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In Vivo Efficacy of Antimicrobials against Biofilm-Producing *Pseudomonas aeruginosa*

Vinay Pawar,[a] Uliana Komor,[a] Nadine Kasnitz,[a] Piotr Bielecki,[e] Marina C. Pils,[b] Benjamin Gocht,[c] Annette Moter,[c] Manfred Rohde,[d] Siegfried Weiss,[a] Susanne Häussler[e]

Molecular Immunology, Helmholtz Centre for Infection Research, Braunschweig, Germany[a]; Mouse Pathology, Helmholtz Centre for Infection Research, Braunschweig, Germany[b]; Biofilm Centre, German Heart Institute Berlin, Berlin, Germany[c]; Central Facility for Microscopy, Helmholtz Centre for Infection Research, Braunschweig, Germany[d]; Molecular Bacteriology, Twincore, Centre for Clinical and Experimental Infection Research, Hannover, Germany[e]

Patients suffering from cystic fibrosis (CF) are commonly affected by chronic *Pseudomonas aeruginosa* biofilm infections. This is the main cause for the high disease severity. In this study, we demonstrate that *P. aeruginosa* is able to efficiently colonize murine solid tumors after intravenous injection and to form biofilms in this tissue. Biofilm formation was evident by electron microscopy. Such structures could not be observed with transposon mutants, which were defective in biofilm formation. Comparative transcriptional profiling of *P. aeruginosa* indicated physiological similarity of the bacteria in the murine tumor model and the CF lung. The efficacy of currently available antibiotics for treatment of *P. aeruginosa*-infected CF lungs, such as ciprofloxacin, colistin, and tobramycin, could be tested in the tumor model. We found that clinically recommended doses of these antibiotics were unable to eliminate wild-type *P. aeruginosa* PA14 while being effective against biofilm-defective mutants. However, colistin-tobramycin combination therapy significantly reduced the number of *P. aeruginosa* PA14 cells in tumors at lower concentrations. Hence, we present a versatile experimental system that is providing a platform to test approved and newly developed antibiofilm compounds.

Most bacterial infections are effectively treated by potent antimicrobial agents and can be quickly overcome. Nevertheless, bacterial infections continue to be a serious threat—mostly when onset of antimicrobial therapy is delayed, resistance of the microbes emerges, or chronic persistence develops (1–3). Although chronic infections are substantially mitigated by intensified antimicrobial therapy, antibiotics usually fail to resolve the infection, and very limited treatment options remain (4, 5). In this phase, bacteria often adopt a biofilm mode of growth: i.e., the bacterial community produces a protective extracellular matrix against antimicrobial therapy and the defense mechanisms of the host (6, 7). As a consequence, intensified treatment becomes insufficient, and surgery is often the ultimate means of infection control.

Despite the medical need, no effective antibiofilm treatment regimens are presently available in the clinic (8, 9). This is largely due to the fact that conventional treatment strategies fail to resolve biofilm infections. Therefore, to prevent biofilms or resolve them, understanding the mode of action of commonly used antibiotics against biofilm-grown bacteria *in vivo* is an essential requirement to develop more effective antimicrobial treatment regimens (10–12). This includes the evaluation of the synergistic activity of antibiotic combinations and the optimization of dosing regimens *in vivo* to maximize their effectiveness. Furthermore, novel compounds with an antibiofilm activity need to be developed. However, most of the novel therapeutic strategies for inhibition of biofilm growth have been developed *in vitro* (13). Obviously, identification of an *in vitro* antibiofilm strategy needs to be followed by the demonstration of *in vivo* efficacy in a suitable animal infection model (14).

Of major clinical relevance are chronic infections of the lung of cystic fibrosis (CF) patients by *Pseudomonas aeruginosa*. Most CF patients acquire *P. aeruginosa* during early childhood and develop chronic biofilm infections with repeated courses of exacerbation (15). Despite intensified antimicrobial therapy, progressive infection and inflammation lead to deterioration of pulmonary function. Thus, chronic *P. aeruginosa* infection remains the major reason for lung failure in these patients (16). To improve present antibiofilm therapies and to test their *in vivo* efficacy, a small-animal model should be employed. However, although the applied models to date (17) result in reduced bacterial clearance as one hallmark of chronic infections, most of them do not exert bacterial growth within biofilms or exhibit a low efficiency of bacterial colonization. This limits their use to study *P. aeruginosa* biofilm infections *in vivo*. We have recently described a model that might accomplish the requirements. It employs transplantable solid tumors in mice that are preferentially colonized by *P. aeruginosa* as well as by other bacteria after systemic administration (18). The microenvironment of the colonized tumor leads to biofilm formation by the bacteria (18–20). Apparently, this microenvironment of the murine tumor resembles very closely that found in the CF lung or in burn wounds (21).

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Address correspondence to Vinay Pawar, vinay.pawar@helmholtz-hzi.de.

V.P., U.K., and N.K. contributed equally to this article, and S.W. and S.H. contributed equally to this article.

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In the present work, we carefully calibrated our murine tumor model and infected mouse tumors with the biofilm-forming PA14 wild-type (WT) strain as well as with two biofilm-defective mutants. Those mutants not only formed poor biofilms *in vitro*, but—albeit they were able to colonize the tumor at comparable CFU—they did not form biofilms *in vivo* within the tumor tissue. This is an important prerequisite for the robust analysis of antibiofilm and antibacterial effects of antimicrobial compounds *in vivo*.

By applying the model, we could demonstrate that ciprofloxacin, tobramycin, and colistin significantly reduced CFU counts of the biofilm-defective *P. aeruginosa* mutants within the tumor tissue, whereas an antibiofilm activity against biofilm-forming wild-type bacteria was observed only at antibacterial concentrations that exceed the normal doses used in the clinics. Importantly, a combination of tobramycin and colistin showed activity already at much lower concentrations, as previously demonstrated (22), indicating synergistic antibiofilm activity. Our results demonstrate that with the mouse tumor model, a versatile model is available that allows the *in vivo* characterization of antimicrobial compounds already in use as well as the evaluation of novel antibacterial substances.

MATERIALS AND METHODS

Bacterial strains and cell lines. The *Pseudomonas aeruginosa* PA14 wild-type strain and transposon mutants PA14 *pqsA*::Tn (defective in 4-quinolone signaling) (23, 24) and PA14 *pelA*::Tn (defective in the production of the Pel exopolysaccharide) (25) were obtained from the Harvard transposon mutant library (26). The phenotype of such mutants was confirmed as shown in Fig. S1 and S2 in the supplemental material. The equal sensitivities of these strains toward the antibiotics employed have also been confirmed (see Table S1 in the supplemental material). CT26 colon carcinoma cells (ATCC CRL-2638) were grown as monolayers in Iscove's modified Dulbecco's medium (IMDM) (catalog no. 42200030; Gibco Life Technologies, Germany) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Integro) and 250 mmol liter^{-1} β-mercaptoethanol (Serva).

Animals. Female BALB/c mice were purchased from Janvier (BALB/cByJRj) at 6 to 7 weeks of age and were allowed to acclimatize to the institutional animal facility for 1 week prior to experiments. All animal experiments were carried out in accordance with the institutional guidelines and are in agreement with the German Animal Welfare Act (Tierschutzgesetz, 1998) and international laws and policies governing the use of animals for scientific purposes (EC Council Directive 86/609, OJ L 358,1, 12 December 1987; and *NIH Guide for the Care and Use of Laboratory Animals*, U.S. National Research Council, 1996). The protocol was approved by the ethical board of the local authorities (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit [LAVES], Oldenburg, Germany). The permission number is 33.9.42502-04-050/09.

Infection of tumor-bearing mice. Seven- to 8-week-old female BALB/c mice were subcutaneously (s.c.) inoculated with 5×10^5 CT26 cells into the abdomen or the flank (Fig. 1a). When the tumor reached an appropriate size, the mice were infected intravenously (i.v.) with 5×10^6 CFU of *P. aeruginosa* in phosphate-buffered saline (PBS).

Antibiotics and dosage. Stock solutions of ciprofloxacin (Hexal, Holzkirchen, Germany), colistin (Grunenthal, Aachen, Germany), and tobramycin (Infectopharm Arzneimittel, Heppenheim, Germany) were prepared fresh in 0.9% saline at a final concentration of 2 mg/ml. Antibiotics were administered i.v. at the indicated time intervals and doses.

Determination of CFU within mouse tissue. To determine CFU counts, mice were sacrificed and organs (tumor, liver, and gut) were homogenized in 0.1% (vol/vol) Triton X-100–PBS using a gentleMACS dissociator. The homogenates were serially diluted and plated on Luria-Bertani (LB) agar plates with ampicillin (0.1 mg/ml).

Histology. Standard histology procedures were followed. Briefly, organs were fixed in 4% buffered formaldehyde for 24 to 48 h and embedded in paraffin, and 3-μm sections were obtained. The sections were stained with hematoxylin and eosin (HE). A combined periodic acid-Schiff-alcian blue reaction (PAS) was conducted according to a standard protocol. The stained sections were analyzed with an Olympus BX51 microscope, and images were captured with an Olympus U-CMAD3 camera using the software ZEN 2009.

FISH. Fluorescence *in situ* hybridization (FISH) was carried out as described previously (27). In brief, tumor samples were fixed for 24 h at 4°C. Samples were embedded in cold polymerizing resin (methacrylate) and sectioned as described elsewhere (28). The hybridization buffer (20 μl) consisted of 0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate (SDS), 20% formamide, and 4′,6-diamidino-2-phenylindole (DAPI). Probes were synthesized commercially and 5′ end labeled with a fluorochrome—either Cy3 (indocarbocyanine) or fluorescein isothiocyanate (FITC) (all from Biomers, Ulm, Germany) and used at a concentration of 20 pmol. Samples were incubated in a dark, humid chamber for 2 h at 50°C. The slides were then rinsed with sterile double-distilled water, dried, and mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) without DAPI. For visualization and identification of *P. aeruginosa*, the specific probe PSM was used (29). For each FISH experiment, positive and negative bacterial control strains were hybridized alongside the tumor samples to ensure specificity. These samples were further analyzed using an epifluorescence microscope (Axioplan 2; Carl Zeiss, Jena, Germany) equipped with narrow band filter sets (AHF Analysentechnik, Tübingen, Germany). Image acquisition was performed with an AxioCam MRm (Zeiss) making use of the AxioVision 4.4 software.

TEM. For transmission electron microscopy (TEM), tumors were fixed in 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose [pH 6.9]) overnight and then cut into cubes 3 to 5 mm in length. Samples were osmificated in 1% aqueous osmium tetroxide for 1 h and washed with cacodylate buffer. Samples were then dehydrated with graded series of acetone (10, 30, 50, 70, 90, and 100%) for 30 min each step. Dehydration in the 70% acetone step was done with 2% uranyl acetate overnight. Samples were then infiltrated with an epoxy resin (1 part acetone/1 part resin, 1 part acetone/2 parts resin, pure resin alternating over 2 days) according to Spurr's formula. Ultrathin sections were cut with a diamond knife, picked up with Formvar-coated grids, counterstained with uranyl acetate and lead citrate, and examined in a TEM910 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at an acceleration voltage of 80 kV. Images were taken at calibrated magnifications using a line replica. Images were recorded digitally with a ProScan slow-scan charge-coupled device (CCD) camera (1,024 by 1,024) (ProScan, Scheuring, Germany) with ITEM software (Olympus Soft Imaging Solutions, Münster, Germany) (30).

Statistics. For all CFU counts and cytokine measurements, the mean values and standard deviations were calculated. Changes were analyzed using Student's *t* test in the statistical program GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

A small-animal model for *P. aeruginosa* biofilm infections. In previous publications, we could show that *P. aeruginosa* is able to efficiently colonize solid murine tumors and to form biofilm-like structures in such tissue (20, 21). Here, we aimed at calibrating the model system to evaluate the effectiveness of antimicrobial compounds and antimicrobial combinations against biofilm-grown bacteria *in vivo*. We therefore determined several parameters of bacterial tumor colonization, like the influence of the size of the inoculum or the tumor to establish robust infections.

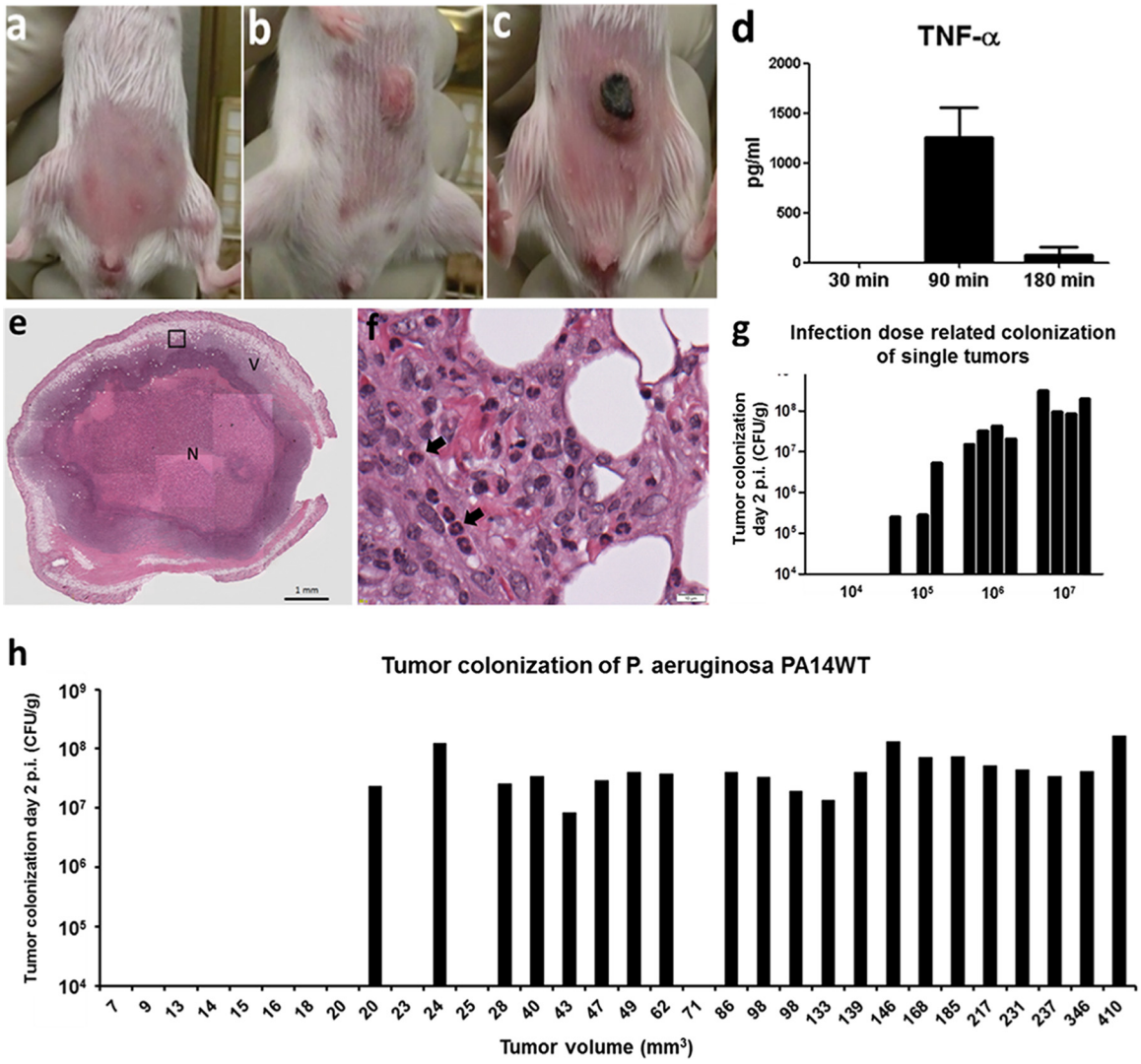

FIG 1 Infection of transplantable solid tumors in mice. (a) A total of 5×10^5 CT26 cells were injected subcutaneously into the abdomen/flank region. (b) After approximately 10 days, tumors reached a size of between 100 and 150 mm^3. (c) Shortly after i.v. *P. aeruginosa* infection, tumors showed macroscopically visible necrotic areas, like the tumor presented here. (d) Induction of cytokines (exemplified by TNF-α) shortly after bacterial application. TNF-α is most likely the most important cytokine for this model system. (e) Overview of hematoxylin-and-eosin (HE)-stained tumor section colonized by *P. aeruginosa*. The black box indicates the area that is enlarged in panel f. N indicates the large necrotic area of the infected tumor, and V indicates the remaining viable part of the tumor after bacterial colonization. (A higher-resolution image was added to Fig. S4 in the supplemental material.) (f) Enlargement of the area from the *P. aeruginosa*-colonized tumor shown in panel e. Tumor-infiltrating neutrophils (black arrows) are identified by their nuclear shape. (g) Efficiency of tumor colonization using different infection doses. Bars represent individual mice. (h) Efficiency of tumor colonization depends on the size of the tumor. Bars represent individual mice.

Transplantable CT26 tumor cells were injected into either the abdomen or flank of syngeneic BALB/c mice (Fig. 1a). After 10 days, these mice were then infected intravenously (i.v.) with 5×10^6 *P. aeruginosa* PA14 cells (Fig. 1b). Roughly 2 to 3 h after application of the bacteria, the tumor changes its macroscopic appearance and turns black due to a severe hemorrhage (Fig. 1c). This reaction is most likely due to the induction of tumor necrosis factor alpha (TNF-α), which was found at high levels in the blood of *P. aeruginosa*-infected mice shortly after bacterial application (Fig. 1d). As a consequence, a large necrotic area forms in the center of the tumor, as shown by histology (Fig. 1e and f). Furthermore, histology revealed a strong inflammatory response and attraction of neutrophils in the colonized tumors (Fig. 1e and f) (20).

Approximately 10 days post-CT26 cell inoculation, tumors normally attain a size of between 100 and 150 mm^3. This appears to be the optimal time point for infection. As depicted in Fig. 1h, tumors with a size smaller than 100 mm^3 carried the risk of not being colonized by bacteria, while in larger tumors, we observed robust bacterial colonization at comparable CFU counts.

Optimal bacterial infection with an appropriate size of the inoculum is also extremely important for robust tumor colonization (Fig. 1g). An inoculum size too low will result in no tumor colonization or colonization of only part of the tumors (Fig. 1g). Probably, too little TNF-α is induced under these circumstances. The presence of too many bacteria, however, results in septicemia and death of the mice (data not shown). Interestingly, the extent of colonization was dependent on the size of the inoculum: i.e., lower

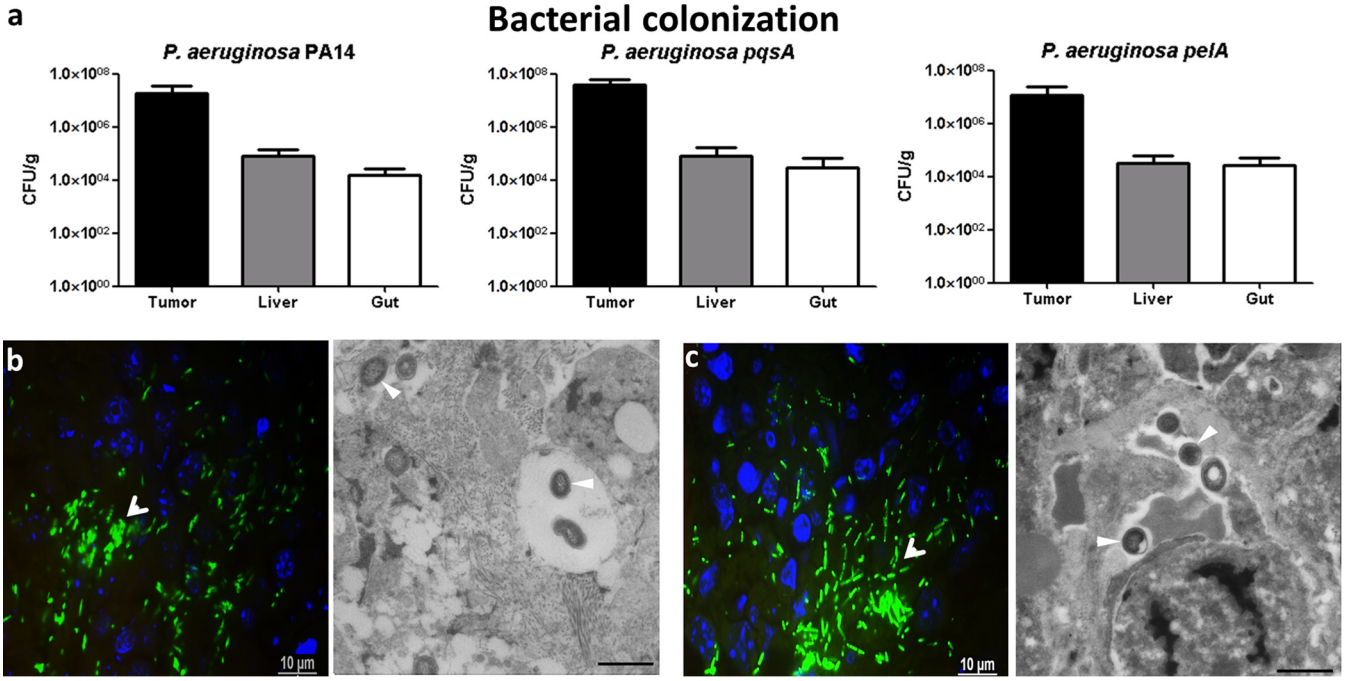

FIG 2 Tumor colonization of biofilm-defective *P. aeruginosa* mutants. (a) Wild-type *P. aeruginosa* PA14 and biofilm-defective mutants (PA14 *pqsA*::Tn and *pelA*::Tn) colonizing different tissues of mice. This experiment includes ≥5 mice per group and was performed at least two times. (b and c) Visualization of *P. aeruginosa* PA14 *pqsA*::Tn (b) and *P. aeruginosa* PA14 *pelA*::Tn (c) by FISH and TEM. Sections of tumor tissue samples were hybridized with a species-specific probe for *P. aeruginosa* (green) for FISH analysis. In TEM images, arrowheads indicate individual bacterial cells. No aggregate formation of bacteria and no visible matrix material were observed.

colonization was observed when lower bacterial doses were used as the inoculum (Fig. 1g). This is different from other bacteria like *Salmonella enterica* serovar Typhimurium, where above a certain threshold, the colonization is mostly independent of the number of bacteria injected (31).

Standard laboratory strains of *P. aeruginosa* PAO1 and PA14 preferentially accumulate and multiply in the neoplastic tissue, while colonization of healthy organs, like liver and gut, is much lower (see Fig. S3a in the supplemental material). Fluorescent *in situ* hybridization (FISH) on tumor sections demonstrates that colonization of *P. aeruginosa* PA14 occurs mostly throughout region of the tumor tissue where still viable cells reside (see Fig. S3b). Biofilm-like structures develop shortly after tumor colonization. Scanning electron microscopy and transmission electron microscopy clearly show the embedment of the PA14 bacteria in a self-produced extracellular matrix (see Fig.S3c and d).

Biofilm-defective mutants colonize the tumor but do not form biofilms in the tumor tissue. Several bacterial factors have been shown to be required for efficient formation of *P. aeruginosa* biofilms *in vitro* (24). However, whether those factors are also crucial for biofilm formation *in vivo* has remained elusive. Hence, we selected two mutants, both of which form poor biofilms *in vitro*. One (a *pqsA* transposon mutant) was defective in the production of the 4-quinolone signal molecules (see Fig. S2 in the supplemental material), and the second (a *pelA* transposon mutant) was defective in the production of the Pel exopolysaccharide (see Fig. S1 in the supplemental material). First, we elucidated whether the biofilm-defective mutants were able to colonize the tumor, liver, and gut at similar rates to the *P. aeruginosa* PA14 WT after i.v. application. The CFU counts (Fig. 2a) as well as the localization of the mutants within the tumors were comparable to those of WT bacteria (Fig. 2b and c). Most importantly, the *in vitro* biofilm-defective mutants were not able to produce an extracellular matrix *in vivo*, as judged by electron microscopy (Fig. 2b and c) (30). They largely remained as single cells in the tumor and were not surrounded by the biofilm-like structures seen for the WT bacteria (see Fig.S3 in the supplemental material).

Efficacy of ciprofloxacin, colistin, and tobramycin against biofilm-grown *P. aeruginosa*. Our test system to screen and characterize antibiofilm antibiotics consisted of the *P. aeruginosa* PA14 WT strain as well as the *pqsA* transposon mutant and was now extended by the *pelA* mutant, both of which are biofilm defective. The robustness of this system was challenged using anti-*Pseudomonas* antibiotics that are routinely used in clinics (ciprofloxacin, colistin, and tobramycin). Treatment of *P. aeruginosa*-infected tumor-bearing mice was initiated 48 h after infection. At this time point, PA14 and the two biofilm-deficient mutant isolates are expected to have reached CFU counts of approximately 10^8/g in the tumors and 100- to 1,000-fold lower counts in liver and gut. Antibiotics were administered i.v. in 3 individual doses at time intervals of 12 h before the mice were sacrificed, and CFU counts were determined for tumors as well as for liver and gut. Neither of the antibiotics successfully reduced bacterial counts of the PA14 wild type in the tumor tissue, despite treatment with a dose of 5 mg/kg ciprofloxacin, 5 mg/kg colistin, or 4 mg/kg tobramycin, respectively (Fig. 3a to c). On the other hand, ciprofloxacin, colistin, and tobramycin reduced the CFU counts very effectively in liver and gut, where lower bacterial numbers are present and the bacteria presumably do not form biofilms (Fig. 3a to c). In contrast, all three antibiotics proved to be effective against the two

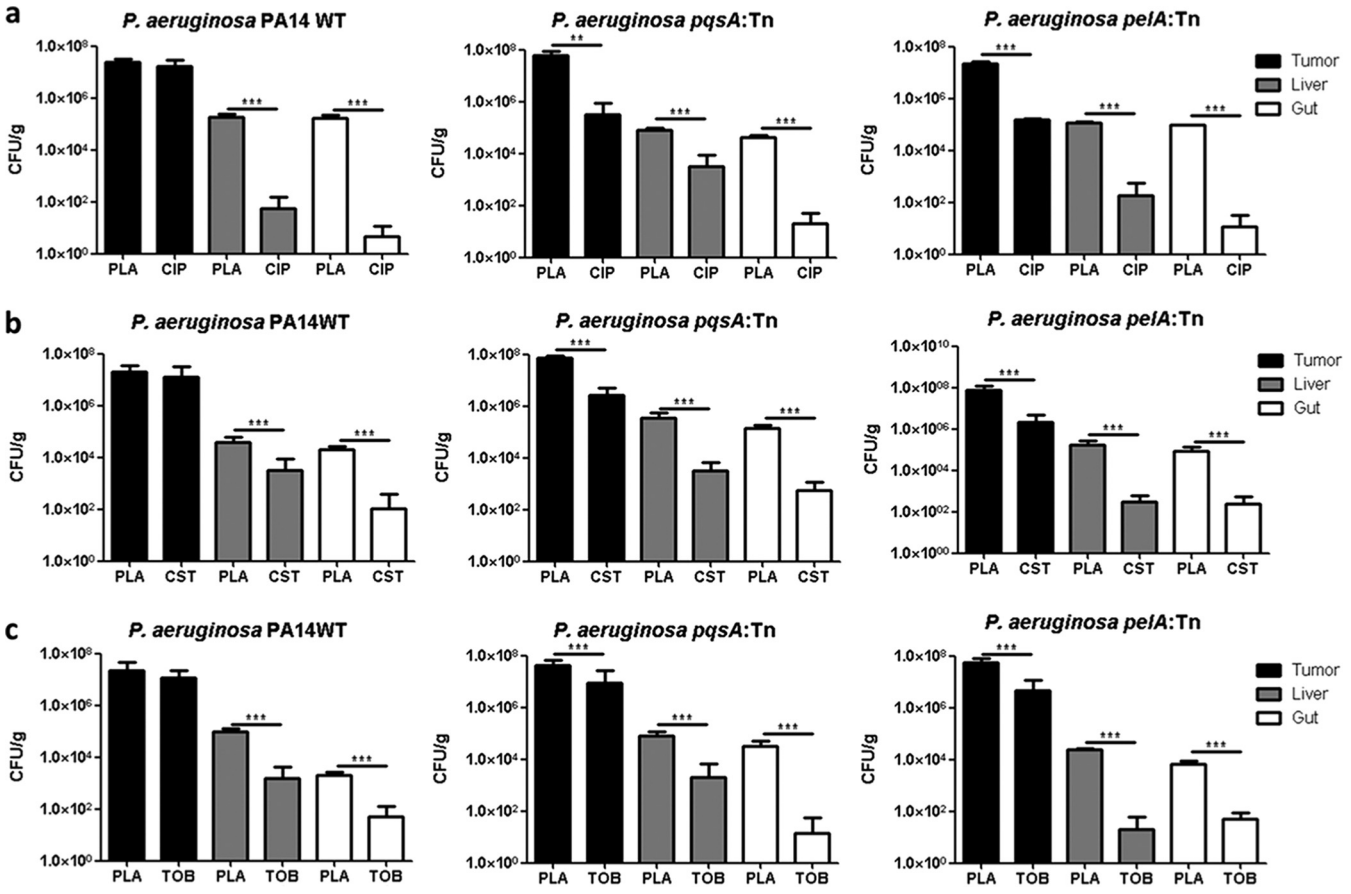

FIG 3 Effect of antibiotics against biofilm-sufficient and biofilm-defective *P. aeruginosa in vivo*. Tumor-bearing mice were infected with PA14 WT, PA14 *pqsA*::Tn, and *pelA*::Tn. Forty-eight hours postinfection (p.i.), mice were treated i.v. with ciprofloxacin (CIP) at a dose of 5 mg/kg (a), colistin (CST) at a dose of 5 mg/kg (b), and tobramycin (TOB) at a dose of 4 mg/kg (c). Control mice received 0.9% saline solution i.v. as a placebo (PLA). One exemplary experiment out of at least three is presented, with $n \geq 4$. **, $P < 0.005$; ***, $P < 0.0005$.

biofilm-defective mutants (Fig. 3a to c). We observed not only a reduction of CFU counts in liver and gut comparable to the wild type but also a significant reduction of bacterial counts in the tumor tissue (Fig. 3a to c). Interestingly, when the treatment doses of the antibiotics were increased to 10 mg/kg, the CFU counts of all strains, including the WT, were reduced at comparable rates even in the tumor (Fig. 4) (data not shown).

Colistin-tobramycin combination therapy. Colistin-tobramycin combinations have been shown previously to be superior to monotherapy against biofilm-grown *P. aeruginosa* in an *in vitro* system as well as in a rat lung infection model and in CF patients (22). We therefore tested the ability of colistin-tobramycin combinations to kill *P. aeruginosa* biofilms in our test system. Tumor-bearing mice were infected with the PA14 WT strain and the *pqsA* transposon mutant. Two days postinfection, the antibiotic treatment was initiated: colistin alone, tobramycin alone, or colistin and tobramycin in combination. Colistin and tobramycin alone did not show a significant effect on the *P. aeruginosa* PA14 WT strain (Fig. 5b and c). Therapy was clearly more effective for the *pqsA* transposon mutant defective in biofilm formation, despite a

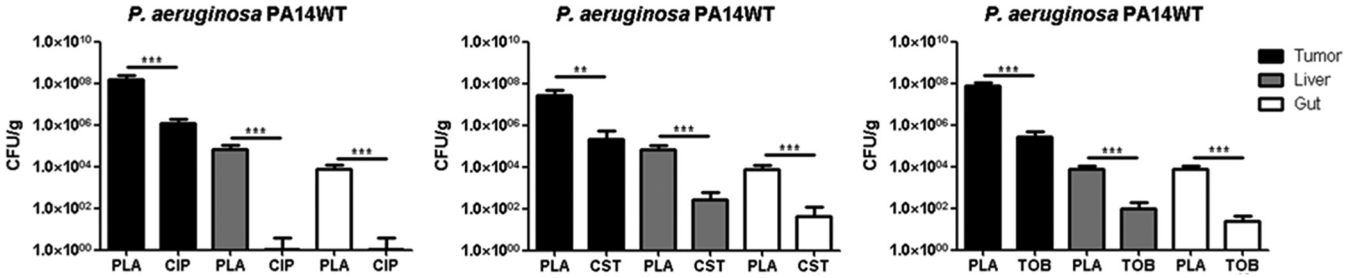

FIG 4 High antimicrobial doses kill biofilm *P. aeruginosa in vivo*. Tumor-bearing mice were infected with the PA14 WT strain, and at 48 h p.i., mice were treated with ciprofloxacin (CIP), colistin (CST), and tobramycin (TOB) i.v. at a dose of 10 mg/kg. Control mice were injected with 0.9% saline solution as a placebo (PLA). One exemplary experiment out of at least three is presented, with $n \geq 5$. **, $P < 0.005$; ***, $P < 0.0005$.

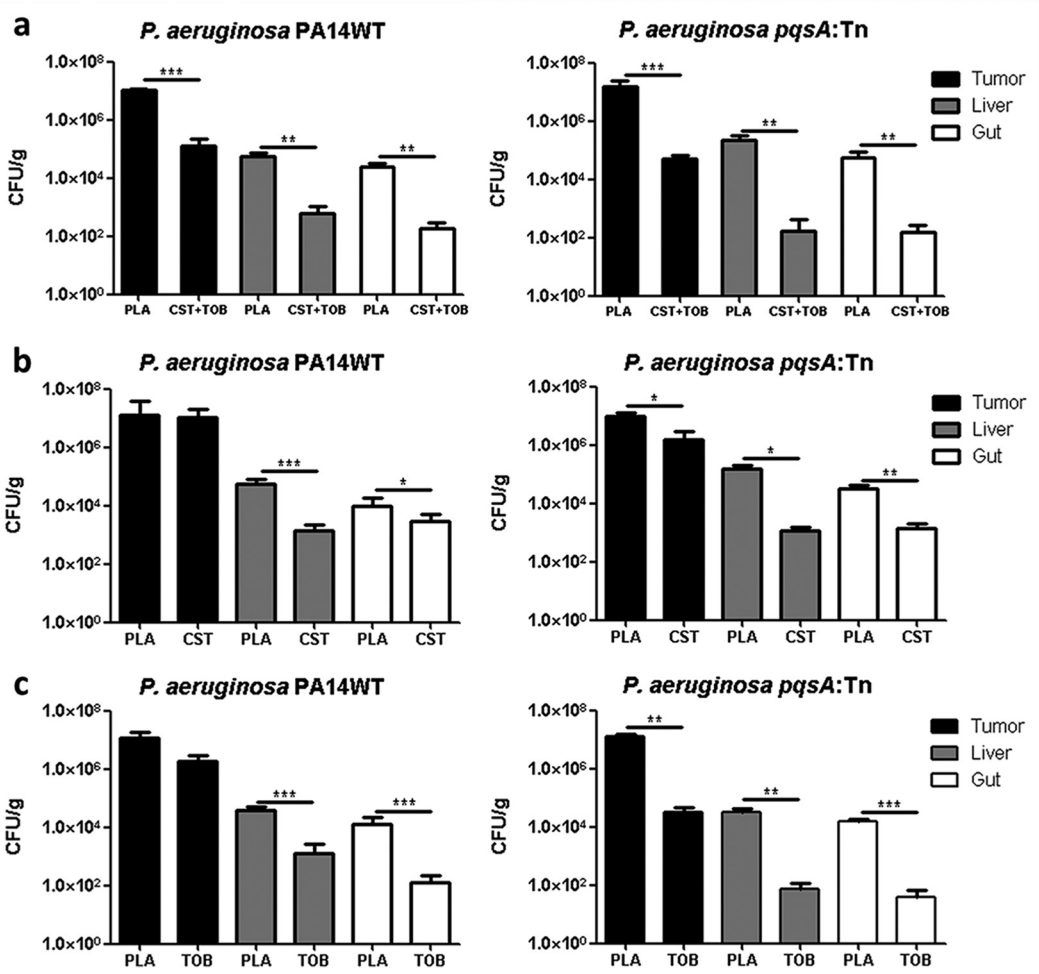

FIG 5 Effective combination therapy against biofilm-producing *P. aeruginosa* and the biofilm-defective *pqsA*::Tn mutant. Tumor-bearing mice were infected with PA14 WT or *pqsA*::Tn, and at 48 h p.i., mice were treated with 2.5 mg/kg of colistin and tobramycin alone or in combination. (a) Colistin-tobramycin in combination (CST+TOB). (b) Colistin only (CST). (c) Tobramycin only (TOB). Control mice were injected with 0.9% saline solution as a placebo (PLA). One exemplary experiment out of at least three is presented, with $n \geq 5$. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

reduction of the administration dose to 2.5 mg/kg colistin and tobramycin, respectively. Again, the bacterial counts of liver and gut from antibiotic-treated groups were reduced significantly by 100- to 1,000-fold. Most interestingly, we found a significant reduction of WT bacterial counts from tumors when treated with a combination of colistin and tobramycin despite low individual antimicrobial doses (Fig. 5a).

DISCUSSION

Despite intensive investigations, we are far from understanding the molecular mechanisms that underlie tolerance of biofilm bacteria to a large variety of adverse environmental conditions, including antimicrobial therapy and attacks by the immune system. Although intensified antimicrobial treatment is applied, chronic *P. aeruginosa* infections are rarely eradicated. In an attempt to more efficiently treat *P. aeruginosa* infections in CF, many European CF care centers start antibiotic treatment early after the first detection of the pathogen (32). Nevertheless, this did not eliminate chronic *P. aeruginosa* infections in CF patients. Therefore, alternative therapeutic strategies are needed. The use of combination therapy involving antibiotics with different modes of action against *P. aeruginosa* is one option in this context. Another option is to foster screens for novel compounds with an effective antibiofilm activity. However, the identification of an antibiofilm activity by *in vitro* systems needs to be followed by a series of steps that include the demonstration of efficacy in a suitable animal infection model (14).

Here, we demonstrate that our tumor model offers a robust and versatile experimental system to monitor the impact of antimicrobials against biofilm-producing *P. aeruginosa*. Matchable tumor growth and equivalent colonization of the tumor tissue with biofilm-forming bacteria can be achieved if standardized protocols are followed. We believe that the invasion of the tumors by *P. aeruginosa* is passive. Most likely, TNF-α produced early during the cytokine storm elicited by the bacterial application is opening the pathological blood vessels of the tumor. TNF-α is known for such activity and might preferentially act on the already leaky blood vessels of the tumor, as shown for *Salmonella* before (33). With the inflowing blood, the bacteria reach the tumor tissue and proliferate. Although the microorganisms reside among large numbers of immigrating neutrophilic granulocytes, these phagocytes did not harm the bacteria under the hypoxic conditions

of the tumor. On the other hand, they might force the bacteria to form biofilms as a protective response to reactive oxygen species (ROS) or antimicrobial peptides produced by these immune cells.

Most importantly, bacteria that *in vitro* are non-biofilm producing colonize the tumor tissue at similar CFU counts as WT bacteria. We could show that two disparate bacterial factors important for biofilm formation *in vitro* are also of relevance *in vivo*. The PA14 *pqsA* transposon mutant is not able to synthesize 4-quinolone signal molecules involved in quorum sensing. The variant *pelA* is unable to synthesize sugars required for formation of the extracellular matrix (25). None of the variant bacteria exhibiting a defect in forming biofilms *in vitro* was able to form such structures in the tumor. This fact is based on electron microscopic analysis of tumor-colonizing *P. aeruginosa*. Thus, it is possible to specifically track biofilm-associated characteristics *in vivo*. This allows the evaluation of the efficacy of classical antimicrobial agents and combinations thereof, as well as novel active compounds against *P. aeruginosa* biofilm infections and to dissect *in vivo* antibacterial from antibiofilm activities.

Here, we tested three commonly used antibiotics, ciprofloxacin, colistin, and tobramycin, in the concentrations that were equivalent to doses used for human patients. Both biofilm-defective variants were sensitive against the activity of all three antibiotics, while the CFU counts of biofilm-forming wild-type bacteria in the tumor tissue did not decline. However, wild-type bacteria were sensitive to such antibiotics when not residing in biofilms, as could be concluded from the data obtained for liver and gut. Here, the CFU counts were strongly reduced as expected. Although the difference in CFU counts of wild-type bacteria in the tumor compared with those of the variants was sometimes only 10-fold, the results were reproducible. In addition, only three applications of the antibiotics were performed. A more extended application would most likely lead to a stronger reduction of the biofilm-defective mutants, while the wild-type bacteria are expected not to be affected. Thus, the most probable explanation for the differential resistance of the bacteria is indeed the ability to form biofilms. Since the two variants that are unable to form biofilms and the wild type are equally sensitive to the three antimicrobials when tested under planktonic conditions, we can conclude that the tumor model has proven to be a valid test model to dissect antibiotic sensitivity of biofilm *P. aeruginosa*.

The key advantages of this model are that first, the model mimics the natural steps of biofilm formation during the establishment of biofilm-associated *P. aeruginosa* infections. Second, the niche within the tumor largely reflects environmental characteristics of the chronically infected CF respiratory tract. The *in vivo* transcription profile of wild-type *P. aeruginosa* isolated from tumors was previously shown to match that of isolates from CF lungs and lesions of patients suffering from burn wounds (21). This provides evidence that the microenvironments of colonized solid tumors and CF lungs or burn wounds are very similar. Third, the transferability of *in vitro* findings on biofilm developmental processes to the *in vivo* situation has been demonstrated. The model therefore allows us to reconsider currently used therapies of chronic *P. aeruginosa* biofilm infections—e.g., in CF patients.

Biofilm infection by *P. aeruginosa* in lungs of CF patients is the leading cause of their morbidity and leads finally to functional failure of this organ (34, 35). Current therapies are facing serious problems due to antibiotic tolerance and resistance against therapy. Thus, this life-shortening infection needs the development of efficient treatment. Antibiotics combination therapy might be more effective under certain circumstances than monotherapies. For instance, a penicillin-aminoglycoside combination is able to eradicate *P. aeruginosa* from biofilms more efficiently than monotherapy (36, 37). In accordance, tobramycin and colistin showed promising activity against *P. aeruginosa* biofilms. This combination of antibiotics was able to eliminate biofilm-residing bacteria of different stages (13). Similarly, the complementary activity of colistin and tobramycin was reported to be superior in the treatment of chronically *P. aeruginosa*-infected CF patients (22). In our tumor model, the synergistic effect of the combination of colistin with tobramycin could be confirmed, although at antimicrobial concentrations that are far below of those required for an effective monotherapy, the combination of colistin and tobramycin significantly reduced the numbers of biofilm-forming WT bacteria in the tumor.

In conclusion, our model will be ideal to investigate steps of natural biofilm formation under *in vivo* conditions and confirm the importance of bacterial factors that have been shown to be critical for biofilm formation processes in *in vitro* systems. The model is also applicable for unraveling the complex interactions that occur during *P. aeruginosa* coinfections with other bacteria—typically with *Staphylococcus aureus* or anaerobes (38). Furthermore, it is possible to evaluate the efficacy of classical antimicrobial agents against *P. aeruginosa* biofilm infections and optimize the combinations and application regimens thereof. Moreover, in the last decade, great efforts have been put forth to find and characterize novel antimicrobial/biofilm compounds (18). Therefore, it becomes clear that murine solid tumors represent a versatile, easy to handle, and robust model system that should help to improve treatment of infections that represent a real threat in the clinics.

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