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Bringing together what belongs together: Optimizing murine infection models by using mouse-adapted *Staphylococcus aureus* strains

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ABSTRACT

Staphylococcus (S.) aureus is a leading cause of bacterial infection world-wide, and currently no vaccine is available for humans. Vaccine development relies heavily on clinically relevant infection models. However, the suitability of mice for *S. aureus* infection models has often been questioned, because experimental infection of mice with human-adapted *S. aureus* requires very high infection doses. Moreover, mice were not considered to be natural hosts of *S. aureus*. The latter has been disproven by our recent findings, showing that both laboratory mice, as well as wild small mammals including mice, voles, and shrews, are naturally colonized with *S. aureus*. Here, we investigated whether mouse-and vole-derived *S. aureus* strains show an enhanced virulence in mice as compared to the human-adapted strain Newman. Using a step-wise approach based on the bacterial genotype and *in vitro* assays for host adaptation, we selected the most promising candidates for murine infection models out of a total of 254 *S. aureus* isolates from laboratory mice as well as wild rodents and shrews. Four strains representing the clonal complexes (CC) 8, 49, and 88 (n = 2) were selected and compared to the human-adapted *S. aureus* strain Newman (CC8) in murine pneumonia and bacteremia models. Notably, a bank vole-derived CC49 strain, named DIP, was highly virulent in BALB/c mice in pneumonia and bacteremia models, whereas the other murine and vole strains showed virulence similar to or lower than that of Newman. At one tenth of the standard infection dose DIP induced disease severity, bacterial load and host cytokine and chemokine responses in the murine bacteremia model similar to that of Newman. In the pneumonia model, DIP was also more virulent than Newman but the effect was less pronounced. Whole genome sequencing data analysis identified a pore-forming toxin gene, *lukF-PV(P83)/lukM*, in DIP but not in the other tested *S. aureus* isolates. To conclude, the mouse-adapted *S. aureus* strain DIP allows a significant reduction of the inoculation dose in mice and is hence a promising tool to develop clinically more relevant infection models.

1. Introduction

Staphylococcus aureus is a human pathobiont that colonizes the anterior nares (Wertheim et al., 2005), but also causes various infectious diseases ranging from mild skin and soft tissue infections to severe infections, *e.g.* sepsis, pneumonia or endocarditis (Lowy, 1998; Tong et al., 2015). The treatment of *S. aureus* infections is complicated by the wide spread of methicillin-resistant *S. aureus* strains (World Health Organization, 2014). This has spurred efforts to develop an anti-staphylococcal vaccine, but so far all candidates have failed in clinical trials (Fowler and Proctor, 2014; Giersing et al., 2016). To develop novel approaches for the prevention and treatment of *S. aureus* infections, it is necessary to gain a better understanding of the host-pathogen interaction and adaptive immune response using a robust and clinically relevant animal model.

Laboratory mice are the most commonly used *S. aureus* infection model for a number of reasons: they are relatively easy and inexpensive to breed, there are several gene knock-out strains available and their

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immune system is already well characterized. However, many virulence factors that are present in human-adapted *S. aureus* isolates such as superantigens, some staphylococcal superantigen-like (SSL) proteins, the phage-encoded immune evasion cluster (IEC) molecules staphylokinase, chemotaxis inhibitory protein and staphylococcal complement inhibitor, or Panton-Valentine leukocidin (PVL) require high doses or they do not work at all in mice (Gladysheva et al., 2003; Haas et al., 2004; Holtfreter and Bröker, 2005; Langley et al., 2005; Löffler et al., 2010; Rooijakkers et al., 2005a, b).

While in the past mice were not thought to be natural hosts of *S. aureus* and therefore their suitability as an *S. aureus* infection model was contentiously discussed (Capparelli et al., 2011; Cuny et al., 2010; McCarthy and Lindsay, 2010; Mulcahy et al., 2012; Mulcahy and McLoughlin, 2016; Salgado-Pabón et al., 2014), we now know better. Our research group recently showed that laboratory mice as well as wild rodents and shrews are naturally colonized with *S. aureus* in their nose. Laboratory mice were predominantly colonized with *S. aureus* isolates of the clonal complex (CC) 1 and CC15, which are common in the human population, as well as CC88, a lineage rarely found in Northern American and European human populations (Ghebremedhin et al., 2009; Monecke et al., 2007; Zhang et al., 2009). In contrast, wild mice, voles, and shrews were colonized with unique lineages, that are not associated with humans, such as CC49, CC1956, and ST890 (Mrochen et al., 2017b). The isolated *S. aureus* strains from both laboratory mice and wild animals showed features of host adaptation, such as the absence of superantigen gene-encoding mobile genetic elements and the IEC-encoding Sa3int phages, both of which are common in human *S. aureus* isolates (Holtfreter et al., 2013; Monecke et al., 2016; Mrochen et al., 2017b; Schulz et al., 2017; Sung et al., 2008; van Wamel et al., 2006).

Mouse- and vole-derived *S. aureus* strains belong to unique clonal complexes and seem to be well adapted to their host. Therefore, we hypothesize, that strains isolated from mice and voles, hereafter called mouse-adapted strains, will show an enhanced fitness or virulence in their natural host, as compared to human-adapted *S. aureus* strains. In fact, we and others previously demonstrated that some mouse-adapted strains, such as JSNZ, WU1, and SaF_1, are better colonizers than the human-adapted *S. aureus* Newman in laboratory mice, leading to persistent colonization of the nasopharynx and gastrointestinal tract (Flaxman et al., 2017; Holtfreter et al., 2013; Sun et al., 2018).

The aim of this study was to test whether mouse-adapted *S. aureus* strains can be used to optimize current infection models. Based on their genotype and their ability to coagulate murine plasma and survive in murine whole blood, we selected three well-adapted *S. aureus* strains representing the lineages CC8, CC49, and CC88 and also included the previously described mouse-adapted strain JSNZ (CC88). These four *S. aureus* strains were compared to the human isolate Newman in murine pneumonia and bacteremia models. Notably, one isolate from a bank vole (*Myodes glareolus*), termed *S. aureus* DIP, was highly virulent in both infection models allowing a reduction of the infection dose by 90%. As a low infection dose represents a more physiological situation, DIP enables us to establish clinically more relevant infection models.

2. Material and methods

2.1. Staphylococcus aureus strains

All murine *S. aureus* isolates were obtained from naturally colonized laboratory mice or wild rodents and shrews as previously reported (Mrochen et al., 2017a, b). JSNZ is a mouse-adapted *S. aureus* strain that was isolated from a C57BL/6 colony at the animal breeding facility of the University of Auckland, New Zealand (Holtfreter et al., 2013). Newman is a human *S. aureus* isolate frequently used for murine infection models (Duthie and Lorenz, 1952). The genetic characterization of the *S. aureus* isolates (*spa* type, virulence genes, phage integrase genes) has been previously reported (Holtfreter et al., 2013; Mrochen et al., 2017a, b; Schulz et al., 2017), except for CC8 isolates, which are described in this study.

The initial genetic characterization of the *S. aureus* isolates (*spa* type, virulence genes, phage integrase genes) was performed as previously described (Holtfreter et al., 2013; Mrochen et al., 2017a, b).

2.2. Laboratory mice

Female BALB/c mice with Specific and Opportunistic Pathogen Free status (7 weeks) were obtained from Janvier Labs (Saint-Berthevin, France). Mice were housed in individually ventilated cages and were fed food and water *ad libitum*.

2.3. Wild mice and voles

S. aureus CC8 strains were isolated from yellow-necked mice (*Apodemus flavicollis*), field voles (*Microtus agrestis*), common voles (*Microtus arvalis*), and bank voles (*Myodes glareolus*). During monitoring studies from 2010 to 2014, animals were collected in the wild by snap trapping according to a standard protocol in Mecklenburg-Western Pomerania (Jeeser) and Thuringia (Gotha) (Fischer et al., 2018). Animals found dead in live traps were also included in the study. All animals were immediately frozen and stored at −20 °C until dissection. Their noses were aseptically removed from the body and frozen again at −20 °C.

2.4. Preparation of bacterial infection stocks

S. aureus strains were grown overnight in Brain Heart Infusion (BHI) medium (Oxoid, Wesel, Germany) at 37 °C and 200 rpm. Next, they were diluted 1:100 in fresh BHI and cultivated at 37 °C with agitation (200 rpm) to the mid-logarithmic growth phase. The bacterial cells were harvested by centrifugation (5 min, 4000 *g*) and resuspended in BHI supplemented with 20% sterile glycerine. The infection stocks were stored at −80 °C until needed. Before infection, bacteria were thawed at room temperature and washed once in sterile phosphate-buffered saline (PBS). Based on the optical density (OD) values at 595 nm, bacteria were diluted in PBS to the desired concentration. To determine the actual infection dose, a small fraction was plated in serial dilutions on Luria-Bertani (LB) agar plates and incubated over night at 37 °C. Colony forming units (CFU) were calculated by standard plate counting.

2.5. Infection models

To induce bacteremia, mice were anesthetized with isoflurane and infected intravenously (*i.v.*) into the tail vein with 3×10^7 CFU of *S. aureus* in 100 µL PBS. For experiments to determine the dose of the vole-derived *S. aureus* strain DIP three different infection doses were used (5×10^7, 5×10^6 and 5×10^5 CFU). To induce pneumonia, mice were anesthetized with isoflurane and inoculated intranasally with 1×10^8 CFU of *S. aureus* in a total volume of 30 µL PBS. For experiments to determine the dose of the *S. aureus* strain DIP three different infection doses were used (2×10^8, 2×10^7 and 2×10^6 CFU). Upon infection, animals were monitored for weight loss and signs of infection twice per day. Based on visual inspection and weight loss, a disease activity index (DAI; Supplementary Table A.1) was determined for each individual mouse. Moribund mice (DAI ≥ 20) were sacrificed according to the German Animal Welfare Act and regarded as 'dead' in survival curves.

2.6. Determination of the bacterial load

The bacterial load was determined by homogenizing lungs (pneumonia) or kidneys (bacteremia) using the homogenizer Precellys 24 (VWR, Darmstadt, Germany) in 1 mL PBS and plating serial dilutions on LB agar. After 21 h of incubation at 37 °C, CFU were calculated by standard plate counting and presented as CFU/organ.

2.7. Detection of cytokines and chemokines

Cytokine and chemokine levels in the homogenized lungs and kidneys were determined by using commercially available kits. The LEGENDplex™ Mouse Th Cytokine Panel (13-plex, Biolegend, San Diego, USA) allows the quantification of interleukin (IL)-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17 A, IL-17 F, IL-21, IL-22, interferon (IFN)-γ and tumor necrosis factor (TNF)-α. The LEGENDplex™ Mouse Proinflammatory Chemokine Panel (13-plex, Biolegend) allows the quantification of MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), Eotaxin (CCL11), TARC (CCL17), MIP-1α (CCL3), MIP-1β (CCL4), MIG (CXCL9), MIP-3α (CCL20), LIX (CXCL5), KC (CXCL1), BLC (CXCL13) and MDC (CCL22). The assay was performed as described in the manual.

2.8. Coagulation assay

The coagulation assay was performed as previously reported (Schulz et al., 2017). Briefly, *S. aureus* strains were grown in Tryptic Soy Broth (TSB) until they reached the early stationary phase. 65 µL of the bacterial cultures (approx. 2.5×10^9 CFU/mL) were mixed in 10 mL glass tubes with 500 µl of murine heparinized plasma (Equitech-Bio, Kerrville, Texas, USA) and incubated at 37 °C without agitation. The coagulation was examined visually at 1, 2, 4 and 18.5 h using a modified coagulation score (Sperber and Tatini, 1975): 0 = no coagulation; 1 = small coagulation flakes; 2 = medium-sized clot; 3 = large clot; 4 = complete coagulation (coagulum sticks to the inverted tube). The scoring was performed in a blinded fashion.

2.9. Whole blood survival assay

The assay was performed according to Kolar et al. with some modifications (Kolar et al., 2011). In detail, *S. aureus* strains were cultured overnight in 10 mL TSB at 37 °C and 200 rpm. The next day, the OD was determined and the whole culture was centrifuged at 4 °C for 10 min at 4000 *g*. After discarding the supernatant, the pellet was washed once in 10 mL PBS and then resuspended in 10 mL PBS. The bacterial suspension was adjusted to 1×10^6 CFU/mL and mixed in a 96-well flat bottom plate with fresh murine heparinized blood at a ratio of 1:4, yielding a total volume of 100 µl. Plates were sealed with a lid and incubated at 37 °C with agitation (200 rpm). 10 µL samples were obtained at 0, 1, 3, 5 and 24 h, sonicated for 3 min to disperse bacterial aggregates and plated in serial dilutions on LB agar to determine the CFU.

2.10. Assessment hemolysin production on sheep blood agar plates

Pore forming toxins such as α-hemolysin (Hla), β-hemolysin (Hlb), phenol soluble modulins and bi-component leukocidins such as γ-hemolysin determine the hemolytic phenotype of *S. aureus* on sheep blood agar (SBA) plates. Whether genomic changes of *S. aureus* strains affect Hla and Hlb production *in vitro* was tested using hemolytic activity on SBA. The CAMP test is commonly used to identify Group B streptococci, which secrete a protein called CAMP factor known to interact with the Hlb of *S. aureus* (Brandt and Spellerberg, 2015). An agar diffusion test on Columbia agar with 5% sheep blood (bioMérieux, Nürtingen. Germany) was used for CAMP testing, with the quality control strain *Streptococcus agalactiae* (ATCC12386), and the Hlb-producing strain ATCC25923 as a positive control. For assay interpretation, note that Hlb enhances lysis by *δ*-hemolysin (Hld) but inhibits lysis by Hla (Traber et al., 2008). After 18 h at 37 °C incubation followed by 4 h at 4 °C, isolates were inspected for CAMP hemolysis.

2.11. Whole genome sequencing of S. aureusstrains

Whole genome sequencing (WGS) of *S. aureus* strains were whole-genome sequenced (WGS) using Illumina MiSeq 300 bp paired-end sequencing with an obtained coverage > 90 × . After quality control using the NGS tool kit13 (70% of bases with a phred score > 20), high-quality filtered reads were used for de novo assembly into contiguous sequences (contigs) and subsequently into scaffolds using SPAdes v3.9. Assembled draft genomes of the isolates were annotated using Prodigal (PROkaryotic DYnamic programming Gene-finding ALgorithm) (Hyatt et al., 2010). Geneious 10.0.5 (Biomatters Ltd., Australia) was used for in-depth comparison of selected genomic loci of strains DIP, muCC88d, JSNZ, and muCC8c with strain Newman (accession no. AP009351).

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accessions QWKQ00000000 (JSNZ), QWKR00000000 (muC88d), QWKS00000000 (DIP), and QWKT00000000 (muCC8c).

2.12. Ethics statement

Wild mice and voles were trapped according to relevant legislation and by permission of the responsible State authorities (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern 7221.3-030/09; Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz 22-2684-04-15-107/09).

Animal infection experiments were approved by the local government of Lower Franconia, Germany (No.: 55.2 2532-2-188). The experiments were performed in accordance with the German Animal Welfare Act (Deutsches Tierschutzgesetz), the EU Directive 2010/63/EU for animal experiments and the Federation of Laboratory Animal Science Associations (FELASA). All animal experiments comply with the ARRIVE guidelines.

2.13. Statistics

Data analysis was performed using the GraphPadPrism6 package (GraphPad Software, Inc., La Jolla, California, USA). Group-wise comparisons were conducted using the Mann-Whitney *U* test, since data were not normally distributed.

3. Results

3.1. Representative mouse-adapted S. aureus strains were selected based on their genotype and phenotypical features possibly involved in host adaptation

To test whether mouse-adapted *S. aureus* strains are more virulent in the mouse model than human-adapted strains, we selected representative strains from a collection of 254 *S. aureus* isolates from laboratory mice, wild rodents and shrews (Mrochen et al., 2017a, b). As previously reported, the *S. aureus* lineage CC88 is predominant in laboratory mice, while CC49, CC88, and CC8 colonize wild mice and other wild small mammals (voles and shrews) (Mrochen et al., 2017b; Schulz et al., 2017). All strains were previously characterized by *spa* typing and multiplex PCRs for bacteriophage genes, as well as virulence and immune evasion genes (superantigens, exfoliative toxins, PVL, IEC genes). As previously reported (Mrochen et al., 2017a, b), mouse-adapted *S. aureus* strains from both laboratory mice and wild small mammals showed features of host and/or niche adaptation. For example, they frequently lack superantigen gene-encoding mobile genetic elements and the IEC-encoding Sa3int phages, both of which are common in human *S. aureus* isolates.

Representative strains from each lineage were selected in a stepwise approach based on their genotype, pro-coagulatory activity, and ability to survive and grow in whole murine blood. First, five representative strains per CC were selected based on having a common *spa* type, as well as a pattern of virulence genes and bacteriophages typical for the respective CC (Table 1). Second, we tested whether these strains differ in their pro-coagulatory activity on murine plasma, as it is

Table 1
Overview of the genotype, virulence genes, and phage pattern of the selected *S. aureus* strains.

Strain	Host	Origin	*spa* type	MLST	non-*egc* SAgs	*egc* SAgs	*nuc*	*gyr*	*agr*	*eta, etd*	*pvl*	*mecA*	Phage integrase	*sak*	*chp*	*scn*	Reference
muCC8a	field vole	Gotha (TH, Germany)	t211	CC8	–	–	+	+	1	–	–	–	–	–	–	–	This work
muCC8b	field vole	Gotha (TH, Germany)	t211	CC8	–	–	+	+	1	–	–	–	–	–	–	–	This work
muCC8c	common vole	Gotha (TH, Germany)	t211	CC8	–	–	+	+	1	–	–	–	–	–	–	–	This work
muCC8d	yellow-necked mouse	Gotha (TH, Germany)	t211	CC8	–	–	+	+	1	–	–	–	–	–	–	–	This work
muCC8e	bank vole	Jeeser (MV, Germany)	t211	CC8	–	–	+	+	1	–	–	–	–	–	–	–	This work
muCC49a	yellow-necked mouse	Jeeser (MV, Germany)	t208	CC49	–	–	+	+	2	–	–	–	5	–	–	–	(Mrochen et al., 2017b)
muCC49b	bank vole	Jeeser (MV, Germany)	t208	CC49	–	–	+	+	2	–	–	–	5	–	–	–	(Mrochen et al., 2017b)
muCC49c	bank vole	Jeeser (MV, Germany)	t208	CC49	–	–	+	+	2	–	–	–	5	–	–	–	(Mrochen et al., 2017b)
muCC49d	yellow-necked mouse	Heimerdingen (BW, Germany)	t4189	CC49	–	–	+	+	2	–	–	–	5	–	–	–	(Mrochen et al., 2017b)
muCC49e/DIP	bank vole	Heimerdingen (BW, Germany)	t4189	CC49	–	–	+	+	2	–	–	–	5	–	–	–	(Mrochen et al., 2017b)
muCC88a	C57BL/6NCrl	Kingston (USA)	t186	CC88	–	–	+	+	3	–	–	–	1	–	–	–	(Schulz et al., 2017)
muCC88b	C57BL/6NCrl	Kingston (USA)	t12341	CC88	–	–	+	+	3	–	–	–	1	–	–	–	(Schulz et al., 2017)
muCC88c	C57BL/6	Vendor (USA)	t186	CC88	–	–	+	+	3	–	–	–	–	–	–	–	(Schulz et al., 2017)
muCC88d	field vole	Gotha (TH, Germany)	t2311	CC88	–	–	+	+	3	–	–	–	1,2,3	+	+	+	(Schulz et al., 2017)
JSNZ	C57BL/6J	Auckland (New Zealand)	t729	CC88	–	–	+	+	3	–	–	–	1	–	–	–	(Holtfreter et al., 2013)
Newman	human	Southampton (UK)	t008	CC8	*a*	–	+	+	1	–	–	–	3,5,6,7	+	+	+	(Duthie and Lorenz, 1952)

Abbreviations: TH, Thuringia; MV, Mecklenburg-Western Pomerania; BW, Baden-Wuerttemberg; SA, gsuperantigen; *egc*, enterotoxin gene cluster; *nuc*, nuclease; *gyr*, gyrase; *agr*, accessory gene regulator; *eta/ etd*, exfoliative toxin a/d; *pvl*, Panton-Valentine leukocidin; *mec*, Amethicillin resistance; *sea/sep*, staphylococcal enterotoxin a/p; *sak*, staphylokinase; *chp*, chemotaxis inhibitory protein; *scn*, staphylococcal complement inhibitor; *MLST*, multi-locus sequence typing. in bold:*in vivo*-used *S. aureus* strains.

known that host-adapted strains can coagulate their host's plasma (Fig. 1A) (Viana et al., 2010). All strains were able to coagulate the murine plasma, but differed greatly in their kinetics. *S. aureus* Newman (CC8) induced surprisingly strong coagulation within two hours, whereas it was delayed for the mouse-adapted CC8 strains. All CC49 isolates showed a strong pro-coagulatory activity. In contrast, the mouse- and vole-derived CC88 isolates, which are genetically more diverse than the CC8 and CC49 isolates, differed drastically in their ability to coagulate murine plasma. While JSNZ and muCC88d caused a strong coagulation, another strain, muCC88a, did not induce any coagulation at all. For each lineage, we selected three strains with strong pro-coagulatory capacities for the subsequent *in vitro* assay.

Third, we tested whether the mouse-adapted strains can survive and replicate in fresh murine blood, which mimics *in vivo* conditions (Fig. 1B). All strains were able to grow in murine blood after an initial drop in the CFU by one log. The mouse-adapted CC8 strains recovered better than Newman. All CC49 isolates showed a similar growth behavior and recovered quickly from the initial drop in CFU. In contrast, the CC88 strains again differed in their survival in murine whole blood.

Finally, four mouse-adapted strains were selected and compared to the human-adapted *S. aureus* Newman in murine bacteremia and pneumonia models: muCC8c, muCC49e (named *S. aureus* DIP, hereafter), muCC88d and JSNZ (Table 1). MuCC8c, DIP, and muCC88d were all isolated from wild voles (see Table 1), while JSNZ originated from laboratory mice.

3.2. Mouse-adapted S. aureus strains differ strongly in their virulence in murine bacteremia and pneumonia models

To compare the four different mouse-adapted *S. aureus* strains with Newman, BALB/c mice were infected intranasally with 1×10^8 CFU (range: 8×10^7–1.3×10^8 CFU; pneumonia) or *i.v.* into the tail vein with 3×10^7 CFU (range 2–3×10^7 CFU; bacteremia). The virulence of the tested mouse-adapted strains differed strongly, as reflected by the survival, disease activity index (DAI), and bacterial load (Fig. 2).

S. aureus muCC88d was least virulent in both infection experiments. All animals survived; furthermore, the bacterial load in the kidneys (bacteremia) and the lungs (pneumonia) was very low compared to the other strains. Nevertheless, animals developed an infection, as reflected by the DAI (Fig. 2B and E). *S. aureus* muCC8c also displayed little virulence in both models with 70% survival in the bacteremia and 80% in the pneumonia model. This isolate showed an unexpectedly high bacterial load in the lungs (median: 2×10^5 CFU), which can hardly be explained by the slightly elevated infection dose (1.3×10^8 CFU). The virulence of *S. aureus* JSNZ was comparable to that of Newman. In the bacteremia model, 30% and 10% of the animals survived the infection with JSNZ and Newman, respectively (Fig. 2A). Bacterial loads in the kidneys and the DAI were comparable. In the pneumonia model, all animals survived the bacterial challenge, but displayed signs of infection with very similar bacterial loads in the lungs (median: 3×10^3 CFU) as well as a comparable DAI (Fig. 2D-F).

Interestingly, the *S. aureus* strain DIP was highly virulent in both infection models. It induced 100% mortality within 42 h in the bacteremia model and within 38 h in the pneumonia model. Compared to the other tested strains, DIP induced the highest DAI. Since all animals died or reached the humane end point, it was not possible to determine the bacterial load in the organs at the end of the experiment. To conclude, DIP appeared to be an interesting candidate for studying *S. aureus* pathogenicity and host immune response in murine colonization and infection models. However, the high virulence of DIP made it necessary to first determine the optimal inoculation dose.

3.3. The vole-derived CC49 isolate DIP is highly virulent in murine bacteremia and pneumonia models

To determine the optimal inoculation dose for DIP, mice were

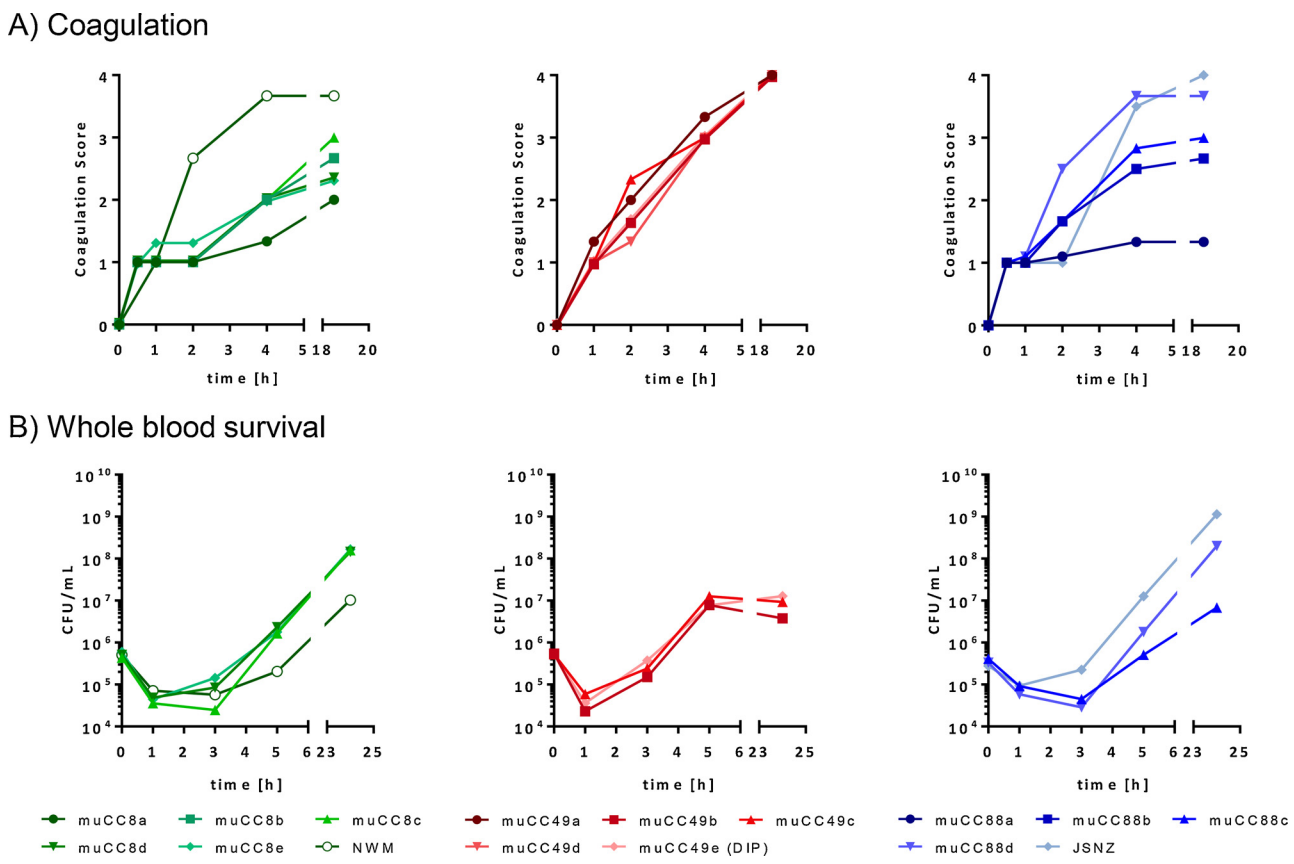

Fig. 1. Selection of *S. aureus* strains for *in vivo* studies. Mouse- and vole-derived *S. aureus* isolates from three different lineages (CC8, CC49, CC88) as well as the human-adapted strain Newman (CC8) were inoculated in 500 µL murine plasma and their pro-coagulatory ability was compared (A). Coagulation was visually assessed after 1, 2, 4 and 18.5 h. The mean values of three technical replicates are shown. Afterwards, three selected *S. aureus* strains from each tested lineage were compared in their ability to grow in murine blood (B). 1×10^6 CFU/mL of bacterial culture were mixed with fresh murine heparinized blood in a ratio of 1:4. CFU/mL blood were determined by plating serial dilutions on LB agar at 0, 1, 3, 5, and 24 h. The mean values of three technical replicates are shown. Abbreviations: NWM, Newman.

infected with three different infection doses of DIP and a standard dose of *S. aureus* Newman. As before, all animals infected with the high dose of DIP (5×10^7 CFU) died from bacteremia (Fig. 3A). A reduction of the infection dose to 1/10 (5×10^6 CFU) led to 50% survival in the bacteremia experiment which was comparable to Newman (30% survival). Another reduction to 5×10^5 CFU further improved the survival to 70%. Mice infected with DIP at 1/10 of the standard dose had a similar bacterial load in the kidneys and a DAI similar to that of Newman-infected mice (Fig. 3B and C). At 1/100 of the standard dose, some mice were able to clear the infection within 78 h. In pneumonia, the standard dose of DIP (2×10^8 CFU) was again highly lethal (Fig. 3D). A reduction of the infection dose to 1/10 led to 100% survival. Nevertheless, the DAI and bacterial load in the lungs were comparable to those of Newman (Fig. 3E and F). At 1/100 of the standard dose, mice developed almost no clinical symptoms despite the presence of bacteria in the lung.

To investigate whether DIP and Newman trigger a similar host immune response, we compared cytokine and chemokine levels in kidneys (bacteremia) and lungs (pneumonia). In the bacteremia model, DIP (at the 1/10 dose) and Newman induced a similar cytokine response in the kidneys at 78 h post infection (Fig. 4A). Only IL-6 was significantly reduced in mice infected with DIP. Furthermore, the cytokine levels of IFN-γ, IL-13, IL-17 A, IL-22 and IL-10 were slightly albeit not significantly higher in DIP-infected animals. The magnitude of the chemokine response was somewhat lower in DIP-infected mice than in Newman-infected animals (Fig. 4B). In the pneumonia model, cytokines and chemokines were generally lower for DIP as compared to Newman (Fig. 5). This correlates well with the slightly lower DAI in the DIP-infected animals.

To conclude, in the bacteremia mo del at 1/10 of the standard infection dose, DIP induced a disease severity, bacterial load and host immune response similar to the human-adapted strain Newman. In the pneumonia model, disease severity as well as cytokine and chemokine levels were slightly lower at 1/10 of the standard infection dose compared to Newman.

3.4. Genotyping provides some clues on the molecular basis for the enhanced virulence of DIP

To elucidate the genetic basis for the enhanced virulence of DIP, we performed *S. aureus* WGS on DIP as well as the other *in-vivo*-tested strains (JSNZ, Newman, muCC8c, and muCC88d). Comparative analysis revealed that only strain DIP harbored the genes for a phage-encoded pore-forming toxin, *luk*F-PV(P83)/*luk*M, previously described in *S. aureus* isolates from ruminants as well as wild rodents and shrews (Table 2) (Barrio et al., 2006; Fechter et al., 2014; Morfeldt et al., 1995; Mrochen et al., 2017b; Novick et al., 1993; Schlotter et al., 2012). The *luk*F-PV(P83)/*luk*M genes were located on an temperate bacteriophage of 44,090 bp length harboring integrase type 5, which is in accordance with our PCR data (Table 1).

Moreover, only Dip yielded a single nucleotide insertion in the commonly 514-bp RNAIII encoding sequence (Table 2), the main effector molecule of the *agr* quorum sensing system. Beside other functions, RNAIII controls i) the switch between early expression of surface proteins and late expression of several exotoxins and ii) acts as a mRNA that encodes the 26-aa Hld (Novick et al., 1993). The non-coding parts

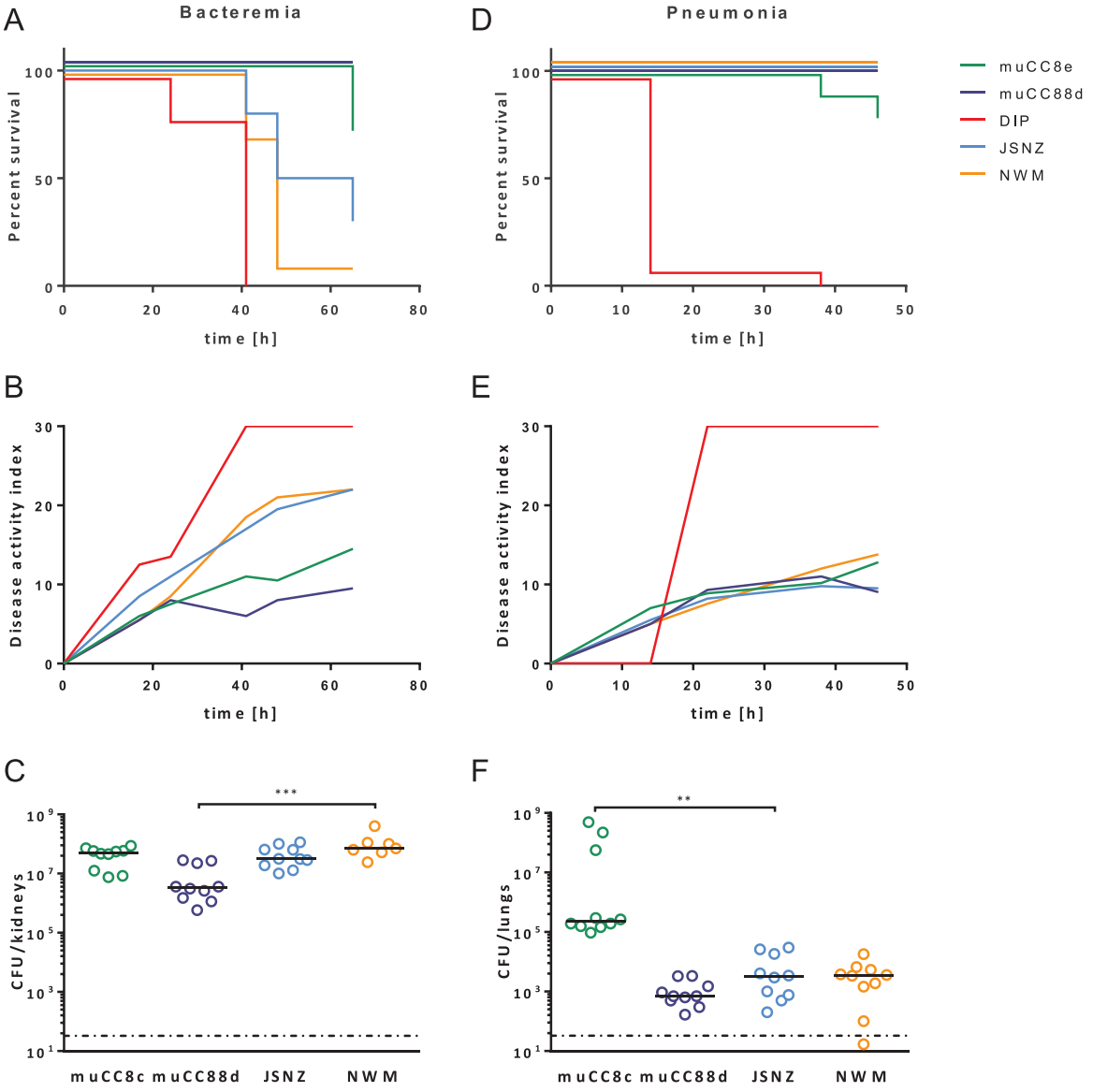

Fig. 2. Mouse-adapted *S. aureus* strains differ in their virulence in murine bacteremia and pneumonia models. BALB/c mice (n = 10 per group) were infected with 1×10^8 CFU (pneumonia) or 5×10^7 CFU (bacteremia) of four selected murine *S. aureus* isolates (muCC8c, muCC88d, DIP, and JSNZ) or the human strain Newman. The exact infection dose was determined by plating as 8×10^7 CFU – 1.3×10^8 CFU for pneumonia and 2–3 $\times 10^8$ CFU for bacteremia. Murine survival was monitored for 48 h (pneumonia; A) or 72 h (bacteremia; D). The health status of infected mice was scored at regular intervals (B, E). Mice were sacrificed when the DAI reached 20. Bacterial loads were determined from homogenized kidneys (bacteremia) and lungs (pneumonia) (C, F). Each point represents a mouse. The line represents the median CFU value. Statistical analysis: Mann-Whitney *U* test. **, $p \leq 0.01$, ***, $p \leq 0.001$. Abbreviations: NWM, Newman.

of RNAIII are the regulatory domains: The 5′ sequence binds to the leader region of *hla* mRNA encoding α-hemolysin to facilitate ribosome recruitment (Morfeldt et al., 1995) while its large 3′ region acts mostly as a repressor domain (Fechter et al., 2014). While the nucleotide insertion at position 28 (+T) in hairpin 2 of 18 in RNAIII alters the reading frame subsequently causing a premature stop codon in the CDS of *hld* gene, the regulatory activity of RNAIII might possibly be still intact, since *hla* expression was not abolished in DIP (Fig A.1). However, it seems likely that this alteration presumably results in loss of function of the Hld toxin (Fig A.2).

We also investigated genes, which encode pro-coagulatory factors, because the murine *S. aureus* strains differed in their coagulatory capacity (Fig. 1). Analysis of the WGS dataset revealed allelic variants of coagulase and chromosomally encoded von Willebrandt factor binding protein (*vWbp*) genes in DIP, with 71.5% and 66% amino acid identity in the corresponding proteins to Newman, respectively. Moreover, we confirmed the presence of a vWbp variant in JSNZ with 70% sequence identity (Sun et al., 2018). Finally, SasD, a LPXAG-anchored surface protein with unknown function, was absent from the Dip genome.

4. Discussion

Animal infection models should mimic the human clinical situation as closely as possible. The discovery that laboratory mice as well as wild rodents and shrews are natural hosts of *S. aureus* has enhanced the status of mice as *S. aureus* colonization and infection models. Here, we report that one of the mouse-adapted CC49 *S. aureus* strains, named DIP, was highly virulent in laboratory mice, allowing a reduction of the infection dose by a factor of 10 compared to the commonly used highly virulent human strain Newman. Therefore, *S. aureus* DIP could be a promising tool to study *S. aureus*-host interactions in the mouse model.

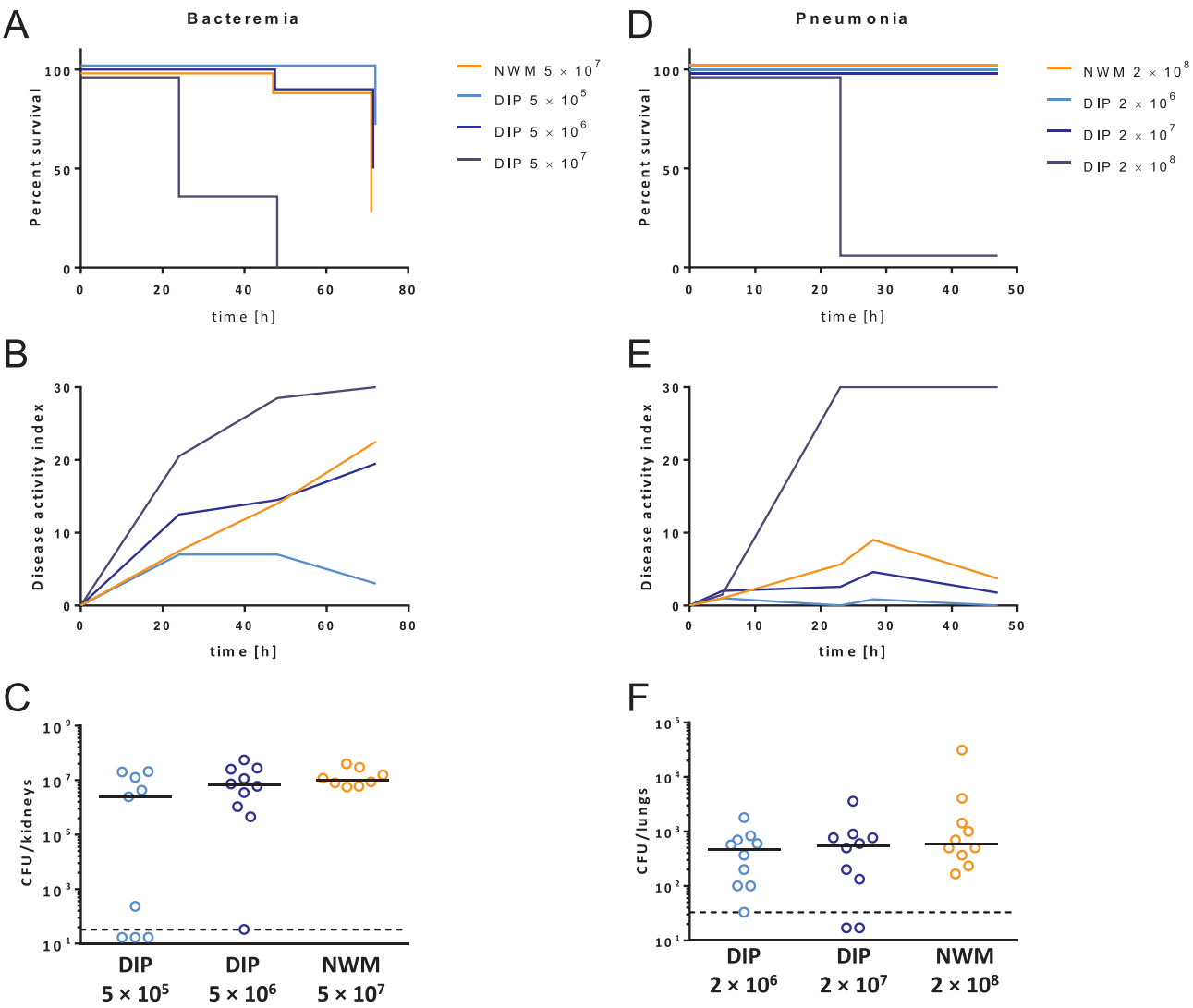

Fig. 3. Dose finding for *S. aureus* strain DIP in a murine bacteremia and pneumonia model. To induce bacteremia, female BALB/c mice (n = 10 per group) were infected *i.v.* into the tail vein with either *S. aureus* strain Newman (5×10^7 CFU) or with three different infection doses of the vole-derived *S. aureus* isolate DIP (5×10^7 CFU, 5×10^6 CFU, 5×10^5 CFU). To induce pneumonia, female BALB/c mice (n = 10 per group) were infected intranasally with either Newman (2×10^8 CFU) or three different infection doses of *S. aureus* DIP (2×10^8 CFU, 2×10^7 CFU, 2×10^6 CFU). Murine survival was monitored for 78 h (bacteremia; A) or 48 h (pneumonia, D). The health status of infected mice was scored at regular intervals (B, E). Mice were sacrificed when the DAI reached 20. Bacterial loads were determined from homogenized kidneys (bacteremia) and lungs (pneumonia) (C, F). Each point represents a mouse. The line represents the median CFU value. Statistical analysis: Mann-Whitney *U* test. Abbreviations: NWM, Newman.

4.1. Pro-coagulatory activity does not predict in vivo behavior of S. aureus isolates

In order to pick the best candidates out of 254 mouse-, vole- and shrew-derived *S. aureus* isolates for our mouse experiments, we developed a step-wise selection process using several *in vitro* assays. Selection criteria included (1) having a typical genotype based on *spa* type, virulence genes and phages, (2) inducing a strong coagulation of murine plasma, and (3) being able to survive and replicate in whole murine blood.

The coagulation assay was chosen because coagulation is an essential virulence trait of *S. aureus*, and some pro- or anti-coagulatory factors, *i.e.* vWbp and staphylokinase, are host-specific (Schulz et al., 2017; Sun et al., 2018; Viana et al., 2010). The pro-coagulatory activity of the tested *S. aureus* isolates did not predict their *in vivo* behavior. We detected similar coagulation rates for Newman and DIP, despite a ten-fold difference in their virulence. In addition, the muCC88d induced strong coagulation but displayed the lowest virulence in both infection models. While the tested five murine CC8 and CC49 isolates showed a clade-specific phenotype in the coagulation assay, the murine CC88 isolates differed drastically in their outcome. The first might be due to lineage-specific variants of pro-coagulatory genes, such as vWbp (Sun et al., 2018); the latter might be explained by the acquisition of additional pro- or anti-coagulatory genes or differential regulation on protein level. In line with this, WGS revealed allelic variants of coagulase and *vWbp* in DIP, and confirmed the presence of a *vWbp* variant in JSNZ (Sun et al., 2018). However, further vWbp variants encoded by mobile genetic elements such as pathogenicity islands known to affect coagulation abilities (Viana et al., 2010) were not detected. Further studies are required to test functionality and host specificity ranges of these DIP-specific variants. In addition, Sun et al recently reported that the pro-coagulatory activity of JSNZ and Newman also depends on protein expression levels. Newman secreted large amounts of coagulase and rapidly agglutinated human and mouse plasma. In contrast, JSNZ secreted less coagulase and encoded an allelic variant of *vWbp* enabling them to agglutinate mouse plasma more readily than human plasma (Sun et al., 2018).

The bacterial survival in fresh murine whole blood was chosen as

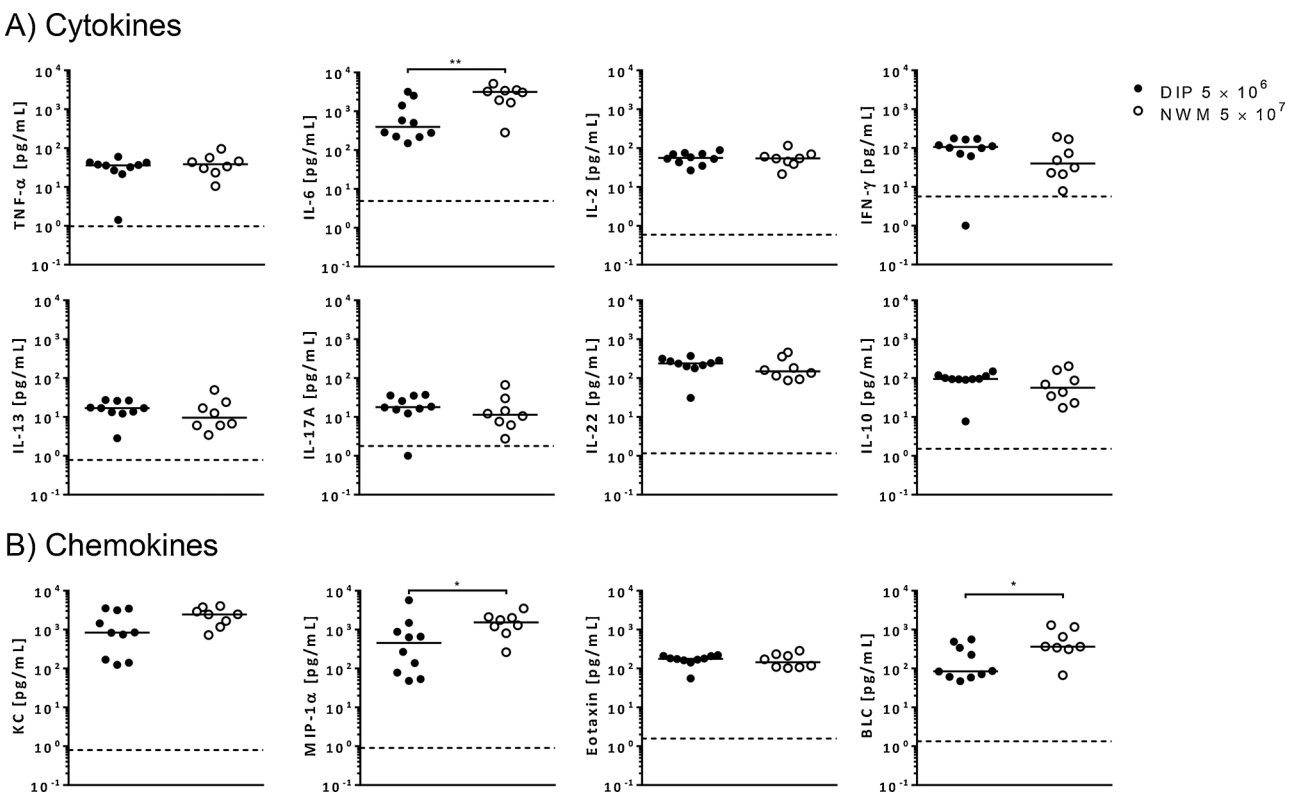

Fig. 4. DIP and Newman trigger comparable cytokine responses in the kidneys during bacteraemia. BALB/c mice (n = 10 per group) were infected *i.v.* into the tail vein with the vole-derived *S. aureus* isolate DIP at 1/10 of the standard infection dose (5×10^6 CFU) or the human *S. aureus* strain Newman (5×10^7 CFU). Cytokine and chemokine levels in the kidneys were determined after 78 h. Each point represents a mouse. DIP induced a similar cytokine response and a slightly lower chemokine response compared to Newman, despite a 10-fold lower infection dose. Statistical analysis: Mann-Whitney *U* test. Abbreviations: NWM, Newman.

read-out system, because blood closely resembles the *in vivo* situation. Bacteria have to evade the attack of the host immune system, *e.g.*, opsonization by antibodies and complement, phagocytosis and killing by neutrophils, and destruction by antimicrobial peptides. In line with the coagulation assay, we observed lineage-specific rather than individual differences in the ability of bacterial isolates to survive and replicate in murine blood. Notably, the mouse- and vole-derived CC49 isolates and JSNZ recovered faster from an initial drop in the CFU than did the CC8 and CC88 isolates. The strong growth of CC49 strain DIP and JSNZ in murine blood correlates well with their enhanced virulence in the bacteremia model. In contrast, Newman showed high virulence in the bacteremia model, but displayed the lowest growth rate. This emphasizes, that the pathogenesis of *S. aureus* infections is far more complex than a simple blood survival assay. Nevertheless, the whole blood survival assay seems to be a relatively good marker for the *in vivo* virulence of a given *S. aureus* isolate.

4.2. Wild rodents represent the predominant host of the S. aureus lineage CC49

Epidemiological data suggest that the lineage CC49 has a rather restricted host range (Mrochen et al., 2017b). With a prevalence of 35%, CC49 is the most prevalent lineage in wild rodents and shrews. Moreover, CC49 represents the third-leading *S. aureus* lineage in pigs (Oppliger et al., 2012; Overesch et al., 2011). A transmission between pigs and humans seems possible, as CC49 has been identified in pig farmers more frequently than in the general population, where they are rare (Deplano et al., 2014; Holtfreter et al., 2016; Oppliger et al., 2012; Overesch et al., 2011; Simpson et al., 2013). Apart from wild mice, voles, shrews, pigs and occasionally humans, CC49 isolates were sporadically detected in squirrels (Simpson et al., 2013), rats (Paterson et al., 2012), horses (Haenni et al., 2015), wallabies (Chen et al., 2016), and wildcats (Monecke et al., 2016). The sparsity of CC49 from humans and other animals, suggests that this lineage has specialized on mice, voles, and shrews as its prime host species. Hence, the observed high virulence of DIP in mice might be a general trait of the CC49 lineage, rather than a strain-specific feature. Further animal experiments comparing animal- and human-derived CC49 isolates could clarify this aspect.

4.3. S. aureus DIP harbours a species-specific pore-forming toxin that might enhance its virulence in mice

A genomic comparison of DIP with the other *S. aureus* isolates that were tested *in vivo* (JSNZ, muCC8c, muCC88d, and Newman) revealed the presence of genes encoding a pore-forming toxin, LukF-PV(P83)/LukM, that might contribute to the enhanced virulence in mice. The *lukF-PV(P83)/lukM* genes have been detected in *S. aureus* isolates from cattle (CC151, CC479, CC133) and recently also wild rodents and shrews (CC49), but not in human isolates (Bar-Gal et al., 2015; Eiff et al., 2004; Mrochen et al., 2017b; Schlotter et al., 2012), suggesting that the toxin acts as a virulence factor promoting infection in particular host species. Indeed, LukF-PV(P83)/LukM binds and kills bovine and murine, but not human neutrophils (Fromageau et al., 2010). More recently, the molecular basis for this narrow host range has been resolved: The cellular receptor for LukMF´, CCR1, is present on bovine, but absent on human neutrophils (Vrieling et al., 2015). In mice, CCR1 is expressed on neutrophils, monocytes, macrophages, and T cells and LukMF´is able to bind to these cell types in the low nanomolar range (Fromageau et al., 2010; Furuichi et al., 2008). To conclude, the production of this pore-forming toxin with high activity on murine immune cells may contribute to the high virulence of *S. aureus* DIP in the murine pneumonia and bacteremia model.

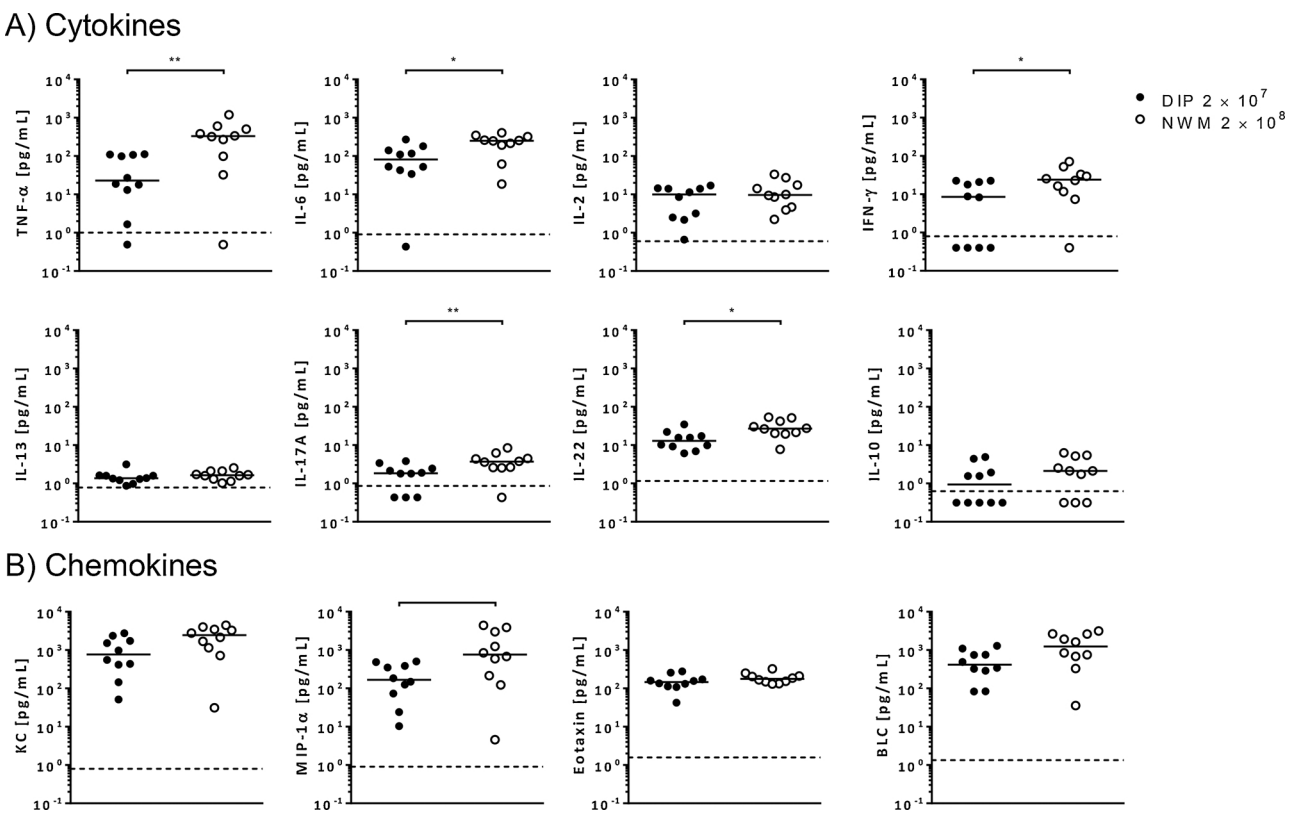

Fig. 5. DIP induces a lower cytokine and chemokine response in the lungs during pneumonia than Newman. BALB/c mice (n = 10 per group) were infected *i.v.* into the tail vein with the vole-derived *S. aureus* isolate DIP at 1/10 of the standard infection dose (2×10^7 CFU) or the human *S. aureus* strain Newman (2×10^8 CFU). Cytokine (A) and chemokine (B) levels in the lungs were determined after 48 h. Each point represents a mouse. At 1/10 of the standard infection dose DIP induced a weaker cytokine and chemokine response than did Newman. Statistical analysis: Mann-Whitney *U* test. Abbreviations: NWM, Newman.

4.4. Some mouse-adapted S. aureus strains are a suitable tool to optimize murine colonization and infection models

Apart from humans, *S. aureus* colonizes a broad range of domestic and wild animals, including mice (Aires-de-Sousa, 2017; Mrochen et al., 2017a, b; Sung et al., 2008). Whole genome studies illustrate that staphylococci adapt to their host by the loss and/or acquisition of mobile genetic elements, the generation of host-specific allelic variants, and the inactivation of superfluous genes (Guinane et al., 2010; Herron-Olson et al., 2007). Since the immune system exerts a strong selective pressure on colonizing and invading pathogens, it is evident that staphylococcal immune evasion factors can be host-specific. The most prominent example for host adaptation are *hlb*-integrating Sa3int phages, which encode the human-specific IEC and are rarely found in animal isolates (Sung et al., 2008; van Wamel et al., 2006). Moreover, vWbp allelic variants have been discovered in *S. aureus* isolates from ruminants and horses, that host-specifically induce coagulation (Viana et al., 2010).

We propose that the use of mouse-adapted *S. aureus* strains in their natural host – the mouse – will provide a more physiological model for studying *S. aureus* host interaction and testing novel prophylactic or therapeutic interventions. Indeed, the murine CC88 strains JSNZ and WU1, as well as the murine CC15 isolate SaF_1 persistently colonize the nasopharynx and gastrointestinal tract of laboratory mice, whereas human-adapted strains are eliminated after intranasal inoculation within a few days (Flaxman et al., 2017; Holtfreter et al., 2013; Sun et al., 2018). Thus, by using certain mouse-adapted strains, researchers have the opportunity for the first time to study bacterial and host factors involved in persistent colonization, as well as novel decolonization drugs and vaccines in an animal model.

In this study, we demonstrate that the bank vole-derived CC49 isolate DIP is highly virulent in murine bacteremia and pneumonia models. In the bacteremia model, the mortality, bacterial load, DAI, and the induced cytokine and chemokine response were comparable between mice infected with Newman and mice infected with DIP at 1/10 of the standard infection dose. Thus, by using DIP it is possible to reduce the infection dose in the bacteremia model in BALB/c mice by one log. In the pneumonia model, the effect was less pronounced: mice inoculated with DIP at 1/10 of the standard infection dose showed the same bacterial load in the lung, but a slightly reduced severity score as well as cytokine and chemokine levels. There was no shift in the balance of the cytokine/chemokine response, *e.g.* from type 1 and 3 to type 2, in either infection model using the mouse-derived *S. aureus* strains. These preliminary data suggest that the pathophysiology of *S. aureus* DIP resembles that of Newman and hence, that, DIP could be a valuable tool for studying *S. aureus* host interactions in murine *S. aureus* infection models.

Apart from DIP, all other tested *S. aureus* strains were comparable to or less virulent than Newman in our pneumonia and bacteremia model. This might suggest that the other strains are not well-adapted to the murine host. However, the absence of the human-specific immune evasion cluster in all strains (except for muCC88d) argues against it. In-depth phylogenetic analyses would be required to determine the degree of host adaptation and to estimate the time point of the host jump. Alternatively, invasive animal models might not be the best read-out for studying mouse-adaptation considering that *S. aureus* is likely adapted for colonization and transmission rather than invasion and life-threatening conditions (Massey et al., 2006; van Wamel, 2017).

4.5. Mice are a relevant model for S. aureus colonization and infection studies

Mice are the experimental workhorse in infection research because

Table 2
Conservation of protein products of select open reading frames in the genomes of *S. aureus* DIP, JSNZ, muCC88d, and muCC8c as compared to Newman.

Newman (Reference genome: AP009351)				DIP			JSNZ			muCC88d			muCC8c		
Group	Protein	Symbol	Gene	Gene		AA	Gene		AA	Gene		AA	Gene		AA
				AV[a]	C (%)	I (%)	AV	C (%)	I (%)	AV	C (%)	I (%)	AV	C (%)	I (%)
Adhesins	Clumping factor A	ClfA	*clf*A	5	100	95.5	2	88.9	89.3	7	90.3	100	4	100	99.0
	Clumping factor B	ClfB	*clf*B	5	91.3	89.0	2	90.7	83.4	2	83.4	90.7	4	100	95.9
	Fibronectin binding protein A	FnBPA	*fnb*A	5	100	80.9	2	100	82.2	2	82.2	100	4	100	99.2
	Fibronectin binding protein B	FnBPB	*fnb*B	5	100	85.1	2	80.9	85.5	2	85.5	80.9	4	100	100
	Iron-regulated surface determinant protein A	IsdA	*isd*A	4	100	96.3	2	100	99.4	2	99.4	100	1	100	100
	Iron-regulated surface determinant protein B	IsdB	*isd*B	4	100	96.3	2	100	98.6	2	98.6	100	1	100	100
	Serine-aspartate repeat-containing protein C	SdrC	*sdr*C	5	93.1	87.2	2	100	95.6	2	95.6	100	4	89.0	95.1
	Serine-aspartate repeat-containing protein D	SdrD	*sdr*D	5	77.7	82.7	2	89.7	89.3	2	89.3	89.7	4	100	97.0
	Serine-aspartate repeat-containing protein E	SdrE	*sdr*E	4	100	97.3	2	94.5	92.7	6	97.2	94.5	NA	NA	NA
	Cell-wall-anchored protein SasA	SasA	*sas*A	4	100	92.1	2	82.3	96.5	2	96.5	82.3	1	100	100
	Cell-wall-anchored protein SasD	SasD	*sas*D	NA	NA	NA	2	100	99.6	2	99.6	100	4	100	86.4
	Cell-wall-anchored protein SasF	SasF	*sas*F	4	100	96.4	2	100	98.0	2	98.0	100	3	100	99.8
	Iron-regulated surface determinant protein H	IsdH	*isd*H	5	100	97.0	2	100	99.6	2	99.6	100	4	100	99.9
	Cell-wall-anchored protein SasG[b]	SasG[b]	*sas*G[b]	5	100	95.9	2	100	75.8	6	75.8	100	4	100	96.8
	Cell-wall-anchored protein SasK[c]	SasK[c]	*sas*K[c]	3	100	98.0	2	100	92.4	2	92.4	100	NA	NA	NA
	Extracellular adherence protein	Eap	*map*	4	99.8	93.0	5	95.6	82.8	2	88.5	100	1	100	100
Coagula-tion	Coagulase	Coa	*coa*	4	94.2	71.5	2	90.6	95.8	2	95.8	90.6	1	100	100
	von Willebrand factor-binding protein	vWbp	*vwb*	3	55.0	66.0	3	100	70.0	2	58.8	99.9	2	92.0	99.0
	Extracellular fibrinogen-binding protein	Efb	*efb*	4	100	98.2	2	100	98.8	2	98.8	100	1	100	100
Leukocidins	Panton-Valentine Leukocidin F-PV (P83)[d]	LukF-PV[d]	*luk*F-PV[d]	2	100	100	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Leukocidin-M subunit[d]	LukM[d]	*luk*M[d]	2	100	99.7	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Leukocidin-F subunit	LukF	*luk*F	4	100	96.4	2	100	97.9	2	97.9	100	1	100	100
	Leukocidin-S subunit	LukS	*luk*S	4	100	99.4	2	100	97.7	2	97.7	100	1	100	100
	Leukotoxin LukD	LukD	*luk*D	5	99.9	98.5	2	99.9	99.4	2	99.4	99.9	4	100	100
	Leukotoxin LukE	LukE	*luk*E	5	100	99.7	2	100	100	2	100	100	4	98.0	100
Hemolysins	Alpha hemolysin	Hla	*hly*	4	100	99.7	FS[f]	FS[f]	FS[f]	5	99.1	100	1	100	100
	Phospholipase C/beta hemolysin[e]	Hlb[e]	*hlb*[e]	5	100	100	2	100	99.7	PI	PI	PI	4	100	100
	Gamma hemolysin component A	HlgA	*hlg*A	4	100	100	2	100	100	2	100	100	1	100	100
	Gamma hemolysin component B	HlgB	*hlg*B	4	100	99.7	2	100	100	2	100	100	1	100	100
	Gamma hemolysin component C	HlgC	*hlg*C	4	100	97.8	2	100	99.7	2	99.7	100	1	100	100
	Delta hemolysin[b]	Hld[b]	*hld*[b]	FS[f]	FS[f]	FS[f]	1	100	100	1	100	100	1	100	100
Proteases	Serine protease SplA	SplA	*spl*A	5	100	95.8	2	100	95.4	2	95.4	100	4	100	98.7
	Serine protease SplB	SplB	*spl*B	3	100	97.5	2	100	100	2	100	100	1	100	100
	Serine protease SplC	SplC	*spl*C	5	100	94.6	2	100	87.4	2	87.4	100	4	100	100
	Serine protease SplD	SplD	*spl*D	5	100	93.7	2	100	97.9	2	97.9	100	4	100	100
	Serine protease SplE	SplE	*spl*E	3	100	97.5	NA	NA	NA	NA	NA	NA	1	100	100
	Serine protease SplF	SplF	*spl*F	4	100	95.4	2	100	100	2	100	100	1	100	100
	Aureolysin	Aur	*aur*	5	100	99.2	2	100	99.6	6	99.8	100	4	100	99.8
	Staphylococcus serine protease A	SspA	*ssp*A	4	95.9	95.1	2	100	98.0	5	97.0	90.6	1	100	100
	Staphylococcus serine protease B	SspB	*ssp*B	4	100	99.7	2	100	99.0	2	99.0	100	1	100	100
IEC	Staphylococcal complement inhibitor	Scin	*scn*	NA	NA	NA	NA	NA	NA	1	100	100	NA	NA	NA
	Chemotaxis inhibitory protein	Chip	*chp*	NA	NA	NA	NA	NA	NA	1	100	100	NA	NA	NA
	Staphylokinase	Sak	*sak*	NA	NA	NA	NA	NA	NA	1	100	100	NA	NA	NA

Abbreviations: AA, amino acid sequence; AV, allelic variant, C, sequence coverage of reference gene; I, identity of AA sequence with reference. FS, frame shift in nucleotide sequence, IEC, immune evasion cluster; NA: not available (gene absent), PI, phage insertion, disrupted by *hlb*-converting bacteriophage.

[a] Allelic variants (AV) were determined by nucleotide sequence-based comparison including sequence data of strain Newman as variant 1.

[b] alternative reference genome: *Staphylococcus aureus* USA300 (CP000255).

[c] alternative reference genome: *Staphylococcus aureus* N315 (BA000018).

[d] alternative reference genome: *Staphylococcus aureus* phage 187, PV83 (AB044554).

[e] alternative reference genome: *Staphylococcus aureus* COL (CP000046).

[f] nucleotide insertion, frame-shift mutation, premature stop codon.

of their numerous advantages, including an overall similar structure of all organ systems, easy animal handling, low demand for space, short generation times, large litter sizes, and low husbandry costs (Buer and Balling, 2003; Haley, 2003; Paigen, 1995; Perlman, 2016; Rosenthal and Brown, 2007). Most importantly, their immune system is extremely well characterized and there are numerous knock-out strains and analytical tools available.

However, using mice as infection model also has its limitations. Humans and mice differ in size, their metabolic rate, reactive oxygen species generation, diet, and some aspects of their cardiovascular physiology (Seok et al., 2013; Xiao et al., 2015). Moreover, laboratory mice are usually inbred. Although the overall structure of the immune system is quite similar between mice and humans, there are still some differences, such as the balance of neutrophils and leukocytes, in addition to the chemokine repertoire (Mestas and Hughes, 2004; Zschaler et al., 2014).

An important argument for using mice as an infection model, is the recent notion that both laboratory mice and wild small rodents and shrews are naturally colonized with *S. aureus* and develop spontaneous infections (Baker, 1998; Blackmore and Francis, 1970; Percy and Barthold, 2007). Like humans, mice are colonized mainly in the nasal cavity, the lower digestive tract and on the skin. Moreover, reports on spontaneous *S. aureus* infections in laboratory mice suggest that the clinical manifestations also resemble the human situation with purulent skin and soft tissue infections being the most common clinical feature (Baker, 1998; Blackmore and Francis, 1970; Percy and Barthold, 2007). Individual cases of facial and mandibular abscesses, necrotizing dermatitis, furunculosis, arthritis, mastitis, pneumonia, and eye infections

have been reported (Baker, 1998; Blackmore and Francis, 1970; Bremell et al., 1990; Clarke et al., 1978; Kiser et al., 1999; Percy and Barthold, 2007).

Alternatives to using mice as an infection model include cotton rats, rabbits, large mammals such as pigs, and humanized mice, each possessing their own advantages and disadvantages. Since DIP was derived from a bank vole, it might also be of interest to investigate *S. aureus* virulence in bank voles (M*yodes glareolus*), which have been previously used in several viral, bacterial and prion-induced infections (Franke et al., 2017; Nonno et al., 2006; Rossow et al., 2014; Tonteri et al., 2013). Importantly, researchers need to know the limitations of each model system and choose the right infection model (or a combination thereof) to address their research questions. For example, some *S. aureus* virulence factors, such as PVL, and superantigens target human and rabbit immune cells, but show no or little effect on murine cells (Holtfreter and Bröker, 2005; Löffler et al., 2010; Schlievert, 2009). Therefore, researchers must use rabbits or humanized mice to study the contribution of these factors to *S. aureus* pathogenesis (Knop et al., 2015; Prince et al., 2017; Tseng et al., 2015).

We argue that despite their limitations, mice represent the best animal model for *S. aureus* colonization and infection studies, because they are natural hosts of *S. aureus*, and their immune response can be characterized down to the smallest detail thanks to knock-out strains and a plethora of analytical tools. Using certain mouse-adapted *S. aureus* strains, laboratory mice can be persistently colonized and the infection dose can be considerably reduced in infection models.

4.6. Summary

Mice and mouse-adapted *S. aureus* must have co-evolved for a long time. The bacteria are well adapted to their host, resulting in a higher fitness and, at least in some cases, higher virulence. Mouse-adapted *S. aureus* strains persistently colonize mice and can cause spontaneous infection. Bringing together what naturally belongs together, rather than using human-adapted strains at unphysiologically high infection doses, is a promising approach to clinically relevant colonization and infection models. Despite some limitations, including the unresponsiveness to human-specific staphylococcal virulence factors, mice represent a valuable model for *S. aureus* colonization and infection studies.

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Conflict of interests

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2018.10.007.

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