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Dormant bacteria within *Staphylococcus epidermidis* biofilms have low inflammatory properties and maintain tolerance to vancomycin and penicillin after entering planktonic growth

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Staphylococcus epidermidis is the most commonly isolated aetiological agent of nosocomial infections, mainly due to its ability to establish biofilms on indwelling medical devices. Detachment of bacteria from *S. epidermidis* biofilms and subsequent growth in the planktonic form is a hallmark of the pathogenesis of these infections leading to dissemination. Here we showed that *S. epidermidis* cells collected from biofilms cultured in conditions that promote cell viability present marked changes in their physiological status upon initiating a planktonic mode of growth. When compared to cells growing in biofilms, they displayed an increased SYBR green I staining intensity, increased transcription of the *rpiA* gene, decreased transcription of the *icaA* gene, as well as higher susceptibility to vancomycin and penicillin. When bacteria collected from biofilms with high proportions of dormant cells were subsequently cultured in the planktonic mode, a large proportion of cells maintained a low SYBR green I staining intensity and increased resistance to vancomycin and penicillin, a profile typical of dormant cells. This phenotype further associated with a decreased ability of these biofilm-derived cells to induce the production of pro-inflammatory cytokines by bone marrow-derived dendritic cells *in vitro*. These results demonstrated that cells detached from the biofilm maintain a dormant cell-like phenotype, having a low pro-inflammatory effect and decreased susceptibility to antibiotics, suggesting these cells may contribute to the recalcitrant nature of biofilm infections.

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INTRODUCTION

Staphylococcus epidermidis is a commensal bacterium that colonizes the skin and mucous membranes, often being the most prevalent staphylococcal species found on human skin (Otto, 2009). The ability to colonize and establish biofilms on indwelling medical devices makes this bacterium the most commonly isolated aetiological agent of nosocomial infections (Uçkay *et al.*, 2009). Moreover, due to the intrinsic resistance of staphylococcal biofilms to antibiotics (Raad *et al.*, 1998), staphylococcal biofilm-originated infections are associated with an increased duration of hospital stays and use of medical resources and, consequently, increased healthcare costs (Dimick *et al.*, 2001; Rogers *et al.*, 2009). Critically ill immune-compromised patients (Bearman & Wenzel, 2005) and premature neonates (Fallat *et al.*, 1998) are the individuals most vulnerable to this opportunistic pathogen.

S. epidermidis biofilm formation involves initial cellular adherence to a surface followed by intercellular aggregation and accumulation in multilayer cell clusters (Otto, 2009). This process is dependent on the synthesis of adhesive extracellular molecules (Götz, 2002), such as the polysaccharide intercellular adhesin (PIA), also known as poly-*N*-acetylglucosamine (PNAG), a major constituent mediating cell-to-cell adhesion in staphylococci (Mack *et al.*, 1994,

Abbreviations: BMDC, bone marrow-derived dendritic cell; MFI, mean fluorescence intensity; PI, propidium iodide; qPCR, quantitative PCR.

1996). The final stage of the biofilm life cycle comprises cell detachment and subsequent growth in the planktonic form, a process that is crucial for *S. epidermidis* biofilm pathogenesis by forming the basis for dissemination of infection (Wang *et al.*, 2011). Therefore, in this study we evaluated physiological changes occurring in *S. epidermidis* bacteria during the shift from the biofilm to the planktonic mode of growth. We show that dormant bacteria within *S. epidermidis* biofilms display a low inflammatory profile and increased tolerance to vancomycin and penicillin upon initiating a planktonic growth.

METHODS

Bacterial strains and growth conditions. *S. epidermidis* strain 9142 (Nedelmann *et al.*, 1998) was used in this study. To establish a 48 h biofilm, a starter culture was grown overnight in tryptic soy broth (TSB) (Merck) at 37 °C with agitation (80 r.p.m.). The optical density of the starter culture at 640 nm was adjusted to 0.250 with PBS and a 10 µl aliquot transferred to a 24-well polystyrene plate (Nunc) containing 1 ml per well of TSB with 0.4 % (w/v) filtered glucose (Merck), further supplemented with 10 mM $MgCl_2$ (Merck). The plates were then incubated for 48 h at 37 °C with agitation (80 r.p.m.). At 24 h of growth, the culture medium was replaced by fresh TSB supplemented with 1 % glucose and 10 mM $MgCl_2$ (TSB 1 %G + Mg^{2+}). Similar culture conditions were used to establish 48 h biofilms with high proportions of dormant bacteria by using growth medium without $MgCl_2$ supplementation, as previously described (Cerca *et al.*, 2011a). The proportions of dormant bacteria within biofilms were assessed by calculating the ratio of culturable bacteria (quantified by tryptic soy agar plating) over the number of viable bacteria (quantified by flow cytometry, using propidium iodide (PI) to discriminate live and dead bacteria, as described below). The proportions of dormant bacteria within the biofilms grown in excess glucose without $MgCl_2$ supplementation typically ranged between 85 and 90 % while $MgCl_2$ supplemented biofilms typically presented less than 5 % of dormant cells.

Preparation of biofilm and planktonic *S. epidermidis* cell suspensions. To assess the bacterial physiological status during the transition from the biofilm to the planktonic mode of growth, 48 h biofilms grown in TSB 1 %G + Mg^{2+} were disaggregated as previously described (Cerca *et al.*, 2011b) and the resulting cell suspensions (typically yielding 3–5 × 10^9 cells ml^{-1}) were diluted in fresh TSB to a concentration of 1 × 10^8 cells ml^{-1}. These cell suspensions were allowed to grow in the planktonic form for a period of 6 h (37 °C, 80 r.p.m.). Simultaneously, the culture medium of 48 h biofilm cultures was washed out and biofilms were allowed to grow for an additional 6 h period in 1 ml of fresh TSB. At the time points 0 h, 1 h 30 min, 3 h and 6 h of growth, an aliquot of bacteria was recovered from each culture (biofilm and planktonic) and used for flow cytometry analysis and gene expression quantification, as described below.

Flow cytometry analysis of bacteria. At the indicated time points, biofilms were washed twice with 1 ml of PBS, and bacteria were then recovered in 1 ml PBS, as previously described (Cerca *et al.*, 2011b). After a 1 : 10 dilution in PBS, an aliquot of 30 µl was transferred to 270 µl of PBS containing 3 µl of quantification microspheres (Invitrogen), SYBR green I (Invitrogen) (1 : 5000 commercial stock) and 5 µg of PI ml^{-1} (Sigma). For the planktonic cultures, an aliquot of 30 µl of cells was transferred from the culture to 270 µl of PBS containing 3 µl of quantification microspheres, SYBR green I (1 : 5000 commercial stock) and 5 µg of PI ml^{-1}. The SYBR and PI concentrations used were optimized as previously described (Cerca *et al.*, 2011b). Bacterial fluorescence analysis was carried out by using a FACScan flow cytometer (Becton Dickinson) containing a low-power air-cooled 15 mW blue (488 nm) argon laser. Data were acquired using CellQuest software (Becton Dickinson) and analysed using Flowjo 7.2.5 software (Tree Star). SYBR fluorescence was detected on the FL1 channel (BP530/30), while PI fluorescence was detected on the FL3 channel (LP650). For all detected parameters, amplification was carried out using logarithmic scales. The concentration of bacteria in the planktonic or biofilm cultures was further determined by acquiring the counts for a specific number of microspheres during flow cytometry analysis of the cell samples.

Quantitative PCR (qPCR). qPCR was used to assess the expression of intercellular adhesin A (*icaA*) and ribose-5-phosphate isomerase A (*rpiA*) encoding genes in *S. epidermidis* bacteria grown in biofilm or planktonic cultures. The primers used were designed with Primer3 software (Rozen & Skaletsky, 2000) using the *S. epidermidis* RP62A genome as a template (GenBank accession no. NC_002976.3). The sequences of the primers used are listed in Table 1. Primer efficiency was determined by the dilution method and by performing a temperature gradient reaction from 54 to 64 °C. The set of primers having the optimal and most similar efficiency values at 60 °C was used. At each time point, total RNA from either biofilm or planktonic cultures was extracted using a FastRNA Pro Blue kit (MP Biomedicals), as described previously (França *et al.*, 2011). Contaminating genomic DNA was removed by treatment with DNase I (Fermentas) for 30 min at 37 °C. The enzyme was then heat inactivated at 65 °C for 10 min in the presence of EDTA. Total RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and stored at −80 °C. Total RNA samples were reverse transcribed in the presence of each reverse primer and RevertAid M-MuLV reverse transcriptase (Fermentas). Control reactions lacking the reverse transcriptase enzyme (no-RT) were included. qPCRs contained 2 µl of 1 : 200 diluted cDNA or no-RT control, 2 µl (containing 10 pmol) of each primer, 6 µl of nuclease-free deionized H_2O and 10 µl of Maxima SYBR green qPCR master mix (Fermentas). The following thermocycler parameters were used: 94 °C for 10 min; 40 cycles of 94 °C for 15 s, 60 °C for 20 s and finally 72 °C for 25 s. To monitor the reaction specificity and primer dimer formation, end products were analysed by melting curves. Relative fold increase was calculated using the $2^{\Delta Ct}$ method, a variation of the Livak method, where $\Delta C_t = C_t$ (housekeeping gene) $- C_t$ (target gene). The data analysis was based on at least two independent experiments.

Evaluation of the susceptibility to vancomycin and penicillin of *S. epidermidis* biofilm bacteria after initiating planktonic growth. *S. epidermidis* bacteria obtained from 48 h biofilms grown in TSB 1 %G + Mg^{2+} were diluted in fresh TSB (1 × 10^8 cells ml^{-1}) and allowed to grow in the planktonic form for a 3 h period. Simultaneously, cultures of 48 h *S. epidermidis* biofilms were allowed to grow for an additional 3 h period in fresh TSB. At this time point, vancomycin (40 µg ml^{-1}) (Sigma), penicillin (40 µg ml^{-1}) (Sigma) or TSB (negative control) was added to the biofilm and planktonic cultures, which were incubated for 30 min. The antibiotic concentrations used resulted in comparable proportions of death in the planktonic cultures. Bacterial death was determined through flow cytometry by assessing the bacterial incorporation of PI, as described above. A similar procedure was carried out using 48 h biofilms grown in TSB 1 %G without magnesium supplementation as a starting point.

Mice. Male BALB/c mice of 6–8 weeks of age were purchased from Charles River and kept at the animal facilities of the Institute Abel Salazar (ICBAS; Portugal) during the experiments. Hiding and nesting materials were provided for enrichment. Procedures involving mice were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC directive and Portuguese rules (DL 129/92). Authorization to perform the experiments was issued

Table 1. Oligonucleotide primers used for qPCR

Target gene	Orientation	Sequence (5′→3′)	TM (°C)	Amplicon size (bp)
16S	FW	GGGCTACACACGTGCTACAA	59.8	176
	RV	GTACAAGACCCGGGAACGTA	59.9	
icaA	FW	TGCACTCAATGAGGGAATCA	60.2	134
	RV	TAACTGCGCCTAATTTTGGATT	59.9	
rpiA	FW	CAACAACGACAAATCGGTCA	60.5	114
	RV	CAATAGATGGCGCTGATGAA	59.8	

FW, Forward; RV, reverse; TM, melting temperature.

by the competent national board (Direção Geral de Veterinária), document number 0420/000/000/2008.

Bone marrow-derived dendritic cells (BMDCs) differentiation. Bone marrow cells were collected from femurs and tibias of BALB/c mice by flushing with cold RPMI 1640 (Sigma). Cells (1×10^6 ml^{-1}) were cultured in six-well plates in RPMI supplemented with 15 % (v/v) J558-cell supernatant, 10 % fetal bovine serum (FBS) (PAA), penicillin (100 I.U. ml^{-1})/streptomycin (100 μg ml^{-1}) (Sigma) and L-glutamine (2 mM) (Sigma), and incubated at 37 °C, 5 % CO_2. Half of the media was renewed every 2 days. On day 6, BMDCs were detached, adjusted at a concentration of 1×10^6 cells ml^{-1}, distributed in 96-well round-bottom plates (100 μl per well) and incubated overnight in supplemented RPMI medium.

Stimulation of BMDCs with bacterial suspensions of *S. epidermidis* obtained from different cultures. Bacterial suspensions were obtained from 48 h biofilms grown in TSB 1 %G or TSB 1 %G + Mg^{2+} as described above. For planktonic cultures, a single colony of *S. epidermidis* was inoculated into 35 ml of TSB and grown overnight at 37 °C in a shaker rotator at 80 r.p.m. Then, cells were recovered, centrifuged for 10 min at 13 000 r.p.m. at 4 °C (Heraeus biofuge fresco; Thermo Scientific) and resuspended in 1 ml of PBS. Before stimulation of BMDCs, all the bacterial inocula were resuspended in RPMI supplemented with 10 % FBS and 2 mM L-glutamine and adjusted to a concentration of 1×10^6 cells ml^{-1}. BMDCs were then stimulated with 100 μl bacterial suspensions (1×10^6 cells ml^{-1}) obtained from biofilms grown in TSB 1 %G or in TSB 1 %G + Mg^{2+}, or from original planktonic cultures. RPMI supplemented media and LPS (1 μg ml^{-1}) (Sigma) were used as negative and positive controls, respectively. After 6 h incubation (37 °C, 5 % CO_2), medium containing bacteria was collected and replaced by fresh medium containing penicillin (200 I.U. ml^{-1}) and streptomycin (200 μg ml^{-1}). At the 6 or 18 h time points, the culture supernatants were removed and stored at −20 °C until use. For the assessment of cell surface markers, BMDCs were collected from the culture plates and washed twice in Hanks's balanced salt solution (Sigma). A subset of 5×10^5 BMDCs were stained per sample. The following mAbs, along with the respective isotype controls, were used (at previously determined optimal dilutions) for immunofluorescence cytometric data acquisition in a Coulter EPICS XL flow cytometer (Beckman Coulter): FITC hamster anti-mouse CD11c (HL3), PE anti-mouse CD80 PE (clone 16-10A1); PE anti-mouse CD86 (clone GL1); PE anti-mouse I-Ad/I-Ed (clone 2G9); PE isotype-matched controls (PE-rat IgG2a,κ, clone R35-95; PE hamster IgG2, κ clone B81-3); all from BD Biosciences Pharmingen.

All the analysed cell samples were pre-incubated with anti-FcγR (a kind gift of Dr Jocelyne Demengeot, Gulbenkian Institute of Science, Oeiras, Portugal) before the staining. Data were analysed by using CELLQUEST software (Becton Dickinson).

Cytokine quantification. IL-6, IL-10, IL-12p70 and TNF-α were quantified using commercially available quantification kits (eBioscience) according to the manufacturer's instructions. Results were read in a Multiskan Ex spectrophotometer (Thermo Electron) using Ascent software (Thermo Electron).

Statistical analysis. All graphs were generated using GraphPad Prism software (GraphPad Software). Means and SDs were calculated. Statistical analysis was carried out by two-way repeated-measures ANOVA with Bonferroni post hoc tests or with one-way ANOVA with Tukey's multiple comparison test. Both tests were performed using GraphPad software. A P value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

S. epidermidis cells that undergo a shift from biofilm to planktonic growth present a high SYBR staining intensity and *rpiA* gene expression

Detachment of bacteria from a biofilm and subsequent growth in the planktonic form is considered a major event in the pathophysiology of biofilm-related infections (Otto, 2013). In *S. epidermidis* this was previously shown to be followed by a transition from a non-aggressive, non-growing and fermentative state (biofilm stage of growth) into a growing, aggressive/inflammatory and respiratory state (planktonic stage of growth) (Yao *et al.*, 2005). SYBR staining intensity was previously found to correlate with *S. epidermidis* respiratory activity, as demonstrated by co-staining studies using SYBR and the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Cerca *et al.*, 2011c). Therefore, having shown this correlation, we used SYBR as a fluorescent probe to evaluate physiological changes of *S. epidermidis* bacteria during the shift from the biofilm to the planktonic mode of growth. For this purpose, 48 h biofilms grown in TSB media supplemented with glucose and magnesium (TSB 1 %G + Mg^{2+}) were prepared and used as the starting point of our study, as these conditions maintain the majority of cells in a culturable state (Cerca *et al.*, 2011a). As shown in Fig. 1, bacterial suspensions were prepared from the 48 h biofilms and allowed to grow in the planktonic form for a further 6 h.

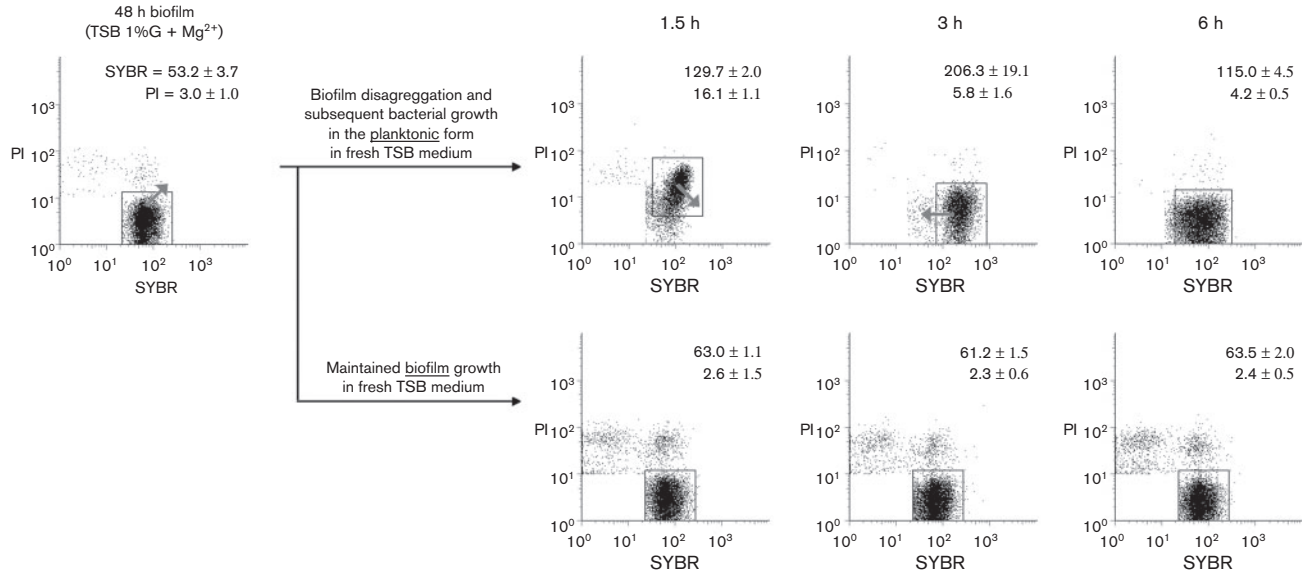

Fig. 1. Evaluation of SYBR staining intensity in *S. epidermidis* bacteria upon the shift from the biofilm to the planktonic mode of growth. Bacteria obtained from low dormancy 48 h biofilms were diluted in fresh TSB (1×10^8 cells ml^{-1}) and allowed to grow in the planktonic form for 6 h. Simultaneously, parallel cultures of 48 h biofilms were allowed to grow for an additional 6 h in fresh TSB. At the indicated time points, bacteria obtained from each culture were stained with SYBR and PI (5 μg ml^{-1}) and the bacterial fluorescence was determined by flow cytometry analysis. Values shown within the bi-parametric dot plots (SYBR vs PI) represent the MFI of the SYBR or PI signal ± SD from bacteria within the respective flow cytometry analysis region. Arrows within the dot plots represent the direction of the shift in the bacterial MFI due to SYBR/PI staining between assessed time points. Results are a representative example of two independent experiments that generated concordant results.

Simultaneously, parallel cultures of *S. epidermidis* biofilms continued to be grown for an additional 6 h in fresh TSB medium. Bacterial cells were then obtained at different time points from either the planktonic or biofilm cultures, stained with SYBR and PI, and analysed by flow cytometry. The bacteria that entered into the planktonic growth phase showed progressive increases in the mean fluorescence intensity (MFI) due to SYBR staining, reaching a detected maximum at the 3 h time point (Fig. 1). In contrast, bacteria that remained growing in the biofilm mode presented a lower and constant SYBR MFI over time (Fig. 1). Our results are in agreement with a previous report showing that the shift from the biofilm to the planktonic mode of growth is accompanied by an increase in the *S. epidermidis* respiratory activity (Yao

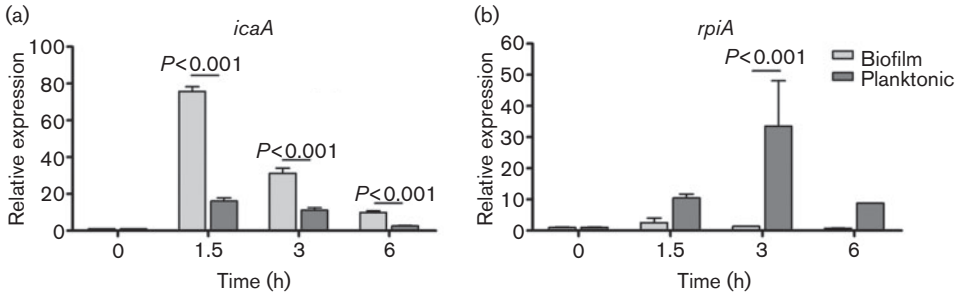

Fig. 2. Evaluation of *icaA* and *rpiA* gene expression over time in *S. epidermidis* bacteria grown in the biofilm or planktonic modes. Bacteria obtained from low dormancy 48 h *S. epidermidis* biofilms were diluted in fresh TSB (1×10^8 cells ml^{-1}) and allowed to grow in the planktonic form for a 6 h period. Simultaneously, parallel cultures of 48 h *S. epidermidis* biofilms were allowed to grow for an additional 6 h period in fresh TSB. At the indicated time points, a sample of bacteria was obtained from each culture and the expression of (a) *icaA* and (b) *rpiA* genes was evaluated by qPCR. Results shown are representative of two independent experiments that generated concordant results. Statistical analysis was carried out by two-way repeated-measures ANOVA with Bonferroni post hoc test.

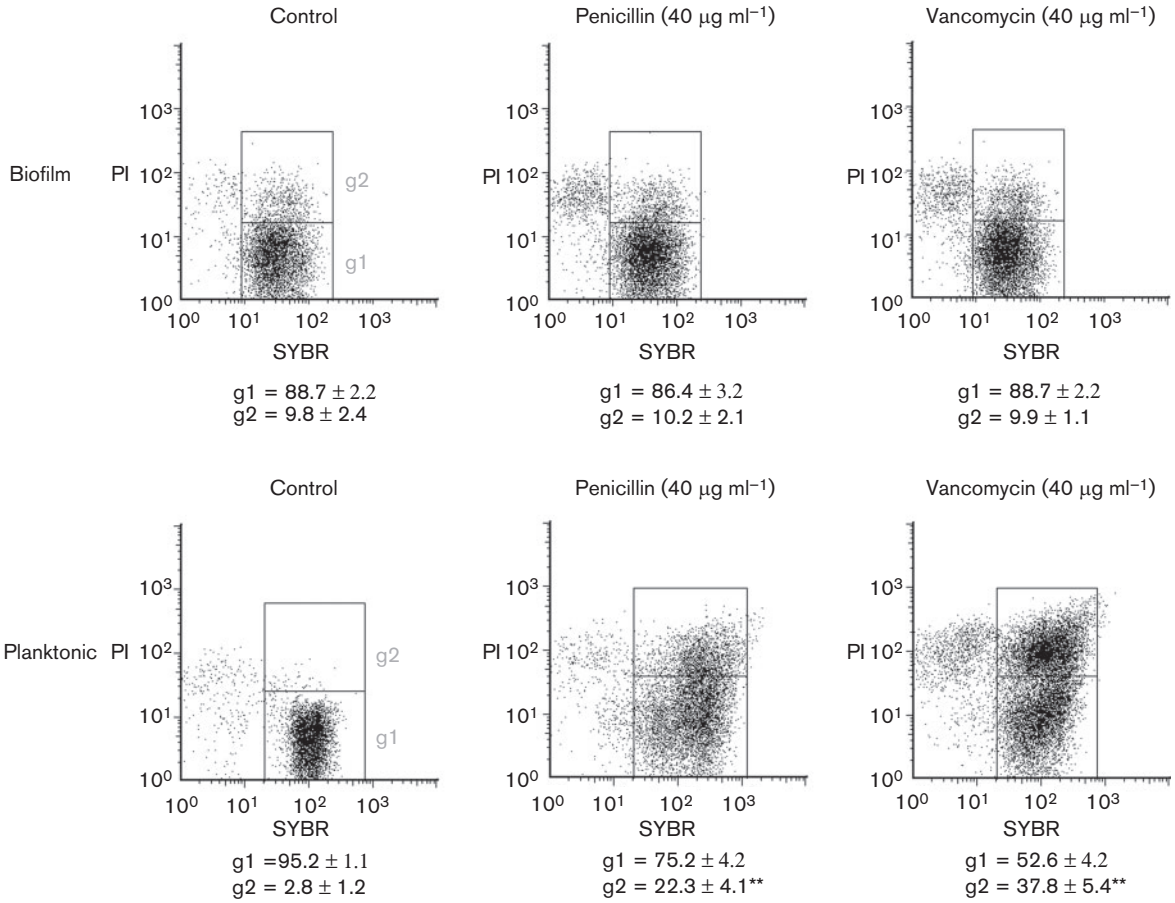

Fig. 3. *S. epidermidis* biofilm bacteria acquire susceptibility to vancomycin and penicillin after initiating a planktonic growth. Bacteria obtained from low dormancy 48 h *S. epidermidis* biofilms were diluted in fresh TSB (1×10^8 cells ml^{-1}) and allowed to grow in the planktonic form for a 3 h period. Simultaneously, parallel cultures of 48 h *S. epidermidis* biofilms were allowed to grow for an additional 3 h period in fresh TSB. At this time point, vancomycin (40 μg ml^{-1}), penicillin (40 μg ml^{-1}) or TSB (negative control) was added to the biofilm and planktonic cultures, which were grown for an additional 30 min. Bacterial death was determined using flow cytometry by assessing the bacterial incorporation of PI. Results shown are representative of two independent experiments that generated concordant results. Statistically significant differences in the proportions of dead PI^+ bacteria (gate g2) between control, vancomycin and penicillin groups ($P<0.01$, ANOVA) are indicated by asterisks (**). Statistical analysis was carried out by two-way repeated-measures ANOVA with Bonferroni post hoc test.

et al., 2005). Interestingly, an increase in PI staining intensity was detected in the bacteria obtained from the planktonic cultures at the 1 h 30 min time point that was not detected on the two later assessed time points. According to previous reports, this transient PI-incorporating state may correspond to temporarily compromised cell membrane integrity due to a fast increase in cell size (Lybarger & Maddock, 2001; Shi *et al.*, 2007). A previous study in which a comparative transcriptome analysis in biofilm and planktonic *Staphylococcus aureus* cells was carried out showed that planktonic bacteria presented an increased respiratory activity and decreased synthesis of poly-*N*-acetylglucosamine as compared with their biofilm counterparts (Resch *et al.*, 2005).

Therefore, in order to further characterize the physiological alterations occurring in *S. epidermidis* bacteria during the transition from the biofilm to the planktonic mode of growth, we evaluated the transcription of *icaA* and *rpiA* mRNA in bacteria obtained from the biofilm or planktonic cultures at different time points. The *icaA* gene encodes a *N*-acetylglucosamine transferase involved in the synthesis of PIA (Heilmann *et al.*, 1996), whereas *rpiA* encodes a ribose-5-phosphate isomerase that participates in the NADPH-generating pentose phosphate pathway (Jeppsson *et al.*, 2002). As shown in Fig. 2(a), and as could be expected, bacteria that remained in the biofilm cultures expressed, over time, significantly higher levels of *icaA* than bacteria that initiated planktonic growth. In contrast, planktonic bacteria expressed significantly higher levels of *rpiA* than their biofilm counterparts (Fig. 2b). The later result, by showing a correlation between $SYBR^{high}$ staining intensity

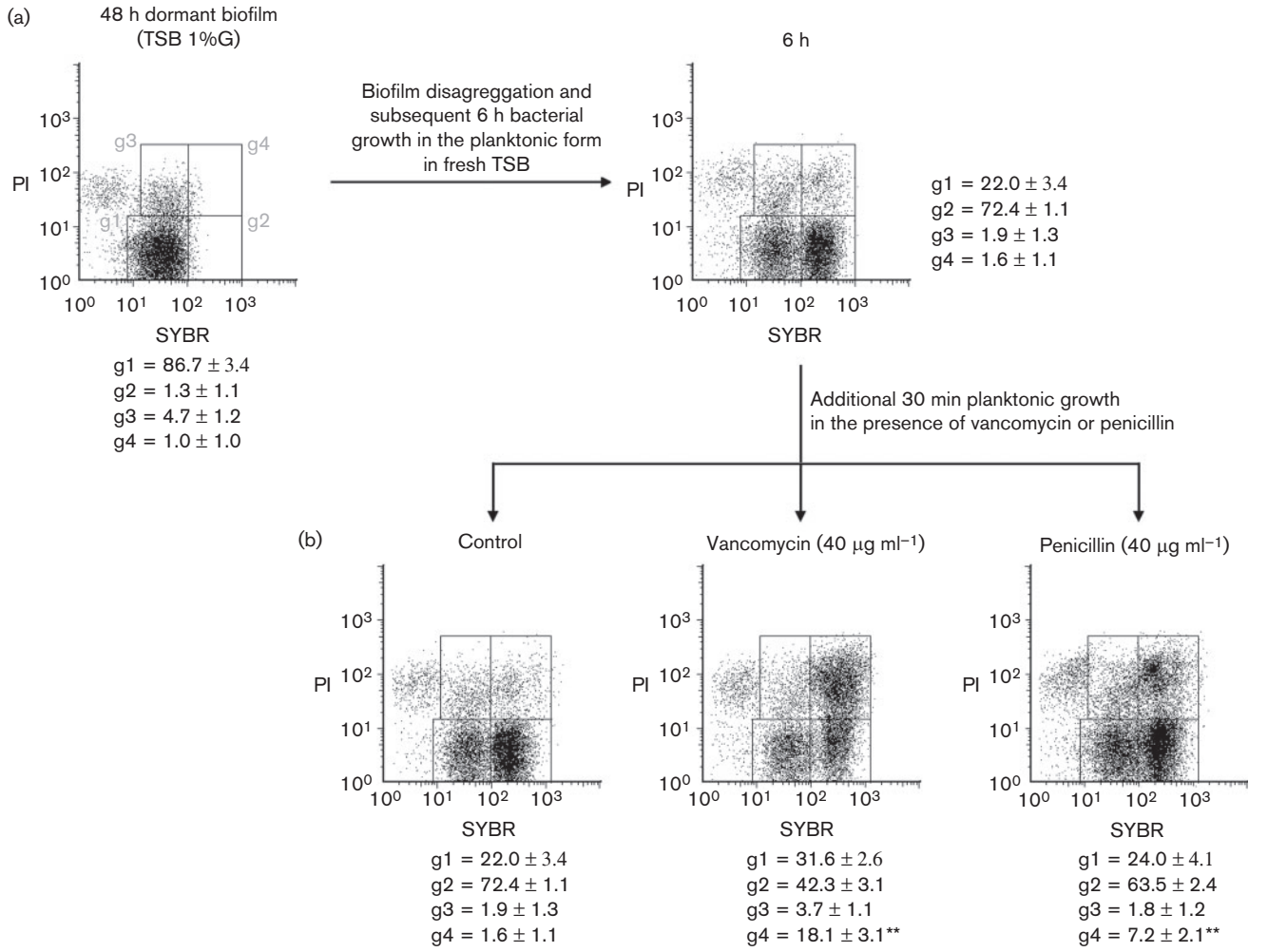

Fig. 4. Dormant bacteria within *S. epidermidis* biofilms maintain tolerance to vancomycin and penicillin after initiating planktonic growth. (a) Cell suspensions obtained from biofilms with high proportions of dormant bacteria were diluted in fresh TSB (1×10^8 cells ml^{-1}) and allowed to grow for a further 6 h period in the planktonic form. At this time point, the bacterial SYBR staining intensity was determined by FACS analysis. (b) Vancomycin (40 μg ml^{-1}), penicillin (40 μg ml^{-1}) or TSB was added to the 6 h planktonic cultures, which were incubated for a period of 30 min. Bacterial death was determined by flow cytometry analysis assessing incorporation of PI. Results shown are representative of two independent experiments that generated concordant results. Statistically significant differences in the proportions of $SYBR^{high}$ PI^+ bacteria (gate g4) between control, vancomycin and penicillin groups ($P<0.01$, ANOVA) are indicated by asterisks (**). No statistically significant differences were found in the proportions of $SYBR^{low}$ PI^+ bacteria (gate g3) between control, vancomycin and penicillin groups.

and increased *rpiA* expression, provides additional evidence for the suitability of using this fluorescent dye as a probe to evaluate the respiratory status of *S. epidermidis* cells.

S. epidermidis biofilm bacteria display a higher susceptibility to vancomycin and penicillin after initiating planktonic growth

The physiological reversibility between biofilm and planktonic bacteria has been identified as an important determinant in their antibiotic tolerant or susceptible profile (Fux *et al.*, 2005). Since we showed that marked physiological alterations occurred in *S. epidermidis* bacteria during the shift from the biofilm to the planktonic mode of growth, we evaluated whether this event was also accompanied by alterations in the susceptibility to vancomycin and penicillin. Since the major differences between biofilm and planktonic cells regarding SYBR MFI and *rpiA* expression were detected at 3 h (Figs 1 and 2), we selected this time point to assess the susceptibility of biofilm and planktonic bacteria to the above-mentioned antibiotics. For this purpose, vancomycin (40 μg ml^{-1}), penicillin (40 μg ml^{-1}) or TSB (negative control) was added to the biofilm and planktonic cultures, which were incubated for a further 30 min. Bacterial death was then quantified by PI incorporation and detected by flow cytometry. As shown in Fig. 3, bacteria within the planktonic

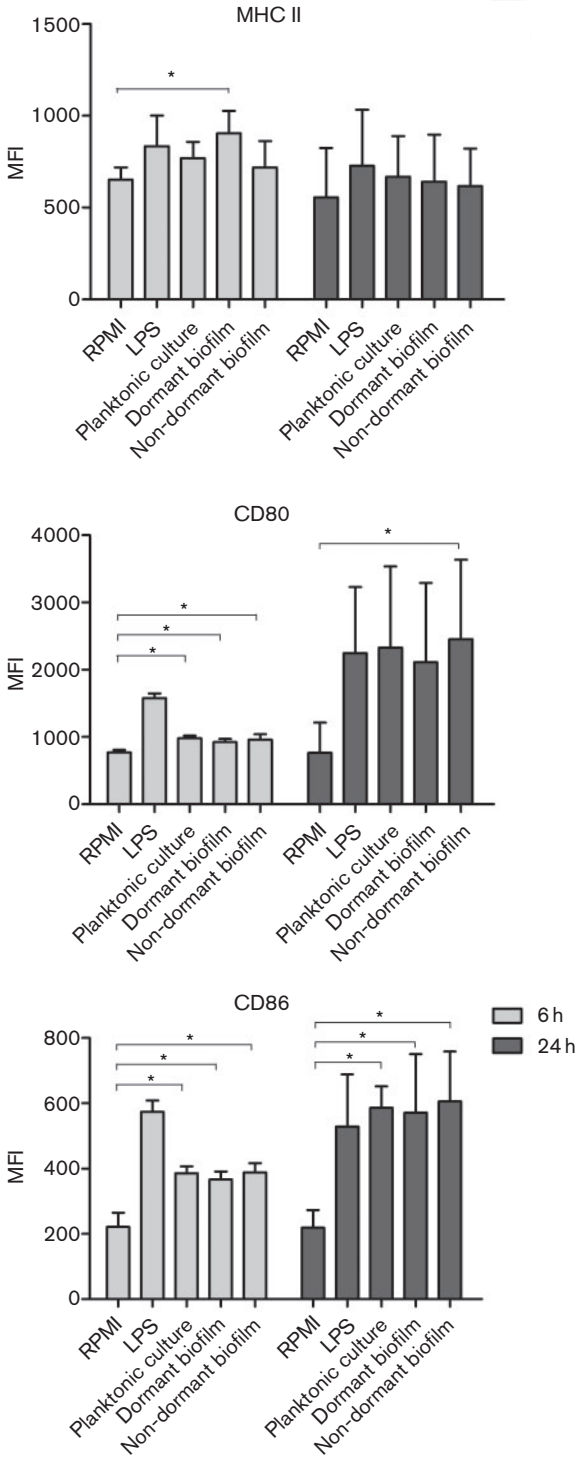

Fig. 5. Increase in co-stimulatory molecules on the surface of BMDCs stimulated with *S. epidermidis* bacteria. Flow cytometric phenotypic characterization of BMDCs (gated as $CD11c^{high}$) at the indicated time points after stimulation *in vitro* with cell suspensions obtained from 48 h biofilms grown in TSB 1 %G (dormant biofilms), 48 h biofilms grown in TSB 1 %G + Mg^{2+} (non-dormant biofilms), original planktonic cultures (planktonic), LPS or medium alone (RPMI), as indicated. BMDCs were stained with specific anti-CD80, anti-CD86 and anti-MHC class II mAbs after previous FcγR blocking. The bars represent MFI values for these molecules corresponding to the mean plus one SD of three samples per group. Results shown are of one out of two independent experiments that generated concordant results. Statistical analysis was carried out by one-way ANOVA with Tukey's multiple comparison test (*$P<0.05$).

cultures were more susceptible to vancomycin or penicillin as evaluated by the marked increase in the proportions of dead bacteria. In contrast, bacteria that remained growing in the biofilm cultures maintained tolerance to the tested antibiotics as indicated by the lack of increased proportions of dead cells in these cultures. This result is in agreement with previous reports highlighting the role of the bacterial physiological status in determining antibiotic tolerance/susceptibility (Fux *et al.*, 2005).

Dormant bacteria within *S. epidermidis* biofilms maintain tolerance to vancomycin and penicillin when cultured in planktonic conditions

It is well established that the majority of bacteria within infectious biofilms may not grow under standard laboratory culture conditions and are in an apparent dormant state (Costerton *et al.*, 2011; Oliver, 2010). Interestingly, the presence of dormant *S. epidermidis* bacteria on explanted central venous catheters was associated with fever in patients with biofilm-related infections (Zandri *et al.*, 2012). This finding highlights the relevance in characterizing physiological alterations that dormant bacteria may undergo upon initiating a planktonic growth. To evaluate this, we established 48 h biofilms with high proportions of dormant bacteria by using TSB + 1 %G without magnesium supplementation (Cerca *et al.*, 2011a). As shown in Fig. 4(a), 6 h after disaggregation of the biofilms and transfer of biofilm cells into planktonic culture conditions, the majority of bacteria displayed a $SYBR^{high}$ staining profile (gate g2), previously shown to correspond to growing non-dormant bacteria (Cerca *et al.*, 2011c). However, a noticeable proportion of bacteria maintained a $SYBR^{low}$ staining intensity (gate g1), corresponding to dormant bacteria. As dormant and non-dormant bacteria could be discriminated by using flow cytometry, we further characterized these bacterial populations regarding susceptibility to vancomycin and penicillin. Thus, vancomycin (40 μg ml^{-1}), penicillin (40 μg ml^{-1}) or TSB (negative control) was added to the 6 h planktonic cultures for a 30 min period, upon which bacterial death was similarly determined by assessing PI incorporation. As shown in Fig. 4(b), no significant increase in the proportions of $SYBR^{low}$ PI^{+} cells (gate g3) was detected upon addition of antibiotics as compared with the control cultures. In contrast, bacteria that underwent a physiological shift, presenting $SYBR^{high}$ staining (gate g4) were more susceptible to these antibiotics, as determined by the marked increase in the proportions of $SYBR^{high}$ PI^{+} cells. Altogether these results indicate that dormant bacteria within *S. epidermidis* biofilms do not

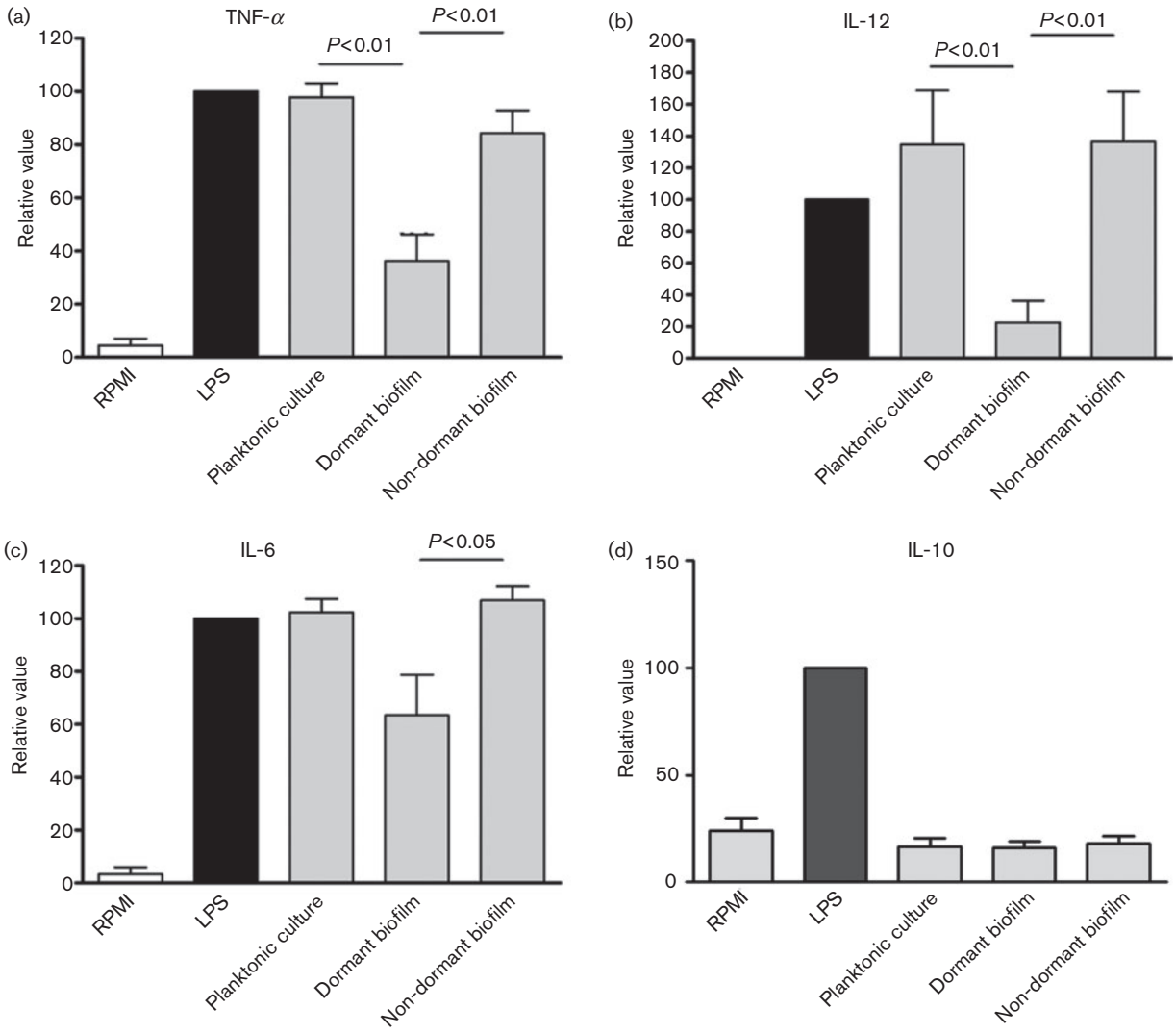

Fig. 6. *S. epidermidis* bacteria obtained from high dormancy biofilms induce a lower activation of murine BMDCs. Cell suspensions obtained from 48 h biofilms grown in TSB 1 %G (dormant biofilms), 48 h biofilms grown in TSB 1 %G+Mg^{2+} (non-dormant biofilms) or original planktonic cultures (planktonic) were used to stimulate BMDCs *in vitro*. The concentration of the proinflammatory cytokines (a) TNF-α, (b) IL-12 and (c) IL-6, and of anti-inflammatory cytokine (d) IL-10, was evaluated by ELISA in the culture supernatants. Results shown for each cytokine are representative of two independent experiments that generated concordant results. Statistical analysis was carried out by one-way ANOVA with Tukey's multiple comparison test.

undergo a physiological shift upon being placed in planktonic culture conditions, maintaining instead a low SYBR staining intensity that was associated with tolerance to vancomycin and penicillin.

S. epidermidis bacteria obtained from biofilms with high proportions of dormant cells induce a low activation of murine BMDCs *in vitro*

As we determined that dormant bacteria within *S. epidermidis* biofilms presented a particular phenotype by maintaining tolerance to vancomycin and penicillin after initiating a planktonic growth, we further explored whether these cells interacted differently with murine BMDCs compared to cells obtained from biofilms with lower proportions of dormant cells. Moreover, cells from both of these conditions have a distinct transcriptomic profile (Carvalhais *et al.*, 2014). Bacterial suspensions were prepared with cells obtained from high dormancy biofilms (TSB 1 %G), low dormancy biofilms (TSB 1 %G+Mg^{2+}) or planktonic cultures and used to stimulate BMDCs *in vitro*. BMDC activation was assessed by flow cytometry, measuring the MFI due to surface staining of co-stimulatory (CD80 and CD86) and MHC class II molecules, and by quantifying the proinflammatory cytokines TNF-α, IL-12 and IL-6, and the anti-inflammatory cytokine IL-10 in the culture supernatants. As shown in Fig. 5, all the

bacterial suspensions induced increased expression of CD80 and CD86 molecules on the surface of BMDCs, as compared with the negative control (medium alone). However, no significant differences were observed among the groups stimulated with the different bacterial suspensions. Also, there was only a marginal effect on MHC class II expression. Although no change in effect was observed for the BMDC surface phenotype induced by the differently grown *S. epidermidis* cells, bacteria obtained from biofilms with higher proportions of dormant cells induced lower production of TNF-α, IL-12 and IL-6 (Fig. 6). A disparate effect on cytokine production by murine dendritic cells upon stimulation with bacterial preparations that induced expression of CD80 and CD86 to indistinguishable levels was also found elsewhere (Xu *et al.*, 2011). It is noteworthy that bacteria obtained from low dormancy biofilms induced cytokine production by BMDCs to the same extent as bacteria obtained from planktonic cultures. These results are in agreement with our previous report showing that *S. epidermidis* bacteria obtained from biofilms enriched in dormant cells induced a low activation of murine macrophages *in vitro* and *in vivo* (Cerca *et al.*, 2011a). Also in agreement, a previous study has shown that staphylococcal biofilms could attenuate the inflammatory response of murine macrophages, as compared to planktonic cell counterparts, by promoting differentiation of these host cells into an M2 phenotype (Thurlow *et al.*, 2011). As shown in Fig. 6, no significant differences were found among any of the *S. epidermidis* cell-stimulated BMDC groups in the production of IL-10. This might indicate that immune evasion of *S. epidermidis* biofilm cells may be mainly due to a lower inflammatory effect rather than by promoting suppressive mechanisms such as the ones dependent on IL-10. Nevertheless, as other innate immune cells, such as myeloid suppressor cells, have been shown to produce IL-10 in the context of *S. aureus* biofilm infections (Heim *et al.*, 2014), a significant role of this cytokine in *S. epidermidis* biofilm infections cannot be ruled out. Further studies would be necessary to better determine to what extent dormant cells may contribute to the differential role of biofilm cells on host mononuclear phagocytes.

Concluding remarks

The physiological shift that occurs in *S. epidermidis* bacteria during the transition from the biofilm to the planktonic mode of growth is considered a major event in the pathophysiology of this bacterium (Yao *et al.*, 2005). Here, we showed that bacteria from biofilms grown in conditions that promoted cell viability displayed increased SYBR staining intensity and *rpiA* gene expression, as well as increased susceptibility to vancomycin and penicillin. In contrast, we also showed that a high proportion of bacteria obtained from biofilms grown in high glucose, a condition promoting dormancy, maintained a low SYBR staining intensity and tolerance to vancomycin and penicillin upon initiating a planktonic growth. These data highlight that biofilms, already intrinsically tolerant to antibiotics (Høiby *et al.*, 2010), may release cells to the surrounding environment that maintain an antibiotic-tolerant profile. This, in turn, may confer on these bacteria an increased likelihood to persist within a host and cause disease. As infectious biofilms may have a high frequency and prevalence of unculturable bacteria (Oliver, 2010), further studies addressing the role of dormant bacteria in the pathophysiology of *S. epidermidis* biofilms would help understand the clinical outcomes of biofilm-related infections.

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