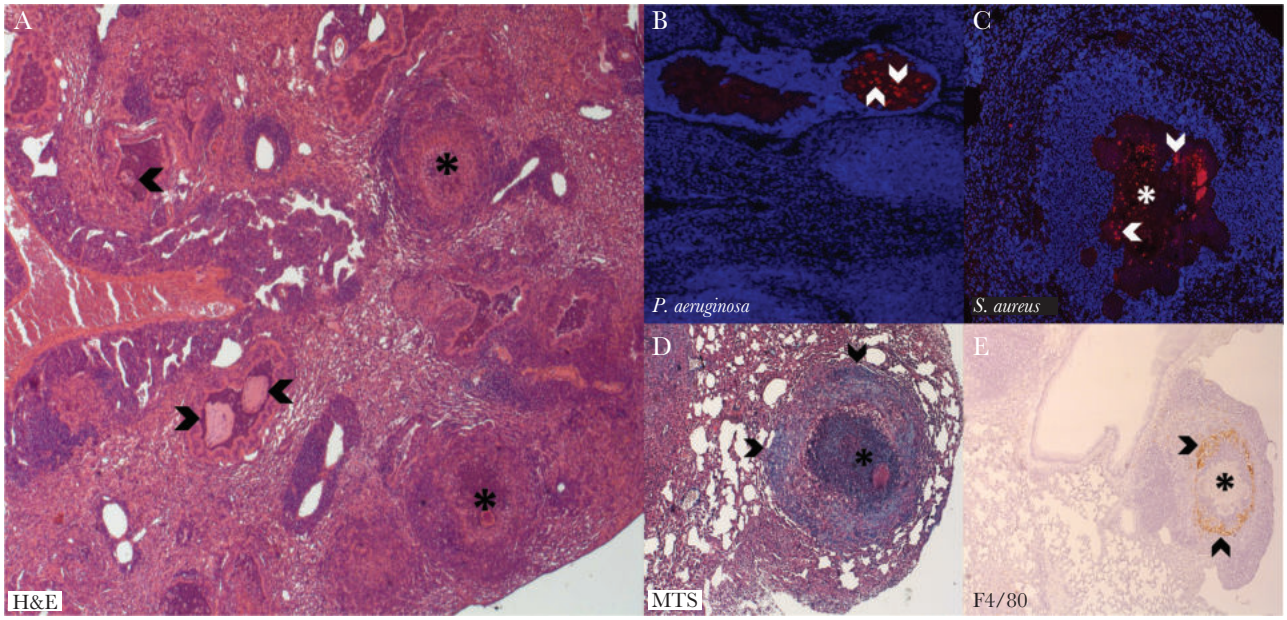


**Figure 5.** Histopathology of murine lungs after infection with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. C57BL/6NCrl (B6) mice were infected with 1–2 × $10^6$ colony-forming units/lung of *S. aureus* Newman or *P. aeruginosa* isolates embedded in agar beads and analyzed 21 days postinfection. The figure exemplifies the murine lungs stained with hematoxylin and eosin (H&E), Masson's trichrome, anti-CD3 monoclonal antibody (mAb), and anti-F4/80 mAb and by immunofluorescence (with specific antibodies against *P. aeruginosa* or *S. aureus*, stained in red, and with 4,6-diamidino-2-phenylindole dihydrochloride, stained in blue), according to the standard procedures. Magnification: (A) ×2.5, and (B–E) ×5. Arrows indicate (A) agar-beads, (B and C) bacterial colonies, (D) collagen deposition, and (E) macrophages. Asterisk indicates abscess-like structures.

whereas it can induce severe damage confined to parts of the lung that is difficult to resolve, as revealed by histopathology.

*S. aureus* is often reported as being the first pathogen infecting the lungs of the CF patients, followed by *P. aeruginosa*. Although the 2 organisms are frequently co-isolated in clinical specimens, several studies demonstrated that *S. aureus* and *P. aeruginosa* have a competitive interaction [32]. Previous studies showed that when cocultured, *P. aeruginosa* thrives better than *S. aureus* [33]. The better survival of *P. aeruginosa* is attributed to its ability to produce respiratory toxins such as pyocyanin, hydrogen cyanide, and alkyl-hydroxyquinoline *N*-oxides that can block the electron transport pathway, thereby inhibiting the growth of *S. aureus* and other pathogenic staphylococci [34, 35]. A prolonged growth of *S. aureus* with *P. aeruginosa* may select for *S. aureus* SCVs altering the population structure of bacterial communities [36], but SCVs have not been detected in our animal model. Using a collection of longitudinal strains isolated from CF patients, we previously showed that *P. aeruginosa* strains outcompeted *S. aureus* [37]. This effect was associated with *P. aeruginosa* early isolates, which in acute infection present higher virulence. On the contrary, *P. aeruginosa* late CF-adapted isolates showed reduced or abolished capacity to outcompete *S. aureus*. Findings in this work expand previous data. In this study, we used the agar-beads mouse model of long-term chronic infection approaching the advanced stage of chronic pneumonia and adopting an experimental design to mimic subsequent infection that occurs in CF patients. Our results did indicate that *S. aureus* and *P. aeruginosa* coexist in the mouse lungs without interfering with each other. These observations, different from the previous models of acute infection and *in vitro* data, could be influenced by the experimental design and the environmental conditions. The microanaerobic growth conditions for bacteria created by agar-beads may change bacterial virulence factors that affect the pathogenesis [38]. Furthermore, we demonstrated that *S. aureus* precolonization represents a risk factor for initial *P. aeruginosa* airway infection as documented by the increasing capacity to establish chronic infection of several *P. aeruginosa* strains. *S. aureus* may cause lesions and patho-physiological changes in the infected lungs that pave the way for *P. aeruginosa* colonization. The severity of these lesions may suggest that even if eradication of *S. aureus* was possible, it might not be sufficient to restore the lung physiology. Furthermore, preinfection with *S. aureus* decreased the incidence of mortality induced by *P. aeruginosa*. The motile *P. aeruginosa* strains can easily disseminate from the lung to the blood by provoking epithelial injury. It may be possible that the infiltrating immune cells induced by previous *S. aureus* infection can block the *P. aeruginosa* spreading from lung to blood.

In patients with CF, *P. aeruginosa* is the leading pathogen that marks the transition to the fatal, chronic stage of the infection. Our model was instrumental in demonstrating the substantial contribution of *P. aeruginosa* when preceded or not by *S. aureus*. Overall, *P. aeruginosa* persistence had a greater effect on

inflammation and damage compared with *S. aureus*. When we investigated *S. aureus*/host interaction and/or *P. aeruginosa*/host interaction in mice in detail, two distinct pathogenic profiles emerged. Although *S. aureus* infected the airways and was then confined in abscess-like lesions in the parenchyma, *P. aeruginosa* was mainly localized in bronchial lumens. *S. aureus* and *P. aeruginosa* infections showed different capacities in shaping the host immune response. *P. aeruginosa* stimulates a much stronger host inflammatory response compared with *S. aureus*. Once the chronic infection has been established, *P. aeruginosa* influences most of the inflammatory responses, independently of *S. aureus.* Coinfection with the two pathogens does not result in an additive effect but rather a follow-on of the *P. aeruginosa* response. The observation supports the hypothesis of long-lasting pathological consequences of *P. aeruginosa* infection.

## CONCLUSIONS

The results presented in this study have real importance to understanding CF airways disease pathogenesis and progression. Our study clearly emphasizes the role of *S. aureus* as a bacterium with high pathogenic potential that enhances *P. aeruginosa* pathogenesis to different extents. To further explore our findings, a panel of different *S. aureus* and *P. aeruginosa* strains, including sequential isolates from CF patients, should be tested in the mouse model. So far, our observations underline the need to assess different strategies to treat *S. aureus* infection in CF patients.

### Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

### Notes

***Author contributions.*** C. C., D. M. C., and A. B. conceived and designed the experiments; C. C., I. B., R. B., M. D. S., C. R., and B. S. performed experiments; C. C., I. B., R. B., M. D. S., and C. R. analyzed data; G. R. performed the histological scoring; C. C. performed statistical analysis; C. C., D. M. C., and A. B. interpreted the experiments results; C. C. prepared the figures; C. C., D. M. C., and A. B. wrote the manuscript. All authors reviewed the manuscript.

***Acknowledgments.*** We thank B. Tümmler (Klinische Forschergruppe, Medizinische Hochschule Hannover, Germany) for supplying the *Pseudomonas aeruginosa* AA2 and AA43 clinical isolates, G. Pier (Harvard Medical School, Boston, MA) for the rabbit antiserum specific for *P aeruginosa* and *S. aureus*, I. De Fino and E. Pedretti for technical assistance, and M. Rocchi and F. Sanvito (Department of Pathology, San Raffaele Scientific Institute, Milano, Italy) for the mouse histological preparations.

Part of this work was carried out in ALEMBIC, an advanced microscopy laboratory established by the San Raffaele Scientific Institute and the Vita-Salute San Raffaele University (Milan).

***Disclaimer.*** The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

***Financial support.*** This study was funded by the Italian Cystic Fibrosis Research Foundation (FFC no. 9/2010) with the contribution of Delegazione di Milano, Delegazione FFC di Villa D'Almè, and Associazione "Gli amici di Claudia", Onlus Cerignola.

***Potential conflicts of interest.*** All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

### References

1. Cutting GR. Cystic fibrosis genetics: from molecular understanding to clinical application. Nat Rev Genet **2015**; 16:45–56.
2. MacKenzie T, Gifford AH, Sabadosa KA, et al. Longevity of patients with cystic fibrosis in 2000 to 2010 and beyond: survival analysis of the Cystic Fibrosis Foundation patient registry. Ann Intern Med **2014**; 161:233–41.
3. Cystic Fibrosis Foundation. Patient Registry. Annual data report 2015. Available at: https://www.cff.org/Our-Research/CF-Patient-Registry/2015-Patient-Registry-Annual-Data-Report.pdf. **August 2016**.
4. Harun SN, Wainwright C, Klein K, Hennig S. A systematic review of studies examining the rate of lung function decline in patients with cystic fibrosis. Paediatr Respir Rev **2016**; 20:55–66.
5. Nguyen D, Singh PK. Evolving stealth: genetic adaptation of *Pseudomonas aeruginosa* during cystic fibrosis infections. Proc Natl Acad Sci U S A **2006**; 103:8305–6.
6. Cigana C, Curcurù L, Leone MR, et al. *Pseudomonas aeruginosa* exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. PLoS One **2009**; 4:e8439.
7. Cigana C, Lorè NI, Riva C, et al. Tracking the immunopathological response to *Pseudomonas aeruginosa* during respiratory infections. Sci Rep **2016**; 6:21465.
8. Kahl BC. Impact of *Staphylococcus aureus* on the pathogenesis of chronic cystic fibrosis lung disease. Int J Med Microbiol **2010**; 300:514–9.
9. Stutman HR, Lieberman JM, Nussbaum E, Marks MI. Antibiotic prophylaxis in infants and young children with cystic fibrosis: a randomized controlled trial. J Pediatr **2002**; 140:299–305.
10. Ratjen F, Comes G, Paul K, Posselt HG, Wagner TO, Harms K. Effect of continuous antistaphylococcal therapy on the

rate of *P. aeruginosa* acquisition in patients with cystic fibrosis. Pediatr Pulmonol **2001**; 31:13–6.
11. Goss CH, Muhlebach MS. Review: *Staphylococcus aureus* and MRSA in cystic fibrosis. J Cyst Fibros **2011**; 10:298–306.
12. Bragonzi A. Murine models of acute and chronic lung infection with cystic fibrosis pathogens. Int J Med Microbiol **2010**; 300:584–93.
13. Diep BA, Gill SR, Chang RF, et al. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. Lancet **2006**; 367:731–9.
14. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ. sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional rsbU strain derived from *Staphylococcus aureus* 8325-4. J Bacteriol **2002**; 184:5457–67.
15. Baba T, Bae T, Schneewind O, Takeuchi F, Hiramatsu K. Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. J Bacteriol **2008**; 190:300–10.
16. Bragonzi A, Paroni M, Nonis A, et al. *Pseudomonas aeruginosa* microevolution during cystic fibrosis lung infection establishes clones with adapted virulence. Am J Respir Crit Care Med **2009**; 180:138–45.
17. Facchini M, De Fino I, Riva C, Bragonzi A. Long term chronic *Pseudomonas aeruginosa* airway infection in mice. J Vis Exp **2014**; 85:e51019.
18. Kukavica-Ibrulj I, Facchini M, Cigana C, Levesque RC, Bragonzi A. Assessing *Pseudomonas aeruginosa* virulence and the host response using murine models of acute and chronic lung infection. Methods Mol Biol **2014**; 1149:757–71.
19. Bragonzi A, Wiehlmann L, Klockgether J, et al. Sequence diversity of the mucABD locus in *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Microbiology **2006**; 152:3261–9.
20. Lorè NI, Cigana C, De Fino I, et al. Cystic fibrosis-niche adaptation of *Pseudomonas aeruginosa* reduces virulence in multiple infection hosts. PLoS One **2012**; 7:e35648.
21. Hirschhausen N, Block D, Bianconi I, et al. Extended *Staphylococcus aureus* persistence in cystic fibrosis is associated with bacterial adaptation. Int J Med Microbiol **2013**; 303:685–92.
22. Voyich JM, Braughton KR, Sturdevant DE, et al. Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J Immunol **2005**; 175:3907–19.
23. Kahl BC, Duebbers A, Lubritz G, et al. Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. J Clin Microbiol **2003**; 41:4424–7.
24. Zolin A, McKone EF, van Rens J, et al. ECFSPR Annual Report 2014. Available at: https://www.ecfs.eu/sites/default/files/general-content-files/working-groups/ecfs-patient-registry/ECFSPR_Annual%20Report%202014_Nov2016.pdf. **November 2016**.
25. Junge S, Görlich D, den Reijer M, et al. Factors associated with worse lung function in cystic fibrosis patients with persistent *Staphylococcus aureus*. PLoS One **2016**; 11:e0166220.
26. Elizur A, Orscheln RC, Ferkol TW, et al. Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* lung infection in patients with cystic fibrosis. Chest **2007**; 131:1718–25.
27. Kim HK, Missiakas D, Schneewind O. Mouse models for infectious diseases caused by *Staphylococcus aureus*. J Immunol Methods **2014**; 410:88–99.
28. Pillarisetti N, Williamson E, Linnane B, et al. Infection, inflammation, and lung function decline in infants with cystic fibrosis. Am J Respir Crit Care Med **2011**; 184:75–81.
29. Gangell C, Gard S, Douglas T, et al. Inflammatory responses to individual microorganisms in the lungs of children with cystic fibrosis. Clin Infect Dis **2011**; 53:425–32.
30. Liou TG, Adler FR, Fitzsimmons SC, Cahill BC, Hibbs JR, Marshall BC. Predictive 5-year survivorship model of cystic fibrosis. Am J Epidemiol **2001**; 153:345–52.
31. Mayer-Hamblett N, Aitken ML, Accurso FJ, et al. Association between pulmonary function and sputum biomarkers in cystic fibrosis. Am J Respir Crit Care Med **2007**; 175:822–8.
32. Nair N, Biswas R, Götz F, Biswas L. Impact of *Staphylococcus aureus* on pathogenesis in polymicrobial infections. Infect Immun **2014**; 82:2162–9.
33. Pernet E, Guillemot L, Burgel PR, et al. *Pseudomonas aeruginosa* eradicates *Staphylococcus aureus* by manipulating the host immunity. Nat Commun **2014**; 5:5105.
34. Filkins LM, Graber JA, Olson DG, et al. Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* drives *S. aureus* towards fermentative metabolism and reduced viability in a Cystic Fibrosis Model. J Bacteriol **2015**; 197:2252–64.
35. Nguyen AT, Oglesby-Sherrouse AG. Interactions between *Pseudomonas aeruginosa* and *Staphylococcus aureus* during co-cultivations and polymicrobial infections. Appl Microbiol Biotechnol **2016**; 100:6141–8.
36. Hoffman LR, Déziel E, D'Argenio DA, et al. Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **2006**; 103:19890–5.
37. Baldan R, Cigana C, Testa F, et al. Adaptation of *Pseudomonas aeruginosa* in Cystic Fibrosis airways influences virulence of *Staphylococcus aureus* in vitro and murine models of co-infection. PLoS One **2014**; 9:e89614.
38. Bragonzi A, Worlitzsch D, Pier GB, et al. Nonmucoid *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a mouse model. J Infect Dis **2005**; 192:410–9.