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The role of programmed death ligand 1 pathway in persistent biomaterial-associated infections

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***Staphylococcus epidermidis* is commonly involved in biomaterial-associated infections. Bacterial small colony variants (SCV) seem to be well adapted to persist intracellularly in professional phagocytes evading the host immune response. We studied the expression of PD-L1/L2 on macrophages infected with clinical isolates of *S. epidermidis* SCV and their parent wild type (WT) strains. The cytokine pattern which is triggered by the examined strains was also analysed. In the study, we infected macrophages with *S. epidermidis* WT and SCV strains. Persistence and release from macrophages were monitored via lysostaphin protection assays. Moreover, the effect of IFN-γ pre-treatment on bacterial internalisation was investigated. Expression of PD-L1/L2 molecules was analysed with the use of FACS. Inflammatory reaction was measured by IL-10, TNF-α ELISAs, and transcriptional induction of TNF-α. Our study revealed that clinical SCV isolates were able to persist and survive in macrophages for at least 3 days with a low cytotoxic effect and a reduced proinflammatory response as compared to WT strains. Bacteria upregulated PD-L1/L2 expression on macrophages as compared to non-stimulated cells. The results demonstrated that the ability of *S. epidermidis* SCVs to induce elevated levels of anti-inflammatory cytokine, IL-10, and reduced transcriptional induction of TNF-α, together with expression of PD-L1 on macrophages and the ability to persist intracellularly without damaging the host cell could be the key factor contributing to chronicity of SCV infections.**

***Keywords*:** PD-L1, *Staphylococcus epidermidis*, small colony variants, biomaterial-associated infections

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Introduction

Biomaterial-associated infection (BAI) represents a serious problem in modern medicine (Boelens *et al*., 2000a; Zaat *et al*., 2010; Subbiahdoss *et al*., 2011). The majority of cases, 40–75%, are usually associated with relatively non-pathogenic coagulase-negative staphylococci (CNS), particularly *Staphylococcus epidermidis* (Boelens *et al*., 2000a; Jones *et al*., 2005). Although *S. epidermidis* infections only rarely develop into life-threatening diseases, they significantly increase morbidity in the affected groups (Boelens *et al*., 2000a; Vuong and Otto, 2002; Zaat *et al*., 2010; Subbiahdoss *et al*., 2011). Their frequency and the fact that they are commonly multi-drug resistant, constitute a serious burden for the public health system (Mack *et al*., 2013). Therefore, increased knowledge of *S. epidermidis* virulence factors and their impact on the innate immune system is important to gain better understanding of the pathophysiology and etiology of the disease.

Two pivotal processes in the pathogenesis of BAI appear to be vital for the success of *S. epidermidis* as a pathogen: i) formation of biofilms (Mack *et al*., 2013), and ii) alterations in the host inflammatory response resulting in a persistent or chronic infection (Boelens *et al*., 2000a).

The formation of bacterial biofilm and its role in BAI have been studied extensively in this context (Al Laham *et al*., 2007; Singh *et al*., 2009). Another mechanism used by bacteria to subvert host defense, to promote a chronic and persistent infection is phenotypic switch to small colony variant (SCV) sub-population that exhibits atypical growth and biochemical features compared to their parent wild-type (WT) population (Proctor *et al*., 2006).

Small colony variants are capable of surviving in the host in whom protective immunity is generated (Proctor *et al*., 2006; Sendi and Proctor, 2009). However, what enables them to evade the host defense system remains a crucial question for an understanding of persistent infections. To answer this question it is not sufficient to simply understand the function of specific virulence determinants. It is obvious that the co-existence of a host and a pathogen also results in reduced immunogenicity of the pathogen (Proctor *et al*., 2006).

Many viral as well as bacterial pathogens, which cause chronic infections, exploit the programmed death 1 (PD-1) - PD-L1/PD-L2 pathway to evade host immune effector mechanisms (Sharpie *et al*., 2007; McNab *et al*., 2011). The interaction of PD-1 with ligands provides an inhibitory signal that regulates T-cell activation to induce and maintain the balance between effective immunity, peripheral tolerance and immuno-pathology (Okazaki and Honjo, 2007; McNab *et al*., 2011). The induction of PD-L1 mostly affects cytokines

production, in particular the production of IL-10, which is known to down-regulate immunopathology and host defense mechanisms (Rodriguez-Garcia *et al*., 2011; Thi *et al*., 2012). Although many regulatory pathways may contribute to the persistence of intracellular bacteria, it will be useful to determine the function of the PD-L1/PD-L2 pathway in such settings (Sharpie *et al*., 2007). The present study aims at determining the levels of expression of PD-L1 and PD-L2 molecules on macrophages cultured *in vitro*, infected by *S. epidermidis* SCV and their parent WT strains isolated from patients with orthopaedic implant loosening. In the research it was also examined how expression of PD-L1 induced by two bacterial phenotypes affects cytokine patterns. The understanding of the role of PD-1 pathway in persistent orthopaedic infections caused by CNS small colony variants may contribute to controlling these increasingly important infections.

Materials and Methods

Isolation and identification of bacteria

Ten *S. epidermidis* strains isolated from 5 patients with orthopaedic implant loosening were studied. The strains represented two phenotypes: small colony variants and their parental wild type strains. All pairs of microorganisms belonged to the same species (*S. epidermidis*) and were isolated from the same patient. The species of each strain was identified by the use of several well-known phenotypic tests: Catalase test, Coagulase test, Gram-stain, and ID32Staph (bioMèrieux). SCVs were defined as pinpoint colonies, $1/10^{th}$ or a smaller size of normal staphylococcal colonies when grown on sheep blood agar. The growth of the SCV colonies was observed after at least 48 h of incubation in air. The identification of SCVs into species was performed with GenoType Staphylococcus assay (Hain Lifescience) based on PCR followed by hybridisation. The assay was performed according to the manufacturer's recommendations.

Auxotrophism of SCVs for haemin, menadione and thymidine was assessed with the use of the disc method (Zahra *et al*., 2013). Bacteria were considered auxotrophic when they demonstrated increased growth around the impregnated disc as compared to the periphery. All included SCV strains were auxotrophic to haemin.

The relationship between the WT and their corresponding SCV strains were assessed with the use of a commercially available DNA fingerprinting assay based on the rep-PCR technology (data not shown).

The bacterial strains used in this study are precisely characterized in our previous publication (Bogut *et al*., 2014). Characterization of the bacterial strains included in this study is presented in Table 1.

Preparation of bacteria

Bacterial strains were incubated to the mid log phase (OD_{600} =0.6) in 5 ml of tryptic soy broth (TSB; Oxoid) at 37°C under constant rotation. The cells were then harvested by centrifugation (5,000 × g for 10 min) and washed twice in 5 ml of Dulbecco's balanced salt solution (DPBS, Pan-Biotech). Bacterial chains and aggregates were broken by mild sonication for 3 × 10 s at 30 W (Bransonic ultrasonic cleaner; G. Heinemann) at a temperature of 20°C. Samples were then centrifuged (5,000 × g for 10 min) and the pellets were resuspended in RPMI 1650 medium (SIGMA Aldrich) without antibiotics or antimycotics. The accuracy of preparation of bacterial samples for phagocytosis assay was routinely verified by plating dilutions on agar plates and counting colonies to determine colony forming units (CFU) per ml.

Cell culture and differentiation

THP-1 (ATCC TIB202, LGC Standards), a human monocytic cell line, was maintained in a continuous culture in RPMI 1640 medium (SIGMA Aldrich) containing 10% heat-inactivated fetal bovine serum (SIGMA Aldrich). THP-1 cells were pretreated with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA; SIGMA Aldrich) for 24 h in 5% CO_2 at 37°C to induce maturation of the monocytes into macrophage-like adherent cells. This procedure was followed by 2 washes (RPMI 1640) and the addition of complete media over 48 h post differentiation.

Invasion of macrophages using lysostaphin protection assay

We decided to use lysostaphin protection assay instead of more commonly used gentamycin protection assay, as most strains used in the study were resistant to high doses of gentamycin.

The preliminary experiments (data not shown) proved that lysostaphin at final concentration of 50 μg/ml, incubated with all WT and SCV strains individually, efficiently killed all bacteria.

Table 1. Bacterial strains used in the study

Isolate no.	Species	Properties
4 WT	*S. epidermidis*	wild-type isolate form aseptic implant loosening
4 SCV	*S. epidermidis*	haemin auxotroph SCV form strain 4 WT (confirmed by rep-PCR)
57 WT	*S. epidermidis*	wild-type isolate form aseptic implant loosening
57 SCV	*S. epidermidis*	haemin auxotroph SCV form strain 57 WT (confirmed by rep-PCR)
21 WT	*S. epidermidis*	wild-type isolate form septic implant loosening
21 SCV	*S. epidermidis*	haemin auxotroph SCV form strain 21 WT (confirmed by rep-PCR)
22 WT	*S. epidermidis*	wild-type isolate form septic implant loosening
22 SCV	*S. epidermidis*	haemin auxotroph SCV form strain 22 WT (confirmed by rep-PCR)
61 WT	*S. epidermidis*	wild-type isolate form septic implant loosening
61 SCV	*S. epidermidis*	haemin auxotroph SCV form strain 61 WT (confirmed by rep-PCR)

For lysostaphin protection assays, macrophages (1×10^6 cells/well) were plated in a 24-well culture plate and infected by bacteria at MOI of 10. Differentiated THP-1 cells and staphylococci were cocultured for 2 h in humidified atmosphere containing 5% CO_2. Phagocytosis was stopped by placing the plate on ice and washing the macrophages twice with ice-cold PBS to remove non-phagocytosed bacteria. The culture medium was replaced by RPMI 1640 with 10% FBS containing 50 μg/ml of lysostaphin (DNA Gdańsk). After 2 h of incubation at 37°C cells were washed once with PBS and the medium was changed again to fresh media without antibiotics and the culture was maintained for up to 3 days.

IFN-γ treatment

Macrophages were preincubated overnight with 100 U/ml IFN-γ (R&D Systems). Then, the cells were exposed to *S. epidermidis* SCV and WT strains at MOI 10. Simultaneously, the non-stimulated cells were incubated with the same *S. epidermidis* strains. After 2 h of phagocytosis, extracellular bacteria were killed by adding lysostaphin (50 μg/ml) to the culture medium and the cultures were continued as described above. Directly after the lysostaphin treatment (2 h time point) and during consecutive days post-phagocytosis (24 h and 72 h time points) intracellular bacteria released by cell lysis were analysed for CFU.

Assay for colony-forming ability of engulfed bacteria

PBS-washed macrophages directly after 2 h of incubation with lysostaphin, and infected cultures during consecutive days post-phagocytosis (24 h and 72 h) were lysed by treatment with ice-cold water. The lysates were plated at serial dilutions in sterile PBS on tryptic soy agar (TSA; Oxoid) plates. The plates were incubated at 37°C up to 3 days and the number of colonies (CFU) was enumerated. In parallel, the presence of viable staphylococci in the conditioned media was determined also by the colony-forming assay (Koziel *et al.*, 2009).

The assessment of cell viability using propidium iodine (PI) staining

Following the internalisation of the bacteria to macrophages, plasma membrane integrity was assessed after 2 h, 24 h, and 72 h post-phagocytosis by PI staining (live/dead). Propidium iodine (SIGMA Aldrich) at the final concentration of 2 μg/ml was added to infected macrophages, followed by incubation for 5 min. in the dark on ice. Next, the macrophages were observed using an Olympus BX 41 fluorescence microscope.

Flow cytometry

For the maximal expression, control non-infected and *S. epidermidis* - infected macrophages (1×10^7 cells/ml at MOI 10) on the 3rd day post-phagocytosis were stained for CD14 (APC-A), PD-L1 (CD274 mouse anti-human FITC-A) and PD-L2 (CD-273 mouse anti-human PE-A) expression using specific mAbs (BD Bioscience). Negative control samples were incubated with isotype-matched mAbs in parallel with experimental samples. The samples were analysed with a FACSCanto II (BD Bioscience) flow cytometer using FACSDiva software version 6.1.3 (BD Bioscience).

Cytokine secretion measured by ELISA

Macrophages (1×10^6 cells/ml) were stimulated by *S. epidermidis* strains (MOI 10) for 24 h. Supernatant from cultures following stimulation was collected and assayed for TNF-α and IL-10 cytokines by ELISA applied in accordance with the manufacturer's protocol. The cytokine-specific ELISAs were: IL-10 (OptEIA IL-10, BD Bioscience) and TNF-α (OptEIA TNF-α, BD Bioscience). The intensity of the reaction colour in samples was measured at 450 nm with an ELISA plate reader (Labsystems Multiscan RC). The detection limits of cytokine-specific ELISA were: IL-10, 2.0 pg/ml; and TNF-α, 1.0 pg/ml.

RNA isolation and TNF-α mRNA analysis

Control unstimulated and *S. epidermidis* WTs and SCVs stimulated macrophages (1×10^6 cells/ml at MOI 10) were lysed at 24 h after treatment and total cellular RNA was extracted using NucleoSpin RNA kit according to the manufacturer's protocol (Macherey-Nagel). TNF-α mRNA was quantitatively measured by chemiluminescence plate assay (Signosis). Briefly, a total of 1 μl of isolated RNA was mixed with TNF Oligo Mix and hybridisation buffer and incubated overnight at 50°C on a hybridisation plate. After the washing step, hybridisation buffer together with multiple biotin molecules were added to the wells and incubated at 50°C for 1 h. After the next washing step, blocking buffer was added to the wells for 15 min. Next, the samples were incubated with streptavidin-HRP conjugate for 45 min. As the last step, substrate solution was added to each well and after short incubation, the chemiluminescence was determined within 5 min. The signal was measured with a luminescent plate reader (Infinite M200 Pro microplate reader, Tecan). The relative intensity of the hybridisation signal for each sample was determined in relation to the TNF IVT RNA standards.

Statistical analysis

All presented data are expressed as means ± standard deviation (SD) for three independent experiments. Differences between groups were evaluated using the Student *t*-test with $P<0.05$ taken as statistical significance at 95% of confidence level.

Results

Persistence of *S. epidermidis* WT and SCV strains inside macrophages

The ability of each clinical *S. epidermidis* strains to invade THP-1 - differentiated macrophages was evaluated in three independent experiments using a lysostaphin protection assay. Our data shows that all tested *S. epidermidis* strains: SCVs as well as their parent WT strains co-cultured with macrophages were internalised. Cultured macrophages engulfed bacteria efficiently within 2 h. To determine the intracellular survival/killing rate of *S. epidermidis* WT and SCV

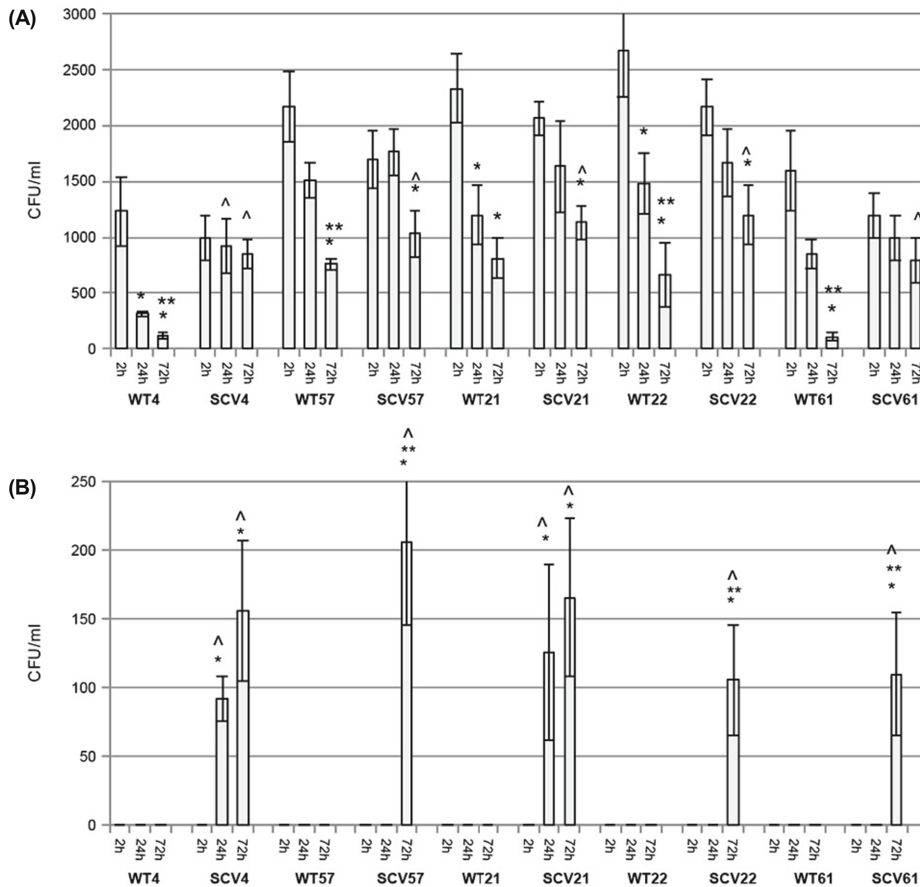

Fig. 1. Internalization and extracellular shift of *S. epidermidis* WT and SCV strains after phagocytosis. Data collected after 2 h, 24 h, and 72 h post-infection. Number of bacteria (CFU) present in cell lysates (A) and conditioned medium (B) are presented. Presence of the bacteria inside macrophages was evaluated using lysostphin protection assay. Data presented are the averages of 3 independent experiments (gray bars). Number of bacteria present in cell lysates (A) and conditioned medium (B) were counted after 2 h, 24 h and 72 h post-phagocytosis. Differences within groups were significant at *P*<0.05 (*) at 95% confidence level when compared with 2h time point post infection. Differences within groups were significant at *P*<0.05 (**) when 24 h time points were compared to 72 h time points post infection. Differences between SCVs and the parental WTs were significant at *P*<0.05 (^) when compared with corresponding time point.

strains, the infected macrophages were lysed at 2 h, 24 h, and 72 h post-phagocytosis and plated onto a TSA medium. Based on the data presented in Fig. 1, the number of viable intracellular bacteria, both WT and SCV, decreased gradually between 2 h and 72 h. The CFU of engulfed SCVs was significantly higher as compared to their parent WT strains in each of the tested pairs at 72 h post-infection (Fig. 1A).

The conditioned medium of the infected cultures was sterile within 24 h post-infection. After which time, two SCV strains (SCV 4, SCV 21) were cultured from the medium. On the third day after internalisation, it was observed that all SCV strains were present in the conditioned medium (Fig. 1B). This may indicate that a small number of surviving intracellular bacteria escaped intracellular milieu and multiplied in the media.

To verify cell viability of macrophages after infection by WT and SCV strains, a fluorescence microscopy analysis was performed. Notably, SCV-infected cells had 100% intact cell membranes within the first 24 h post infection (PI negative staining). After 72h post infection, 4% of cells were noted dead (PI positive staining). The integrity of plasma membrane of macrophages infected with WT strains of *S. epidermidis* was decreased during time: within the first 2 h post infection, 3% of cells were noted dead, after 24 h the number decreased to 5% , and after 72 h post-infection up to 10% of cells were dead.

The effect of IFN-γ pre-treatment on *S. epidermidis* WT and SCV strains internalisation by macrophages

Interferon-γ plays a central role in host defense against intracellular pathogens (Boelens *et al.*, 2000b; Schroder *et al.*, 2004). Therefore, it was decided to investigate the effect of this cytokine on intracellular survival of the staphylococci strains. As shown in Fig. 2, the number of bacterial CFU in macrophage lysates was significantly decreased within 24 h post-infection with nonculturable bacteria present after 72 h post-infection. Interestingly, all tested SCV strains of *S. epidermidis* were present in the conditioned medium after 24 h and 72 h post-phagocytosis, whereas WT strains were absent in the media collected from IFN-γ pre-treated cultures.

IFN-γ induced a significant decrease in the number of all tested *S. epidermidis* strains, both WT and SCV. These results demonstrate that the ability of macrophages to eliminate pathogens is increased when the signalling pathway initiated by IFN-γ is activated (Schroder *et al.*, 2004).

The induction of co-inhibitory molecules PD-L1/PD-L2 by *S. epidermidis* SCV and WT strains

The PD-L1 (CD274) and PD-L2 (CD273) pathway deliver negative signals to balance T cell activation and tolerance (Keir *et al.*, 2008). To test whether *S. epidermidis* also impact negative regulatory pathways the expression of PD-L1 and PD-L2 co-inhibitory molecules on macrophages after *S. epi-*

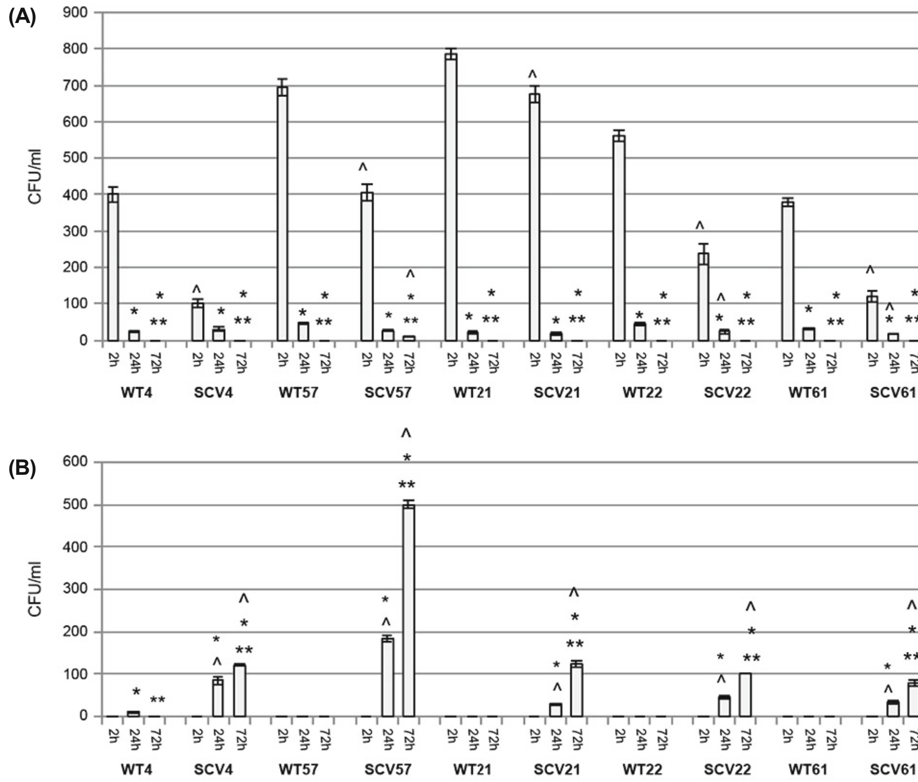

Fig. 2. Effect of IFN-γ treatment on *S. epidermidis* WT and SCV strains internalization by macrophages. Data presented at 2 h, 24 h, and 72 h post-infection in cell lysates (A) and conditioned medium (B). Macrophages were preincubated overnight with 100 U/ml IFN-γ, then the cells were exposed to *S. epidermidis* strains. Presence of the bacteria inside macrophages was evaluated using lysostphin protection assay. Data presented are the averages of 3 independent experiments. The number of bacterial CFU in macrophage lysates (A) and in conditioned medium (B) were counted after 2 h, 24 h and 72 h post-phagocytosis. Differences within groups were significant at $P<0.05$ (*) at 95% confidence level when compared with 2 h time point post infection. Differences within groups were significant at $P<0.05$ (**) when 24 h time points were compared to 72 h time points post infection. Differences between SCVs and the parental WTs were significant at $P<0.05$ (^) when compared with corresponding time point.

dermidis WT and SCV strains treatment was measured. Presence of the molecules were measured after 72 h post-phagocytosis for their maximal expression. As shown in Fig. 3, PD-L1 and PD-L2 expressions were significantly upregulated by *S. epidermidis* WTs and SCVs on human macrophages *in vitro* ($P<0.05$) as compared to non-stimulated cells.

Pre-treatment of the cultures with IFN-γ upregulated PD-L1 on macrophages but the significance was marginal (*P*<

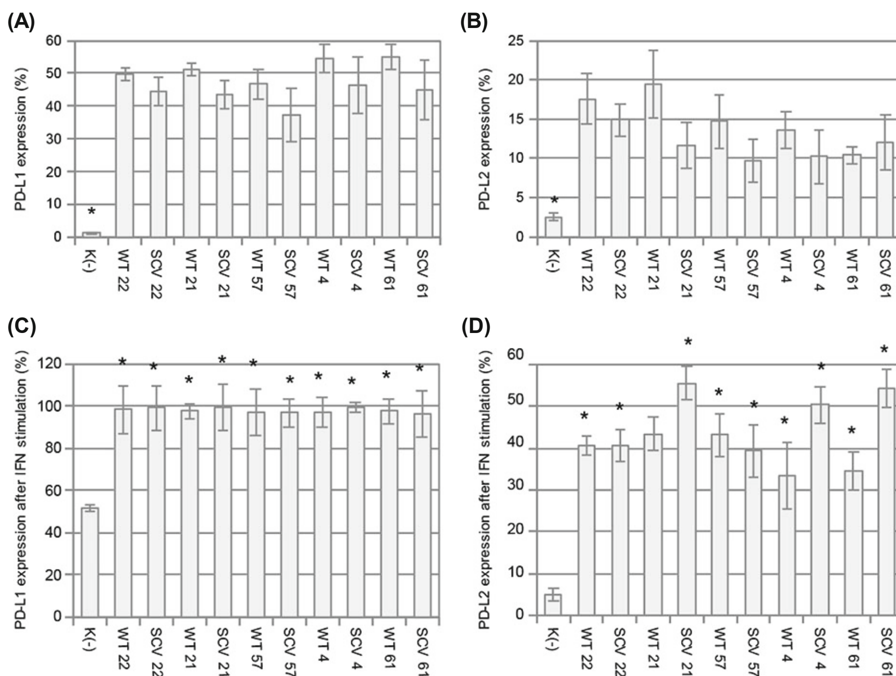

Fig. 3. PD-L1 and PD-L2 expression on macrophages after stimulation with *S. epidermidis* SCV and WT strains. THP-1-differentiated macrophages were treated with *S. epidermidis* SCV and WT strains without (A), (B) and with overnight pre-incubation with IFN-γ (C), (D). All data represents the mean±SD from three independent experiments. (A) Expression of PD-L1 and (B) PD-L2 was significantly higher on macrophages stimulated with *S. epidermidis* strains comparing to control group of unstimulated macrophages (K-) (*$P<0.05$). (C) IFN-γ increased expression of PD-L1 on macrophages with marginal significance ($P<0.02$). But all SCV and all WT strains significantly increased the expression of PD-L1 as well as (D) PD-L2 on the cells, when compared with macrophages stimulated with bacteria alone (*$P<0.05$).

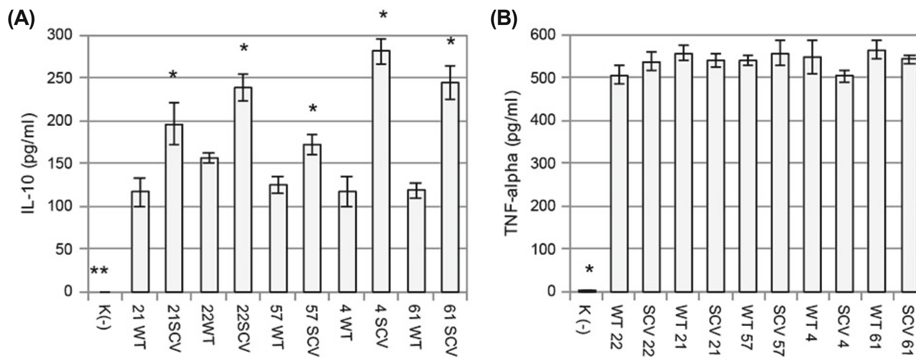

Fig. 4. Effects of *S. epidermidis* SCV and WT strains on the induction of IL-10 (A) and TNF-α (B). Cytokines (A, IL-10; B, TNF-alpha) were measured by ELISA 24 h after stimulation with *S. epidermidis* SCV and WT. Shown are the averages of at least 3 independent experiments. (A) Statistical significance was noticed between negative control of unstimulated macrophages: K(-) and all bacterial strains (***P*<0.05) as well as between all tested *S. epidermidis* pairs (**P* <0.05). (B) all *S. epidermidis* WT and SCV strains stimulated TNF alpha synthesis at significantly higher level than measured in control group (**P*<0.05).

0.02). However, when IFN-γ pre-treated macrophages were stimulated with *S. epidermidis* SCV and WT strains, a significant increase in the expression of PD-L1 as well as PD-L2 on the cells was observed, as compared to macrophages stimulated with bacteria alone (*P*<0.05).

The induction of pro-inflammatory and anti-inflammatory cytokines by *S. epidermidis* SCV and WT strains

The above-mentioned findings showed that PD-L1 and PD-L2 were expressed on macrophages stimulated with WT strains and SCV strains of *S. epidermidis*. This led to a question whether these expression patterns were associated with specific cytokine profiles. It has been shown that pro-inflammatory cytokine TNF-α and anti-inflammatory IL-10 are involved in the induction of PD-L1 expression (Rodriguez-Garcia *et al*., 2011). Therefore, in the next step the levels of these two cytokines were evaluated in the supernatants from macrophages culture stimulated with different *S. epidermidis* WT and SCV strains for 24 h.

As shown in Fig. 4A, the level of IL-10 was significantly increased by incubating the macrophages with bacteria (*P*< 0.05). Moreover, it was observed that SCVs significantly enhanced the synthesis of IL-10 as compared to WTs (*P*<0.05). In contrast, the extracellular levels of TNF-α (Fig. 4B) did not differ significantly between bacterial strains, however they induced significant up-regulation of the cytokine relative to untreated macrophages (*P*<0.05). These results suggest that during *S. epidermidis* stimulation of macrophages the extracellular levels of TNF-α, as well as IL-10 are significantly increased. It was also noted that in response to SCVs stimulation, macrophages produced significantly more anti-inflammatory cytokine IL-10, in comparison with their parent WT strains.

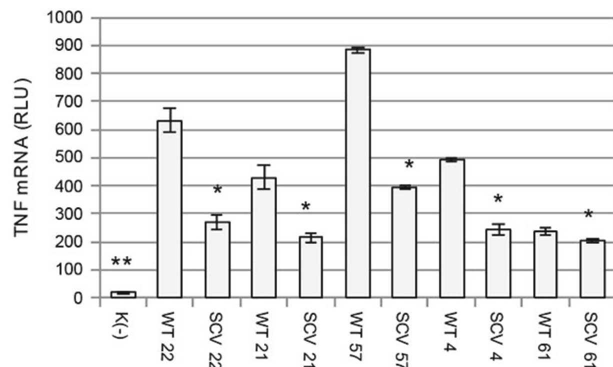

Fig. 5. Effect of *S. epidermidis* SCV and WT strains on transcripional induction TNF-α. TNF mRNA was measured by chemiluminescence hybridization assay. All data represents the averages of at least 3 independent experiments±SD. Statistical significance was noticed between negative control of unstimulated cells (K-) and bacterial strains (***P*<0.05) and between all SCVs and WTs pairs (**P*<0.05).

The transcriptional induction of TNF- α by *S. epidermidis* WT vs SCV strains

To determine the impact of SCVs on transcriptional regulation of TNF-α in human macrophages, chemiluminescence hybridisation assay for the above-mentioned cytokine was performed. As shown in Fig. 5, mRNA for TNF-α was induced by WT, as well as SCV strains of studied bacteria. The level of TNF-α gene transcription was significantly enhanced in all *S. epidermidis* WT-stimulated macrophage cultures as compared to SCVs (*P*<0.05).

Discussion

A serious problem associated with the use of implanted medical devices is the occurrence of bacterial infections due to staphylococcal species, in particular *S. epidermidis* (Boelens *et al*., 2000b; Zaat *et al*., 2010; Mack *et al*., 2013). The staphylococcal BAI are characterised by their chronic nature associated with reduced susceptibility to antibiotics and facultative intracellular growth of invading bacteria. These infections are particularly associated with the SCV phenotype (Tuchscherr *et al*., 2010; Grant and Hung, 2013).

The present study supports the opinion that switch to the SCV phenotype could be a mechanism exploited by the wild type strains to facilitate their survival within the host and contribute to the recurrence and persistence of the infection (Tuchscherr *et al*., 2010). First, SCVs are able to survive the phagocytosis by macrophages. Second, they have the potential to disable host immunity through the induction of co-inhibitory molecule PD-L1 and modulation of cytokine profile.

Macrophages have been shown to contribute significantly to the initial steps of BAI (Subbiahdoss *et al*., 2011). These cells arrive within minutes to hours at an implant site where they confront invading pathogens as first, activating cellular

functions to destroy phagocytosed bacteria (Subbiahdoss *et al*., 2011). However, it has been postulated that professional phagocytes may also be an important reservoir for a small group of microorganisms that can persist inside these cells evading host defense mechanisms and contributing to pathogenesis of BAI (Boelens *et al*., 2000a; Koziel *et al*., 2009; Zaat *et al*., 2010; Subbiahdoss *et al*., 2011). Apart from typical intracellular pathogens, SCVs of staphylococci were found to be capable of surviving inside macrophages and being more resistant to intracellular host defenses than WT strains (Koziel *et al*., 2009; Fraunholz and Sinha, 2012; Shi and Zhang, 2012). In the present study, using THP-1 - differentiated macrophages it was proved that *S. epidermidis*, both WT and SCV strains can survive intracellularly for at least 3 days without significant alterations in cells' viability. This observation corresponds to clinical findings which state that BAI caused by *S. epidermidis* usually progresses less aggressively than an infection developed by more virulent bacteria, such as *S. aureus* (Subbiahdoss *et al*., 2011). Several studies focusing on BAI have pointed out that *S. epidermidis* has low virulence and infections caused by this bacterium are most commonly implicated in delayed septic loosening of joint prostheses or aseptic loosening (Vuong and Otto, 2002; Zaat *et al*., 2010; Subbiahdoss *et al*., 2011).

To compare the intracellular fate of SCVs and their corresponding WT strains, macrophages were infected by both types of pathogens and the number of viable intracellular bacteria was counted after 2 h, 24 h, and 72 h post infection. The conducted studies revealed that the number of engulfed bacteria of both phenotypes fell gradually in their numbers between 2 h and 72 h post infection. We observed that the number of SCVs shortly after internalization (2 h) was lower comparing to the WT strains. This suggests that SCVs have impaired ability to initiate contact with cells when compared with WT strains. Moreover, the recovery rate after 2 h, 24 h, and 72 h post-phagocytosis differed between strains, suggesting that the tested strains are differently adapted to the intracellular milieu. Interestingly, the number of WT strains decreased in large amounts within the first 24 h post-phagocytosis, whereas the number of SCVs was barely reduced during the study time. Moreover, all tested SCV strains were present in the conditioned medium after 72 h with a slight alteration in macrophages' viability, whereas an extracellular shift was not observed in case of the WTs. These findings can be explained by the fact that SCVs are particularly adapted to the intracellular environment for long-term persistence (Tuchscherr *et al*., 2010). Tuchscherr *et al*. (2010) suspects that due to a slow growth rate of SCVs they release fewer products from cell turnover, such as bacterial DNA and cell wall components, which are known to activate host cells. The fact that after some time bacteria can exit the intracellular compartment appearing in the media is supported by clinical observations in which infections by SCV phenotypes persisted asymptomatically for many years, until the time when certain physical conditions of the patients disturbed the balance between the bacteria and the host response giving rise to recurrent infections (Boelens *et al*., 2000a; Tuchscherr *et al*., 2010; Subbiahdoss *et al*., 2011).

IFN-γ is probably the most important cytokine for the immune response to intracellular bacteria which stimulates intracellular killing of the infecting agent (Schroder *et al*., 2004). Since the survival of bacteria inside macrophages seems to be a pivotal process in the pathogenesis of BAI, in the present study it was determined whether the immunomodulatory effect of IFN-γ has an impact on intracellular survival of staphylococcal strains. It was found that the survival of *S. epidermidis* in THP-1 – differentiated macrophages primed with IFN-γ was greatly reduced, with no difference observed between the wild type and corresponding SCV strains. It can thus be speculated that IFN-γ prevents intracellular persistence of *S. epidermidis* (Boelens *et al*., 2000b). It should be noted that rapid production of IFN-γ is the key factor in resistance of the host to intracellular pathogens (Boelens *et al*., 2000b). This fact might explain the observation that the numbers of internalised bacteria in IFN-γ pretreated macrophages were low even within 2 h post-phagocytosis in relation to the number of bacteria engulfed by untreated macrophages. In the present study it was noticed that bacteria with SCV phenotypes were able to escape from IFN-γ primed macrophages into the conditioned medium after 24 h of incubation and doubled their number during the following 48 h. The escape from the macrophages appears to be critical for avoiding rapid effects of the IFN-γ activation as evidenced by the significant killing of the WT strains.

Recent studies suggest that the PD-1/PD-L1 pathway is exploited by pathogenic microbes during a persistent infection as a strategy for impairment of protective immunity, carrying inhibitory signals to activated T-cells (Sakai *et al*., 2010; Rowe *et al*., 2012; Wang *et al*., 2012). To explore the role of PD-L1/L2 in BAI, PD-L1, and PD-L2 expressions on macrophages in response to *S. epidermidis* WT and SCV strains isolated from patients with orthopaedic implants loosening were investigated. It was found that macrophages expressed low, constitutive levels of PD-L1 and that they were markedly up-regulated upon *in vitro* exposure to both phenotypes of bacteria. The expression of PD-L2 in the absence of stimulation was higher than PD-L1 expression and was only weakly enhanced upon bacterial stimulation. These results are in agreement with previous observations in monocytes, where PD-L1 was up-regulated after LPS treatment (Wang *et al*., 2012). This study suggests that PD-L1 may play a role in the pathogenesis of BAI developed by *S. epidermidis*. As both phenotypes of applied bacteria were able to elevate the level of PD-L1 expression on macrophages after 72 h, it was concluded that both phenotypes can persist intracellularly in macrophages. Such high expression of PD-L1 on phagocytic cells has already been described during chronic viral infections (Rodriguez-Garcia *et al*., 2011). However, the above-described data indicate that the number of SCVs persisting inside macrophages was insignificantly reduced during the study time, whereas CFUs of engulfed WTs were noticeably decreased. Several studies have demonstrated that in most cases the up-regulation of PD-L1 is associated with persistent antigen presence, and clearance of the antigen can result in a reduction of PD-L1 expression (Sharpie *et al*., 2007; Hofmeyer *et al*., 2011). This, in turn, may indicate that *S. epidermidis* SCVs are better adapted for prolonged intracellular persistence and evasion of host defense.

PD-L1 expression can provide either immune activation or suppression signals, and these divergent roles are most likely

controlled by differences in the cytokine profile (Rowe *et al.*, 2012). In this study, both SCVs and their related WT strains evoked cytokines production by macrophages. TNF-α and IL-10 were produced following incubation with both phenotypes. But only SCVs showed significantly increased IL-10 production as compared to WT strains, whereas there was no significant difference in TNF-α levels.

An early host defense against intracellular pathogens is the induction, within infected cells, of TNF-α gene transcription (Baer *et al.*, 1998). In this study, we examined the effect of *S. epidermidis* WT and SCV strains infection on the transcription of the TNF-α genes in THP-1 differentiated macrophages. Although all tested bacterial strains induced the transcription of TNF-α, the transcription of genes was significantly lower in macrophages stimulated with SCV strains than in case of WT strains. Therefore, SCVs were able to infect these cells with lower, compared to WTs, triggering the transcription of genes encoding proteins important in immediate cellular defenses. Our results showed that for TNF-α, the expression levels and protein levels differ. One explanation of this may be the fact that we measured the level of secreted TNF-α instead of its total intracellular concentration.

One factor that inhibits TNF-α expression is IL-10, an anti-inflammatory cytokine that is known to suppress expression of several proinflammatory cytokines (Baer *et al.*, 1998). The findings indicate that IL-10 functions as an autocrine feedback signal that attenuates the production of proinflammatory cytokine, TNF-α, in activated macrophages (Wang *et al.*, 2012). These results may partly explain how bacterial SCVs are able to establish persistent intracellular infections and escape acute host responses that have evolved to combat infections by intracellular pathogens.

To conclude, the ability of *S. epidermidis* SCVs to induce an elevated level of anti-inflammatory cytokine, IL-10, and reduced transcriptional induction of TNF-α, together with expression of PD-L1 on macrophages and the ability to persist intracellularly without damaging the host cell could be the key factor contributing to the chronicity of SCV infections.

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