Tuchscherr, L., Medina, E., Hussain, M., Völker, W., Heitmann, V., Niemann, S., Holzinger, D., Roth, J., Proctor, R.A., Becker, K., Peters, G., Löffler, B.

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(2011) EMBO Molecular Medicine, 3 (3), pp. 129-141

EMBO Molecular Medicine

Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection

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Keywords: bacterial intracellular persistence; chronic infections; host inflammatory response; *S. aureus* SCVs; ***S. aureus*** virulence factor expression

DOI 10.1002/emmm.201000115

Received September 29, 2010
Revised December 15, 2010
Accepted December 16, 2010

→See accompanying
Closeup by Christian Garzoni and William Kelley
DOI 10.1002/emmm.201100123.

Staphylococcus aureus is a frequent cause for serious, chronic and therapy-refractive infections in spite of susceptibility to antibiotics *in vitro*. In chronic infections, altered bacterial phenotypes, such as small colony variants (SCVs), have been found. Yet, it is largely unclear whether the ability to interconvert from the wild-type to the SCV phenotype is only a rare clinical and/or just laboratory phenomenon or is essential to sustain an infection. Here, we performed different long-term *in vitro* and *in vivo* infection models with *S. aureus* and we show that viable bacteria can persist within host cells and/or tissues for several weeks. Persistence induced bacterial phenotypic diversity, including SCV phenotypes, accompanied by changes in virulence factor expression and auxotrophism. However, the recovered SCV phenotypes were highly dynamic and rapidly reverted to the fully virulent wild-type form when leaving the intracellular location and infecting new cells. Our findings demonstrate that bacterial phenotype switching is an integral part of the infection process that enables the bacteria to hide inside host cells, which can be a reservoir for chronic and therapy-refractive infections.

INTRODUCTION

Even though anti-microbial therapy was introduced over 70 years ago, bacterial infections are still difficult to treat and represent serious and costly clinical problems. In particular, *Staphylococcus aureus* is known as a frequent cause for several types of chronic and recrudescent infections, *e.g.* osteomyelitis, endovascular diseases and chronic lung infections (Lowy, 1998). Actually, in some clinical cases, *S. aureus* infections are reported to persist asymptomatically with relapses occurring months or even years after anti-microbial treatment and apparent cure of the infections (Greer & Rosenberg, 1993; Kipp et al, 2003; Proctor et al, 1995; von Eiff et al, 1997a). The emergence of antibiotic-resistant bacterial strains, such as methicillin-resistant *S. aureus* (MRSA), only partly accounts for this poor outcome, as many relapsing and therapy-refractory

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infections are caused by pathogens that have been shown to be susceptible to antibiotics *in vitro*. Although antibiotic-resistant strains increase the likelihood of treatment failures (David & Daum, 2010), the overall outcomes are often similarly poor in drug-susceptible *S. aureus*, which must not be neglected as a pathogen causing potentially lethal infection (Kern, 2010; Plouin-Gaudon et al, 2006; Sheehy et al, 2010). Yet, the mechanism(s) for bacterial persistence despite host defenses and *in vitro* effective antibiotic therapy are not fully understood.

Clinically, chronic and therapy-refractory staphylococcal infections have been particularly associated with an altered bacterial phenotype, the small colony variants (SCVs; Kahl et al, 1998; Proctor et al, 1995, 2006; Sendi et al, 2006). Subpopulations of SCVs have been found in a wide variety of bacteria (Haussler et al, 1999; Proctor et al, 2006; Wellinghausen et al, 2009), but they have been most extensively studied in *S. aureus*. In general, SCVs form small colonies on agar plates (about 10 times smaller than the parent strain) due to their slow growth rate and reduced metabolism, which also explains their decreased susceptibility to a variety of antibiotics (Chuard et al, 1997; von Eiff, 2008). They express a changed pattern of virulence factors, including a reduced expression of exotoxins, such as α-haemolysin (α-toxin) and an increased expression of adhesins, such as the fibronectin-binding proteins (FnBPs; Vaudaux et al, 2002). Furthermore, SCVs are frequently auxotrophic for menadione and haemin, compounds involved in the biosynthesis of electron transport chain elements, or thymidine (Proctor et al, 2006; Sendi & Proctor, 2009; Vaudaux et al, 2002).

As SCVs recovered from clinical specimens are often not stable, but can rapidly revert to their wild-type phenotype when subcultivated (Becker et al, 2006), most knowledge and laboratory work on SCVs has been obtained with stable site-directed mutants in the electron transport system that mimic the SCV-phenotype, *e.g. hemB* and *menD* mutants (von Eiff et al, 1997b, 2006). Consequently, data on the development and dynamics of SCVs are largely missing. SCVs appear well adapted to the intracellular environment (Sendi & Proctor, 2009; Tuchscherr et al, 2010) and there are even some indications that SCVs could be induced by the intracellular milieu (Vesga et al, 1996). Further reported mechanisms leading to the formation of SCVs (*in vitro* and *in vivo*) include prolonged exposure to subinhibitory concentrations of antibiotics (Massey et al, 2001; Mitchell et al, 2010a; von Eiff et al, 1997a) or to exoproducts from other bacteria, *e.g. Pseudomonas aerugenosa* (Mitchell et al, 2010b). Moreover, there is growing evidence that the formation of SCVs could also be due to regulatory mechanisms, involving global regulators (*e.g. sigB*, *sarA* and *agr*), Clp ATPases (Kahl et al, 2005; Mitchell et al, 2008, 2010a) or non-protein-coding RNAs as regulatory molecules (Abu-Qatouseh et al, 2010).

There are many questions open regarding the signals and factors that induce the formation of *S. aureus* SCVs. However, the central question that needs to be addressed before is whether the development of SCVs is only a rare, marginal or laboratory phenomenon (possibly due to gene mutations) or if the formation of SCV is an integral part of the normal bacterial life cycle that is required for adaptation and persistence. In the latter case, a dynamic and reversible formation of SCVs has to be assumed. The phenomenon of rapid phenotype switching of genetically identical cells (bet-hedging strategy) has been described for a variety of other microorganisms (Beaumont et al, 2009; Epstein, 2009; Finkel & Kolter, 1999). Particularly, in a stressfull and fluctuating environment stochastic differentiation into distinct phenotypes can provide a strong advantage, which allows for bacterial persistence (Balaban et al, 2004; Lewis, 2007; Rotem et al, 2010). In our studies, we used several *in vitro* and *in vivo* models of *S. aureus* long-term infections and characterized the recovered bacteria for their virulence potential and their dynamic capacity to revert to the wild-type phenotype. We demonstrate for the first time in *S. aureus* that phenotype switching and SCV formation are indispensably (possibly as part of a bet-hedging strategy) related to chronic infections, which have to be considered as an important immune escape mechanism.

RESULTS

Different staphylococcal strains can survive within cultured host cells for up to 28 days accompanied by phenotypic diversity and changes in virulence factor expression

To test the capacity of *S. aureus* to persist intracellularly, we infected an epithelial cell line (A549 cells, ATCC CCL-185 and human lung adenocarcinoma) with either the highly virulent *S. aureus* strain 6850 isolated from a bacteremic patient with metastatic bone infection (Balwit et al, 1994) or with the strain 628 isolated from the nasal cavity of an asymptomatic carrier (Table 1) and analysed the infected cell cultures weekly for 28 days. Directly after infection with wild-type phenotypes, we found a high number of intracellularly located bacteria and electron microscopy revealed that the cytoplasm of all examined cells contained phagosomes filled with bacteria. During the course of the infection, the number of intracellular bacteria was dramatically reduced, but with both wild-type isolates we always found a small amount of living bacteria that persisted within host cells for up to 28 days (Fig 1A). Analysing the persisting bacteria revealed a high phenotypic diversity, showing normal, small and very small colonies, which resemble SCVs. In the course of the infection, the percentage of small and very small colonies greatly increased, reaching up to 90% after 28 days (Fig 1B).

We next determined whether phenotypic switching during chronic infection affects bacterial auxotrophism and the expression of virulence factor genes; changes which are well known for stable SCV mutants (*e.g.* site-directed mutants in the electron transport system, such as strain IIb13, Table 1; Sendi & Proctor, 2009; Vaudaux et al, 2002). In some, but not all, small colonies recovered, we detected auxotrophism for thymidine, menadione and/or haemin (data not shown). To analyse gene expression, we performed polymerase chain reaction (PCRs) directly from whole cell lysates of infected host cells to avoid changes in gene expression during a subcultivating step (see method described by Garzoni (Garzoni et al, 2007)). We found

Table 1. Bacterial strains and phenotypes used in this study

Strain	Phenotype	Properties	Reference or source
628	Wild-type	Wild-type isolate from nose	This study
6850	Wild-type	Wild-type isolate from osteomyelitis (ATCC53657)	(Balwit et al, 1994; Proctor et al, 1984)
Colonies from 6850 recovered after 28 days from cell culture model	SCV (dynamic)	SCV obtained from a cell culture A549 experiment after 28 days	This study
Colonies from 6850 recovered after 30 days from animal model	SCV (dynamic)	SCV obtained from mice experiment after 30 days (from bone)	This study
IIb13	SCV (stable)	*hemB* mutant auxotroph SCV from strain 6850 (6850, *hemB*:*ermB*, SCV)	(Vaudaux et al, 2002)
KM4	Wild-type	Complemented mutant. IIb13 + pCE12	(von Eiff et al, 1997b)
A26026V	SCV (dynamic)	Wild-type isolate from osteomyelitis	This study

Different *S. aureus* strains and phenotypes were plated on blood agar plates to determine the phenotypes.

the expression of FnBPA (*fnbA*) highly up-regulated and the expression of α-haemolysin (*hla*) down-regulated during the course of the infection (Fig 1D). It is well established that a functioning global accessory gene regulator (*agr*), which controls the expression of many virulence factors, is required to initiate an infection and to provoke a strong inflammatory reaction (Grundmeier et al, 2010; Traber et al, 2008). In our experiments with A549 cells, we detected an initial increase in *agr* expression (days 1–7) followed by a down-regulation of *agr* 14 days post-infection, when the acute infection phase is most likely overcome (Fig 1C). All these changes in gene expression (up-regulation of *fnbA*, down-regulation of *hla* and *agr*) were similarly reported for stable SCV-mutants with defects in the electron transport system (Sendi & Proctor, 2009; Vaudaux et al, 2002; von Eiff et al, 1997b).

To analyse the host cell response during the infection course and during the bacterial adaption process, we measured the expression of the chemokines RANTES, I-TAC and of the adhesion molecule ICAM-1 at the different time points (1–28 days). From previous work we already know that these pro-inflammatory factors are highly up-regulated after endothelial infection with different *S. aureus* wild-type isolates (Grundmeier et al, 2010; Tuchscherr et al, 2010). Consistently, we detected a significant up-regulation of all three factors directly after infection and 7 days post-infection, whereas after 14 days and longer intervals the levels of both chemokines and ICAM-1 returned to control values (Fig 1D). These results show that persisting bacteria can switch to a state, where they largely avoid activation of the host innate defense system.

The phenomenon of phenotype switching and changes in virulence factor expression is similar in different host cell types and in a long-term murine infection model

The phenomenon of staphylococcal phenotype switching and changes in virulence factor expression (Fig 1) was not restricted to the infection of a defined host cell type, as similar results were observed with different human primary non-professional phagocytes, such as human umbilical endothelial cells (HUVECs) or osteoblasts (Fig 2). Here, the long-term infection experiments could be performed for only 1 week, as primary cell cultures were not stable for longer time-periods. In both cell types we found similar results in host cell responses, involving up-regulation of chemokines and the adhesion molecule ICAM-1 (Fig 2D). Although the acute pro-inflammatory response was very pronounced in primary cell cultures, all factors rapidly returned to control levels within 3–4 days post-infection.

By contrast, in professional phagocytes, such as macrophages, all intracellular bacteria were cleared within 3 days, indicating that persistence of highly virulent strains is restricted to non-professional phagocytes (Fig 2A).

Moreover, we could, for the first time, demonstrate phenotype switching and the formation of SCVs in a haematogenous murine model of chronic *S. aureus* infection. For this, we recovered disseminated bacteria in various organs, such as kidneys and bones, during the course of 4 weeks (Fig 3A). In all organs analysed, the infecting bacteria developed an increased phenotypic diversity, including the development of small and SCV phenotypes (Fig 3B). To investigate virulence factor expression, we recovered the infecting bacteria via magnetic beads and used them directly (without subcultivating step) to extract RNA and perform PCRs. We detected the same changes in gene expression, up-regulation of *fnbA* and down-regulation of *hla* and *agr* (Fig 3C), as shown in the different cell culture models (Figs 1 and 2). These results demonstrate that phenotypic diversity and SCVs appear and develop similarly in various long-term staphylococcal infection-models *in vitro* and *in vivo*.

To determine if changes in the host inflammatory response paralleled the changes of the bacterial phenotypes, serum was collected at 2, 7, 14 and 30 days of infection and assayed for IL-6, TNF-α and IL-10. *S. aureus* infection in this mouse model was associated with significantly elevated serum concentrations of pro-inflammatory cytokines IL-6 and TNF-α, but also with the anti-inflammatory IL-10 compared with uninfected controls during the first two weeks of infection (Fig 3D). Levels of IL-6 peaked early and remained high up to 14 days (Fig 3D, left panel), indicating that this cytokine is the most proximal inflammatory mediator triggered by *S. aureus* infection. Levels of TNF-α peaked at day 14 of infection followed by a sharp decline (Fig 3D, middle panel). The production of the anti-inflammatory cytokine IL-10 also peaked at day 14 of infection (Fig 3D, right panel) and may serve to control the inflammatory

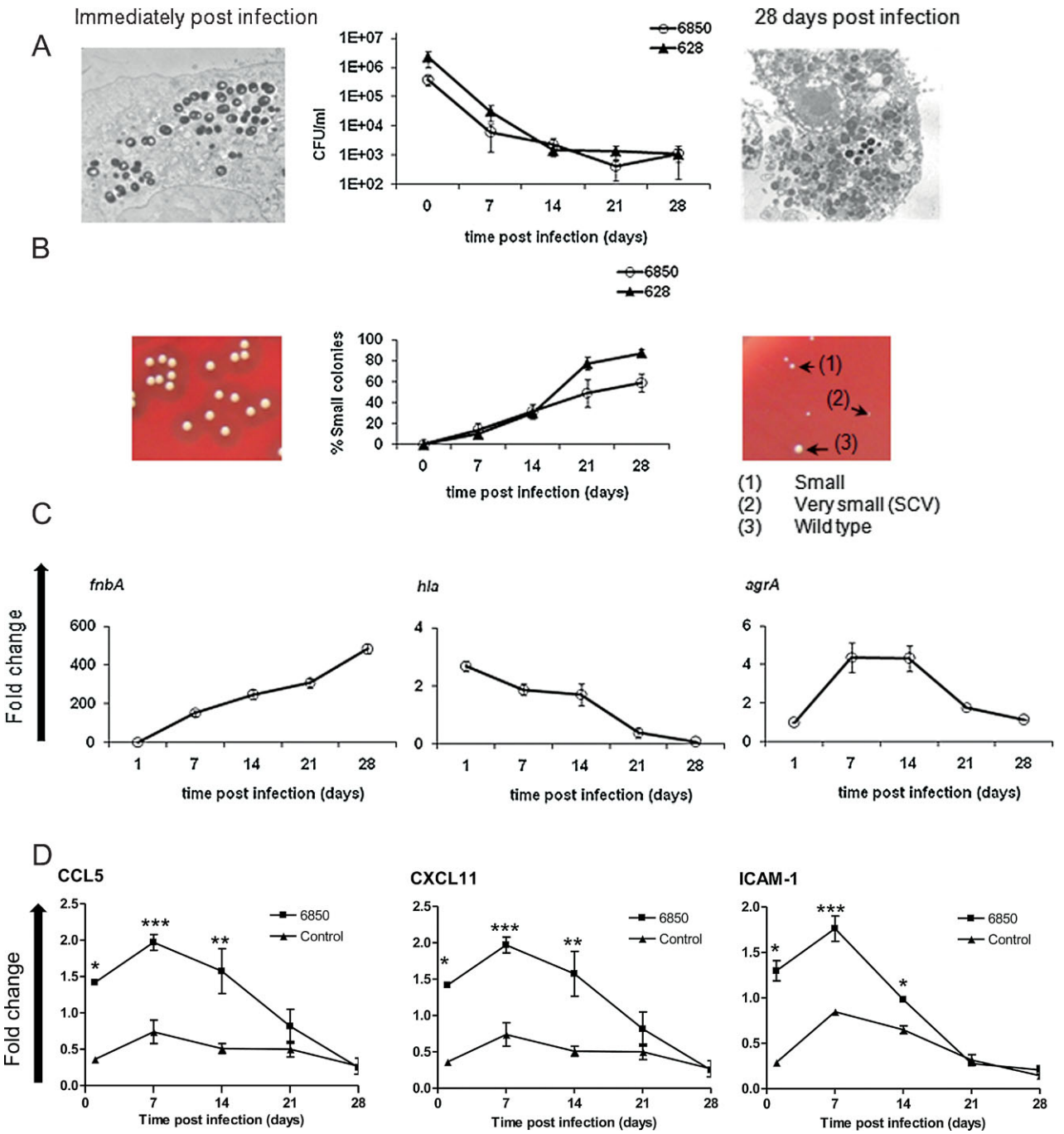

Figure 1. Staphylococci survive within cultured host cells for 28 days and change phenotypes and virulence factor expression.

A. Epithelial cells (A549) were infected with different *S. aureus* strains (6850 or 628) and analysed for 28 days. The number of viable intracellular persisting bacteria was determined weekly by lysing host cells, plating the lysates on agar plates and counting the colonies that have grown on the following day ($n = 3$, ±SEM). Electron micrographs of infected cells were performed directly after and 28 days post-infection showing morphological intact staphylococci within epithelial cells.

B. Percentage of small and very small (SCV) phenotypes (<5 and <10-fold smaller than those of the wild-type phenotype, respectively) recovered during the time-course of 28 days ($n = 3$, between 200 and 500 colonies examined in each sample, ±SEM). Photographs of recovered colonies were performed directly after and 28 days post-infection showing appearance of small and SCV colonies.

C. Changes in bacterial gene expression (strain 6850) for fibronectin binding protein A (*fnbA*), α-haemolysin (*hla*) and *agr* during the course of infection were determined by real-time PCR ($n = 5$, ±SD).

D. Changes in host cell response measured by the expression of CCL5, CXCL11 and ICAM-1 in the time course after infection with strain 6850 ($n = 6$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ in comparison with values from uninfected cells.

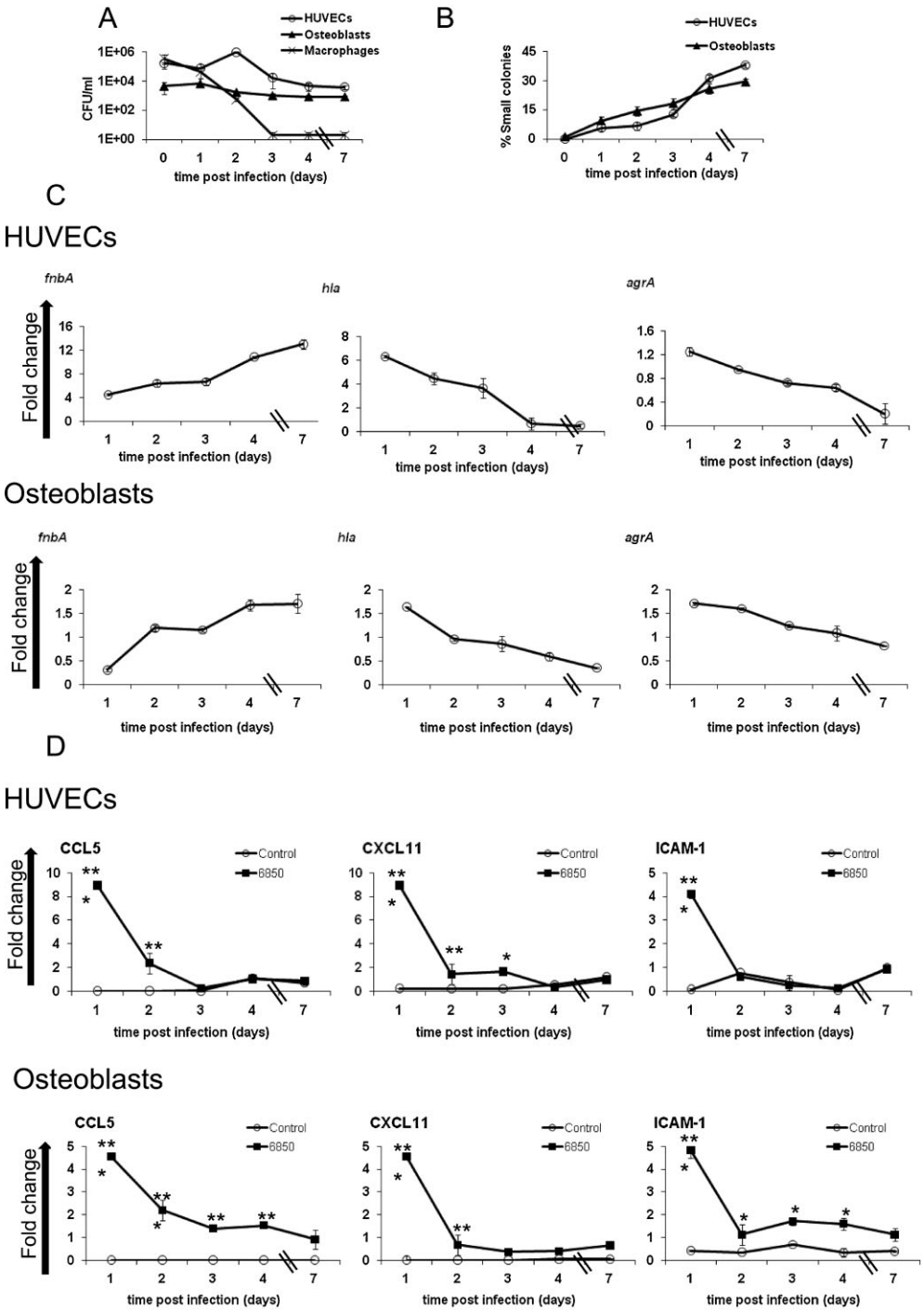

Figure 2. Staphylococci survive within different types of primary isolated human host cells.

A. Primary isolated human cells (HUVECs, osteoblasts and macrophages) were infected with the wild-type *S. aureus* strain 6850 and analysed for seven consecutive days. The number of viable intracellular persisting bacteria was determined daily and was dramatically reduced in HUVECs and osteoblasts, but for both cell types, we found living intracellular bacteria through 7 days post-infection. In macrophages, all intracellular bacteria were cleared within 3 days indicating that persistence of highly virulent strains is restricted to non-professional phagocytes.

B. The percentage of small and SCV phenotypes recovered during the time-course of 7 days was determined as described (Fig 1B) and reaches up to 40% after 7 days for HUVECs and osteoblasts.

C. Changes in bacterial RNA expression for fibronectin binding protein A (*fnbA*), α-haemolysin (*hla*) and *agr* during the course of infection were determined as described ($n = 5$, ±SD; Fig 1C) and revealed similar changes following infection of different cell types.

D. Changes in host cell response measured by the expression of CCL5, CXCL11 and ICAM-1 ($n = 6$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ in comparison with values from uninfected cells.

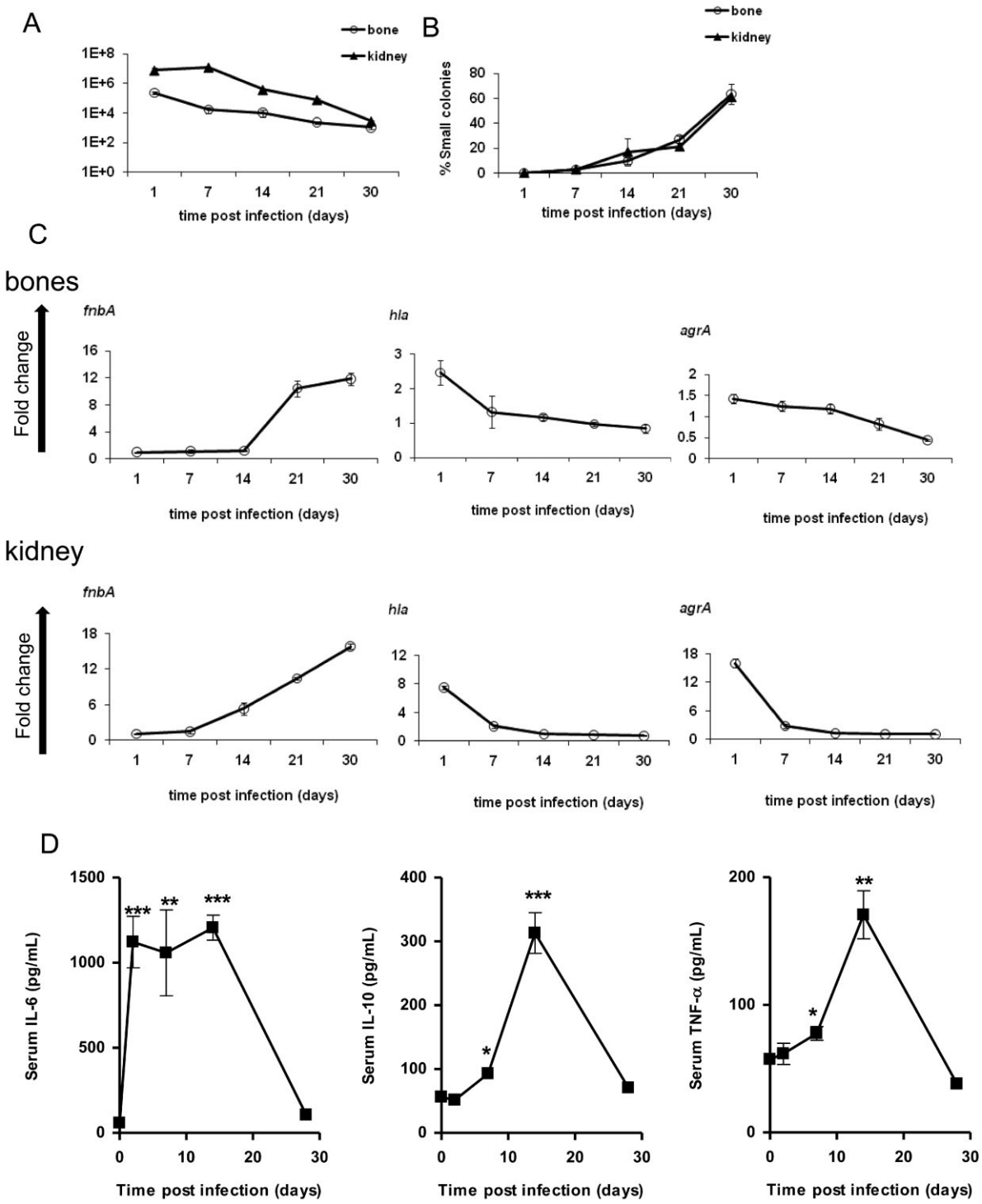

Figure 3. Staphylococci persist in tissues of infected animals while changing phenotypes and virulence factor expression.

A. Bacterial loads in the kidneys (triangles) and tibias (circles) of C57BL/6 mice were analysed at progressing times after intravenous inoculation with 5×10^5 CFU of *S. aureus* 6850. Each point represents the mean $\pm$ SD of five mice per group.

B. The percentage of small and SCV phenotypes recovered during the time-course of 28 days was determined as described (Fig 1B).

C. Changes in bacterial RNA expression for fibronectin binding protein A (*fnbA*), α-haemolysin (*hla*) and *agr* during the course of infection were determined by extracting bacteria from bone tissue via magnetic beats, isolating RNA and performing real-time PCR ($n = 5$, $\pm$SD). The results revealed similar changes as in the *in vitro* cell culture experiments.

D. Levels of IL-6 (left pane), IL-10 (middle panel) and TNF-α (right panel) in serum of *S. aureus*-infected mice at progressive times after bacterial inoculation. Each point represents the mean $\pm$ SD of five mice per group. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ in comparison with values from uninfected mice.

reaction by reducing the production of pro-inflammatory cytokines. By day 30 of infection, the level of the different cytokines has already returned to homeostatic values (Fig 3D). These observations indicate a parallelism between the recession of the host systemic inflammatory response and the switching of *S. aureus* towards adapted phenotypes, such as SCVs.

Staphylococci recovered from clinical subacute infections display phenotypic diversity and typical changes in virulence factor expression

To find out whether this phenomenon of phenotype switching also applies to human infections, we collected six different clinical specimens from *S. aureus* endovascular, soft tissue and

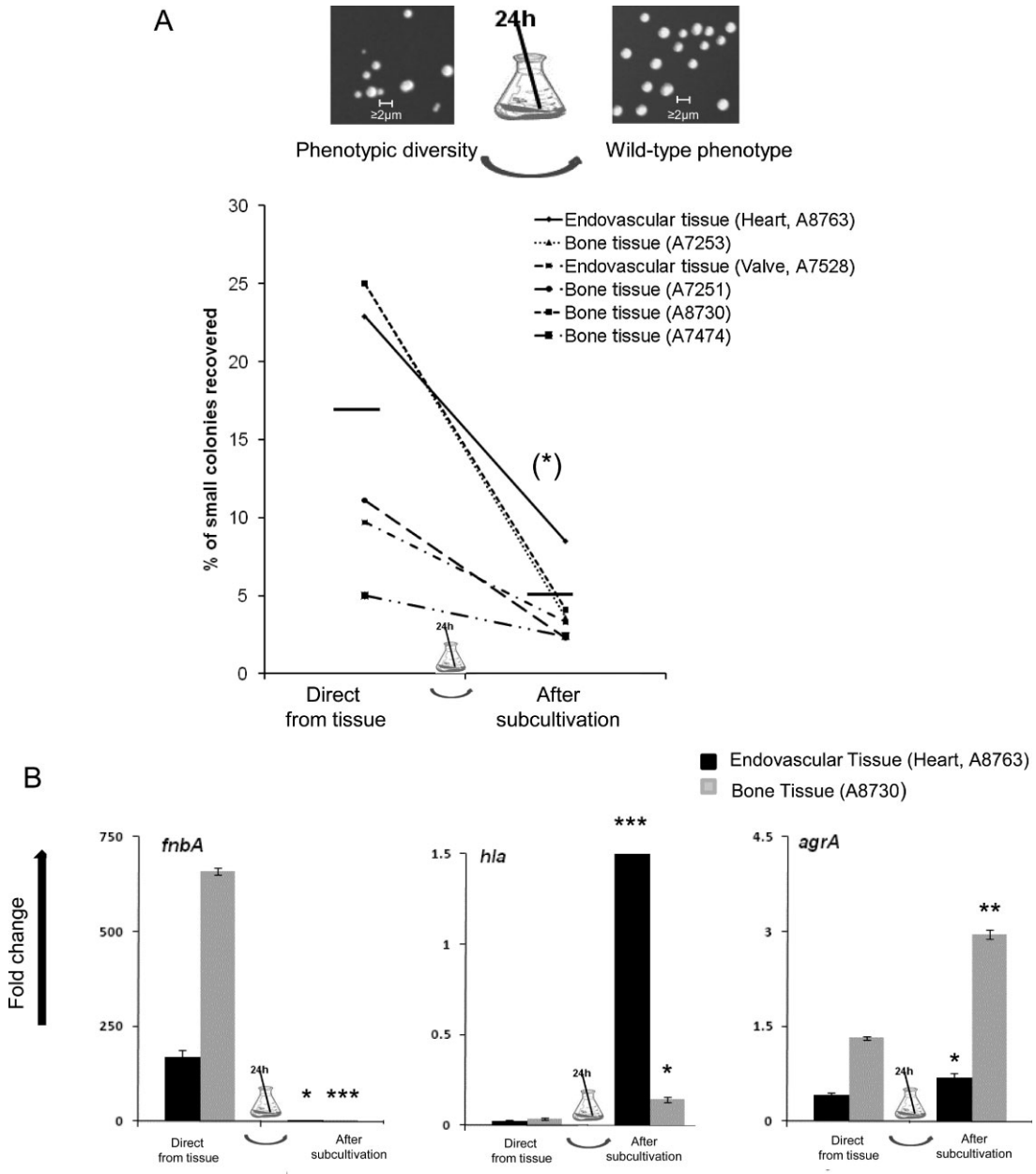

Figure 4. Staphylococci obtained from clinical specimens revealed changes in phenotype switching and virulence factor expression. We collected six different tissue specimens from *S. aureus* endovascular, soft tissue and bone infections, including chronic infection courses (see Supplementary Table 2 of Supporting Information). We analysed bacteria directly from the tissue samples and after one subcultivating step in rich medium (BHI, 24 h, shaking).

A. To determine bacterial phenotypes within infected host tissue, we plated homogenized tissue samples on agar plates and defined the percentage of the small and SCV phenotypes. These results were compared to the corresponding bacteria plated after one subcultivating step ($n = 6$, $^{*}p \leq 0.05$).

B. Bacterial RNA expression for fibronectin binding protein A (*fnbA*), α-haemolysin (*hla*) and *agr* was analysed in bacteria directly extracted from two representative documented chronic infections (heart and bone tissue) with magnetic beats. The results were compared to RNA expression in the corresponding bacteria, which were subcultivated for 24 h and grown to the late exponential phase for another 4 h ($n = 5$, ±SEM, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$).

bone infections (Table S1 of Supporting Information). Directly plating homogenized tissue samples on agar plates revealed diverse phenotypes, including small and SCV phenotypes, such as detected in the long-term cell culture and animal experiments. In the different clinical samples, the percentage of small and SCV phenotypes ranged around 17%, whereby almost all of them rapidly reverted to their wild-type form upon only one subcultivating step for 24 h in rich medium (Fig 4A). To analyse changes in gene expression in bacteria directly isolated from infected tissues in comparison to the corresponding bacteria that had regained their wild-type phenotypes, we selected two representative clinical specimens from endocarditis and osteomyelitis with documented chronic courses. Here, we found the expression of *fnbA* up-regulated and the expression of *hla* and *agr* down-regulated in bacteria directly extracted from infected tissues (Fig 4B), similar to the findings from the different long-term infection models (Figs 1–3).

Recovered SVCs are highly dynamic and can revert to their wild-type fully virulent phenotype within 24 h

It was very remarkable that almost all SCV colonies recovered from the different infection models and clinical samples were not stable, but reverted to their wild-type phenotype during one subcultivating step in rich medium between 8 and 24 h (Fig 5A). Testing both phenotypes before and after the subcultivating step (24 h) revealed that all SCVs were significantly more invasive in host cells than the reverted wild-types. Similar results were obtained with the stable SCV *hemB* mutant IIb13 and its

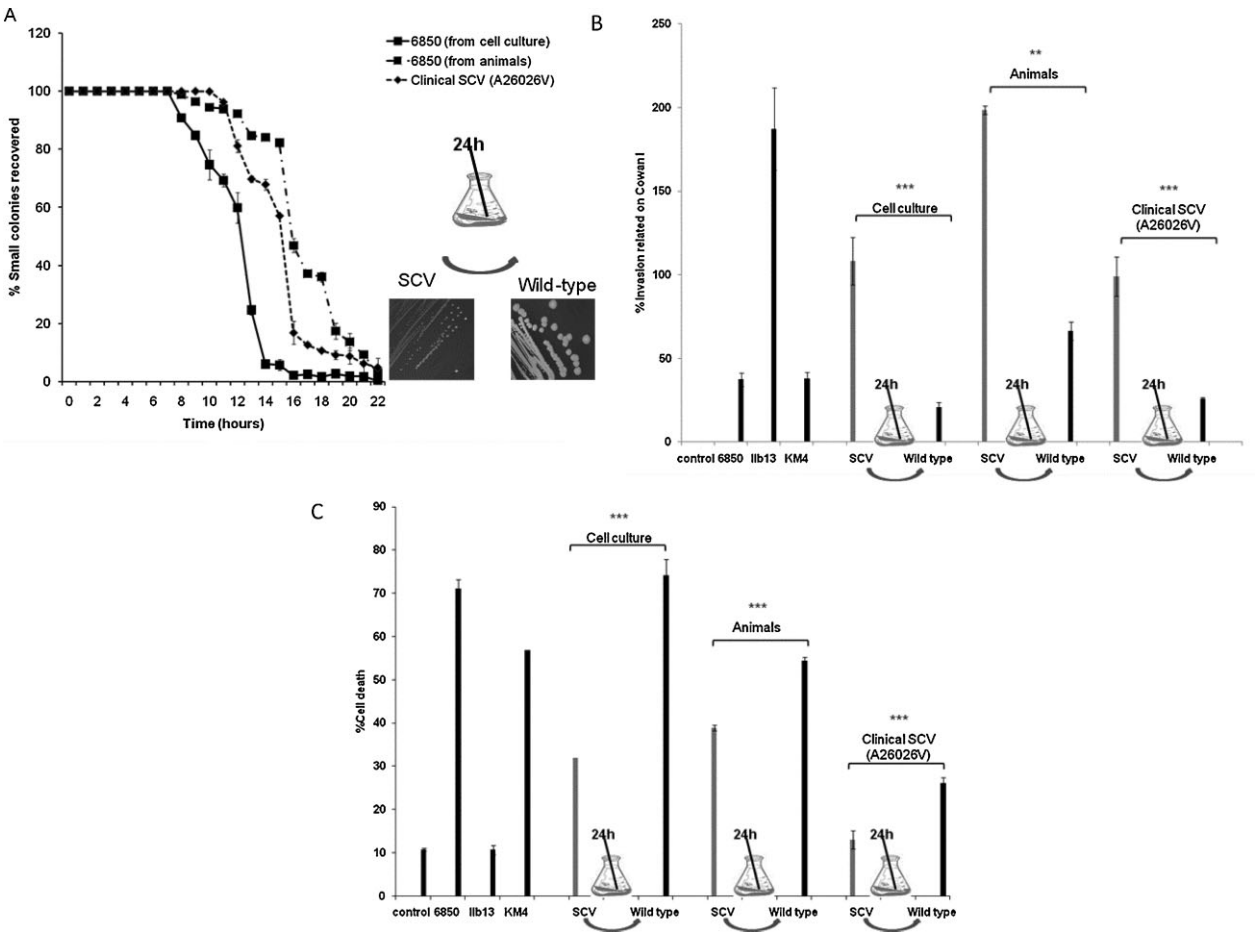

Figure 5. SCVs recovered from infection models and clinical specimens rapidly revert to their fully virulent wild-type phenotype.

A. SCV phenotypes recovered from *in vitro* (cell culture; Fig 1) and *in vivo* (animals; Fig 3) chronic infection models and from an osteomyelitis patient (A26026V) were subcultivated in BHI at 37°C with shaking and every hour samples were plated on agar plates to determine the percentage of small and SCV phenotypes.

B, C. Endothelial cells (HUVECs) were infected with different staphylococcal strains and phenotypes, including strain 6850, its isogenic *hemB* SCV mutant (IIb13), the complemented wild-type mutant (KM4), SCV phenotypes from 6850 recovered after 28 days from *in vitro* (Fig 1) and *in vivo* (Fig 2) chronic infection models and clinical SCVs isolated from osteomyelitis patients (A26026V). All SCVs were compared to their corresponding reverted wild-type phenotypes, which were gained by a 24 h subcultivating step in rich medium (BHI).

B. The invasiveness of the different bacterial phenotypes was determined by a flow cytometric invasion assay.

C.

complemented mutant KM4 (Fig 5B). The enhanced invasiveness can be attributed to the increased expression of *fnbs* in SCVs. Furthermore, we tested the cytotoxic activity of the different phenotypes. Infecting new cell cultures illustrated that the reverted phenotypes were as cytotoxic as the wild-type isolate 6850, whereas the recovered SCV colonies showed significant less cytotoxicity (Fig 5C). These findings on invasion and cytotoxicity were even reproduced by testing SCVs from an osteomyelitis patient, which were highly invasive and did not induce cell death upon infection, such as the stable SCV mutant IIb13 (Table 1). However, when the bacteria started to regain their wild-type phenotype by subcultivation in rich medium (24 h), they showed significantly less invasion and enhanced cytotoxicity (Fig 5B and C).

DISCUSSION

Chronic and therapy-refractive infections have been largely associated with the SCV phenotype, which has been most extensively studied in *S. aureus* (Kahl et al, 1998; Proctor et al, 1995, 2006; Sendi et al, 2006). However, as clinical SCVs are often unstable and difficult to detect, most published work on SCVs has been performed with stable site-directed *S. aureus* mutants in the electron transport system (von Eiff et al, 1997b, 2006) or with SCVs generated by *in vitro* gentamicin selection, which display a stable SCV phenotype (Balwit et al, 1994; Pelletier et al, 1979). Using these mutants, we recently demonstrated that SCVs are very well adapted to the intracellular environment, as they cause much less pro-inflammatory effects and can persist in higher numbers within host cells than wild-type phenotypes (Tuchscherr et al, 2010). The intracellular persistence confers most likely firm protection against many anti-microbial treatments and against the host immune system, which renders intracellular SCVs a potential reservoir for chronic infections.

However, up to now the significance of SCVs in chronic *S. aureus* infections as an integral part of the bacterial life cycle has not yet been established. In this work, we show that phenotype switching and the formation of SCVs are indispensably related to chronic infections and are even induced by the intracellular milieu of different host cell types. We found that in various *in vitro* and *in vivo* long-term infection models, the staphylococci gradually but consistently changed their phenotypes and their virulence factor expression towards SCVs. We detected the expression of FnBPA (*fnbA*), which is crucial for bacterial invasion of host cells (Sinha et al, 1999), highly up-regulated in persisting bacteria, indicating that this subpopulation may promote its own uptake by new host cells immediately after been released from aged host cells. By contrast, the expression of α-haemolysin (*hla*), which is an important pro-inflammatory and cytotoxic protein (Bhakdi & Tranum-Jensen, 1991; Grundmeier et al, 2010; Haslinger-Löffler et al, 2005) and of the *agr* operon (Novick, 2003), which regulates the expression of many exotoxins, was significantly down-regulated during the long-course of the infection suggesting that persisting bacteria try to preserve the integrity of their host cells and to be unnoticeable to the host immune surveillance. This was also apparent during *in vivo* infection where the remission of the inflammatory reaction paralleled the bacterial phenotypic switching towards SCVs.

From previous work it is already known that a functioning bacterial *agr*-system is important to initiate an infection and to provoke an inflammatory response (Grundmeier et al, 2010; Traber et al, 2008). Infecting the cell line A549, we observed an initial increase in *agr* expression followed by later down-regulation of *agr* (after 14 days, Fig 1C). The high levels of *agr* were paralleled by elevated expression of pro-inflammatory mediators in the host cells for up to 2 weeks (Fig 1D). Infecting primary cell cultures (HUVECs and osteoblasts), the high values of *agr* (Fig 2C) as well as host chemokines and adhesion molecule (Fig 2D) were only detected in the first days post-infection, followed by a rapid decrease in gene expression. The differences between the cell line and the primary cells could be explained by varying host defenses and bacterial degradation mechanisms. In this regard, autophagy, a tightly regulated process that contributes to cell growth, development and homeostasis, is also known as an ubiquitous physiological pathway in eukaryotic cells to eradicate invading pathogens (Lerena et al, 2010). It is reasonable to suspect that autophagic mechanisms are less active and slower in cells derived from carcinomatous tissue, such as the cell line A549, than in primary cells. [Some novel anti-cancer therapies even aim to induce the autophagic activity of cancer cells to inhibit uncontrolled cell growth (Fan et al, 2008)]. If so, the bacteria might need to adjust their virulence factor expression more quickly in primary cells than in carcinomatous cell lines, which might explain our findings that *agr*-expression is much faster down-regulated when primary cells are infected than the cell line A549. Taken together, the expression of *agr* appears to have alternating functions during the course of the infection process. On the one hand, it has been demonstrated that *S. aureus* requires *agr* directly post-infection to inhibit autophagosomal maturation and bacterial degradation (Schnaith et al, 2007). On the other hand, *S. aureus* needs to down-regulate *agr* during the later infection process to avoid killing of the host cells (Haslinger-Löffler et al, 2006; Schnaith et al, 2007). In this work we show that infecting staphylococci are able to dynamically adjust their *agr* and subsequent virulence factor expression, which most likely represents an important mechanism to subvert the host innate immunity and to develop adapted phenotypes for long intracellular persistence.

Most importantly, we found that almost all small and SCV phenotypes recovered from the different infection models and clinical specimens were not stable, reverting rapidly (within 24 h) to their wild-type phenotypes. Therefore, most persisting bacteria are ready to regain their fully virulent phenotype and to attack the host whenever they leave the intracellular environment. Up to now, there is only little known about the mechanisms that enable *S. aureus* to switch phenotypes and develop forms that are adapted for long-term persistence within host cells. For some other microorganisms stochastic switching to reversible states of low metabolic activity -'dormancy'- have been described (bet-hedging strategy; Beaumont et al, 2009;

Epstein, 2009; Lewis, 2010). The mechanisms of persister formation are incompletely understood, but dormancy programs are highly redundant and are not dependent on a single gene or mechanism. Especially, stress responses may act as general activator of persister formation, and it is commonly agreed that this bet-hedging strategy is used to overcome unfavourable and fluctuating environmental conditions (Finkel & Kolter, 1999; Lewis, 2010). During an infection and particularly within host cells bacteria are most likely exposed to a combination of many stress factors, such as low pH (Lam et al, 2010), cationic peptides, limited nutrition and reactive oxygen species. Here, we demonstrate for *S. aureus* infections that these factors induce the formation of highly dynamic SCVs, which resemble dormant phenotypes that favour bacterial persistence. Furthermore, clinical observations suggest that phenotype switching and the development of SCVs contributes to the pathogenic process, as a high phenotypic diversity was only observed with disease, but not in colonizing *S. aureus* (Goerke et al, 2007). By adopting different phenotypes during an infection, *S. aureus* have the option of attacking the host or hiding within the host cells. This infection strategy relates to the intracellular location as a source for recurrent infections and most likely represents an important immune escape mechanism. Clinically, this can explain treatment failures in spite of sufficient activity of the anti-microbial substances *in vitro*, as SCVs usually have an increased anti-microbial resistance profile and many anti-microbial agents do not reach sufficient high doses intracellularly (Chuard et al, 1997; Malouin et al, 2005; von Eiff, 2008). Consequently, new therapeutic options should also aim at targeting the bacterial adaptation strategies to ensure complete bacterial eradication after anti-microbial treatment.

MATERIALS AND METHODS

Cell culture infection models

Various types of host cells (the cell line A549, ATCC CCL-185, human lung adenocarcinoma and primary isolated human cells, HUVECs, osteoblasts and macrophages) were isolated as outlined before (Cooper et al, 2002; Jaffe et al, 1973; Mosser & Edwards, 2008) and were infected with different *S. aureus* strains as previously described (Tuchscherr et al, 2010). Briefly, primary cells were infected with an multiplicity of infection (MOI) of 50 and the cell line with an MOI of 100. After 3 h cells were washed and lysostaphin (20 μg/ml) was added for 30 min to lyse all extracellular or adherent staphylococci, then fresh culture medium was added to the cells. For primary cells, the washing, the lysostaphin step and medium exchange was repeated daily and for the cell line A549 every 2–3 days to remove all extracellular staphylococci, which might have been released from the infected cells. To detect live intracellular bacteria host cells were lysed in 3 ml H_2O (for 25 cm^2 bottles) or 20 ml H_2O (for 175 cm^2 bottles). To determine the number of colony forming units (CFU), serial dilutions of the cell lysates were plated on Müller-Hinton (MH) and blood agar plates and incubated overnight at 37°C. The colony phenotypes were determined on blood agar plates, recovered colonies were tested for the specific *S. aureus* clones (*nuc*-PCR, pulse field gel electrophoresis) and about 50 small or very small colonies were tested for auxotrophism as described (Vesga et al, 1996).

Flow cytometric invasion and cell death assays

For the flow cytometric invasion assay, A549 cells were plated at 2×10^5 cells in 12-well plates the day before the assay. Cells were washed with phosphate buffered saline (PBS), then 1 ml of 1% human serum albumin (HSA), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) in F-12 medium (Invitrogen cat. 21127-022). The bacteria were grown in brain heart infusion broth (BHI), washed, fixed by formalin (3%)-fixed and labelled by FITC (Invitrogen cat F143). The bacterial suspension (OD 1, 540 nm) was prepared as described (Sinha et al, 1999) and was added to cells. Culture dishes were preincubated for 30 min at room temperature to allow sedimentation of bacteria and were then shifted to 37°C for 3 h. After 3 h incubation at 37°C, with 5% CO_2, the cells were subsequently resuspended (trypsin/ethylenediaminetetraacetic acid (EDTA)) and bacterial uptake was assessed by flow cytometry as described (Juuti et al, 2004). The invasiveness of the laboratory strain Cowan I was set as 100%. In order to analyse different phenotypes, the bacteria were grown in brain heart infusion (BHI broth; for wild-type phenotypes) or in Müller-Hinton (MH broth; for SCVs) and we followed the same protocol as described before. The reversion of the SCVs to wild-type phenotypes was performed by incubation of bacteria for 24 h in BHI. Analysis of cell death was performed by measuring the proportion of hypodiploid nuclei and/or PI-staining of the cells as described (Haslinger-Löffler et al, 2006). For analysis of different phenotypes, the bacteria were grown in BHI (for wild-type phenotype) or in MH (for SCVs). The reversion of the SCVs to wild-type phenotypes was examined by incubation of the bacteria for 24 h in BHI.

Chronic infection model in mice

C57BL/6 female mice (8 weeks-old) were purchased from Harlan-Winkelmann (Borchen, Germany), housed in microisolator cages and kept under pathogen-free conditions. Mice were inoculated with 5×10^5 CFU of *S. aureus* 6850 in 0.2 ml of PBS via a lateral tail vein. Mice were sacrificed by CO_2 asphyxiation and bacteria were enumerated in the kidneys and tibias by preparing homogenates of these organs in PBS and plating 10-fold serial dilutions on blood agar. All animal experiments were approved by the local ethical board (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany).

Recovery of bacteria from murine tissues and clinical specimens using magnetic beads

Anti-staphylococcal antibodies were used to coat magnetic beads (Dynabeads M-280 sheep anti-rabbit IgG, invitrogen cat 112.04D). Each tissue was incubated for 1 h at room temperature with coated beads to recover the bacteria. Finally, the bacteria were isolated from anti-staphylococcus-coated beads by citric acid 0.1 M (pH 2–3) and neutralized by 2 M Tris–HCl (pH 9.5), following the manufacture instructions.

Cytokine assays

The serum levels of IL-6, TNF-α and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA) according to the recommendations of the manufacturer (BD Pharmingen, San Diego,

The paper explained

PROBLEM:

Chronic and therapy-refractive bacterial infections are a serious and costly clinical problem in spite of susceptibility to antibiotics *in vitro*. In different types of chronic and therapy-refractory infections, altered bacterial phenotypes, such as SCVs, have been found. Yet, it is largely unclear whether the ability to interconvert from the wild-type to the SCV phenotype is only a rare clinical and/or just laboratory phenomenon or is essential to sustain an infection.

RESULTS:

Here, we performed different *in vitro* and *in vivo* long-term infection models with *S. aureus*, a major human pathogen. We demonstrate that a highly dynamic phenotypic diversity, including SCV phenotypes, is an integral part of infections. Our findings highlight an aspect of the *S. aureus* infection process that pathogens are extremely versatile and can rapidly alter their virulent potential. We show that bacteria, which are generally known to be cytotoxic, utilize phenotypic switching to hide inside host cells. Here, pathogens are most likely protected against many anti-microbial treatments as well as the host defense system and can be a reservoir for chronic and recrudescent infections.

IMPACT:

This strategy has important clinical implications. Targeting phenotype switching would be a novel therapeutic and preventive strategy to render the bacteria more vulnerable to host response and anti-microbial treatment, which could enhance complete bacterial clearance after an acute infection.

CA, USA) using matched antibody pairs and recombinant cytokines as standards. Briefly, polystyrene microtitre plates (Costar, Fernwald, Germany) were incubated overnight at 4°C with purified anti-IL-6, anti-TNF-α or anti-IL-10 antibodies (BD Pharmingen) diluted in coating buffer. Wells were blocked with 10% fetal calf serum (FCS)-PBS and serum samples and standards (serial concentrations of recombinant murine IL-6, TNF-α or IL-10) were added and incubated overnight at 4°C. Bound cytokine was detected with either biotinylated anti-IL-6, anti-TNF-α or anti-IL-10 antibodies (BD Pharmingen), followed by streptavidin-peroxidase conjugate and developed with ABTS. The optical densities of samples and standards were measured at 405 nm with a correction wavelength of 650 nm.

Extraction of RNA

For RNA extraction, we used the kit RNeasy Mini kit (Qiagen cat. 74524). The RNA extraction was performed following the manufacture instruction and the suggestions of the protocol described by Garzoni et al (Garzoni et al, 2007).

Real-time PCR

Real-time PCR was performed by using the RNA isolated from infected cells, infected tissues or different bacterial isolates. The cDNA was obtained using the kit QuantiTect reverse transcription (Qiagen, 205313) and iQ™SYBR®Green Supermix (Bio-Rad cat 170-8882) was used. The reaction mixtures were incubated for 15 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 55°C and 30 s at 72°C using the iCycler from Bio-Rad. PCR efficiencies, melting-curve analysis and expression rates were calculated with the Bio-Rad iQ5 Software. In order to analyse the expression of all the bacterial genes and host cell responses, the primers were used, which are listed in Table S2 of Supporting Information (Grundmeier et al, 2010; Tuchscherr et al, 2010).

Ethics Statement

The isolation of human cells and the infection with clinical strains were approved by the local ethics committee (Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Medizinischen Wilhelms-Universität Münster). For our study, written informed consent was obtained (Az. 2010-155-f-S).

Mice were maintained under standard conditions and according to institutional (HZI) and European guidelines and handled in strict accordance with good animal practice and all efforts were done to minimize suffering. All animal experiments were approved by the ethical board Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg in Germany. Permit: 'Einfluss genetischer und immunologischer Wirtsfaktoren bei der Resistenz/Empfindlichkeit gegenüber *S. aureus*-Infektionen' (Az. 33.42502/07-04.05).

Statistical analysis

Statistical analysis for real-time PCR and cell experiment was performed by an unpaired two-tailed *t*-test test. A value of $p \leq 0.05$ was considered significant in all cases. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

Author contributions

LT, EM, GP and BL designed experiments. LT performed cell culture experiments and analysis of gene expression with help of VH and SN. EM performed animal experiments. DH and JR performed experiments with professional phagocytes. WV performed electron microscopy. MH helped to extract staphylococci from tissue. BL wrote the paper with input from GP, KB and RAP.

Acknowledgements

We thank B. Schuhen and M. Brück for excellent technical assistance and C. Garzoni for advice to extract RNA. We are also grateful to F. Schaumburg who provided us clinical

specimens. This work was supported by grants of the Transregional Collaborative Research Centre 34 (C12), the Interdisciplinary Center for Clinical Research (IZKF Münster; Löf2/030/10), the Bundesministerium für Bildung und Forschung (BMBF; grant 01KI0700 'SkInStaph' and grant 0315830B 'Medizinische Infektionsgenomik'), the SFB 492/B9 'Extracellular Matrix' and by a grant of the Deutsche Forschungsgemeinschaft (HA 3177/2-1).

Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

For general information about *S. aureus* and staphylococcal disease:
http://www.textbookofbacteriology.net/staph.html

webpage of the working group of B. Löffler:
www.klinikum.uni-muenster.de/index.php?id=3908#c10367

Accompanying Closeup by Christian Garzoni and William Kelley
DOI 10.1002/emmm.201100123.

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