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1 Live intramacrophagic *Staphylococcus aureus* as potential responsible for antibiotic therapy
2 failure: observations in an *in-vivo* mouse model of prosthetic vascular material infections

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Running Title

Intramacrophagic *Staphylococcus aureus* in vascular infection

ABSTRACT

Objective: evaluating the significant role played by biofilms during prosthetic vascular material infections (PVMIs). Methods: we developed an *in-vivo* mouse model of *Staphylococcus aureus* PVMI allowing its direct observation by confocal microscopy to describe: (i) the structure of biofilms developed onto a Dacron[®] vascular material, (ii) the localization and the effect of antibiotics on these biostructures and (iii) the interaction between bacteria and host tissues and cells during PVMI. Results: in this model, we highlight that the biofilms structures are correlated to the activity of antibiotics. Furthermore, live *S. aureus* bacteria were visualized inside the macrophages present at the biofilm sites while antibiotics do not penetrate in these immune cells. Conclusion: this intracellular situation could represent one explanation of the only limited effect of antibiotics but also of the possibility of PVMIs relapse after antibiotic therapy.

1 Introduction

2 *Staphylococcus aureus* biofilm development plays a significant role in the difficulties
3 encountered when treating prosthetic vascular material infections (PVMIs). Previous literature
4 data provided evidence that *S. aureus*, classically considered as an extracellular pathogen, can
5 also invade and survive inside immune cells, including the phagocytic cells. Such survival
6 mechanism could be responsible for the lack of antibiotics efficiency and the possibility of
7 relapse of chronic infections.¹ This hypothesis was suggested from *in vitro* co-culture models
8 and data reporting such *in vivo* interactions between *S. aureus* and mammalian tissues in the
9 particular setting of PVMIs are scarce.

10 We recently developed an *in-vivo* mouse model of *S. aureus* PVMI evaluating the efficacy of
11 different antibiotic regimen on six clinical and collection *S. aureus* strains.² While antibiotics
12 MICs were similar for all strains, their antibacterial efficacy was overall limited and strain-
13 dependent. For instance, mice infected with Methicillin-Resistant *S. aureus* (MRSA) BCB8
14 were cured with daptomycin monotherapy while this antibiotic demonstrated no efficacy for
15 Methicillin-Susceptible *S. aureus* (MSSA) ATCC 27217. To better understand these
16 surprising results, we used the same PVMI model to visualize *in situ* the *S. aureus* biofilms
17 structures whether treated or not with antibiotics. In particular, we focused on the interaction
18 between immune cells and bacteria embedded in biofilms to address whether intracellular
19 position of *S. aureus* could be an explanation of the lack of antibiotic efficacy in PVMIs.

21 Materials and methods

22 Based on our previous data² on six *S. aureus* clinical and collection strains, we selected the
23 two most representative bacterial strains: one Methicillin-Susceptible *S. aureus* (MSSA)
24 ATCC 27217 and one Methicillin-Resistant *S. aureus* (MRSA) BCB8 isolated from blood
25 cultures. Both strains were fully sensitive to the antibiotics tested (MIC were: daptomycin =

1 0.125 mg/L for MRSA and 0.25 mg/L MSSA, vancomycin = 1 mg/L for both strains and
2 rifampicin <0.006 mg/L for both strains). Antistaphylococcal agents were provided by drug
3 companies and prepared following label instructions: vancomycin (Sandoz, Levallois-Perret,
4 France), daptomycin (Novartis Pharma SAS, Rueil-Malmaison, France) and rifampicin
5 (Sanofi-Aventis, Paris, France). The *in-vivo* experiments were approved by the French
6 ministry of research review board and have been described elsewhere.² Briefly, at least 4
7 Four-weeks old female Swiss mice (RjOrl/SWISS, Janvier laboratory St Berthevin, France)
8 were used for the experiments. Sterile squares of Dacron[®] were implanted into a subcutaneous
9 pocket created in the centre of the mice back after general anaesthesia. Two days later, a
10 saline solution containing 10⁷ colony forming units (cfu) of *S. aureus* was transcutaneously
11 inoculated onto the graft surface. Antibiotics treatment started two days later. All the
12 antibiotics used were administered at dose regimens resulting in serum concentrations similar
13 to those obtained in humans and through the respective classical routes used in mice model.
14 Mice were randomized into different groups: no treatment (controls); vancomycin group
15 (subcutaneous injection (SC), 110 mg/kg/12 h);³ daptomycin group (50 mg/kg/24 h, SC);⁴
16 rifampicin group (30 mg/kg/12 h, intraperitoneal);⁵ vancomycin-rifampicin group; and
17 daptomycin-rifampicin group. Mice were treated for 48 h and then euthanized following
18 international guidelines. Immediately after the procedure, Dacron[®] patches were removed and
19 visualized with a confocal laser scanning microscope (Leica TCS SP5 Microsystems, France).
20 Images were acquired using a ×63 oil immersion objective with a numerical aperture of 1.4.
21 For all the experiments, the size of the confocal images was 512 x 512 pixels (either 215 by
22 215 μm² or 82 by 82 μm²), recorded with a z-step of 1 μm and a 3x zoom. For each biofilm, at
23 least four different regions were analysed and biofilms structures were compared by direct
24 observation. For this purpose, nucleic acids (both bacteria and eukaryotic cells) were stained
25 with Syto9[®] (Invitrogen), able to penetrate into all cells, and propidium iodide (PI,

Invitrogen), which can only penetrate into damaged-membrane cells. Syto9® and PI were excited with an Argon laser at 488 and 543 nm, respectively, and their fluorescence emissions were collected between 500-600 nm for Syto9® and between 640-750 nm for PI.

To visualize simultaneously the bacteria, the immune cells (neutrophils and macrophages), and the antibiotics, all were specifically stained to well discriminate their fluorescence emission. Neutrophils were stained with Ly-6G®/mouse specific marker (GR-1, Pacific Blue™ conjugate RB6-8C5), excited using Argon laser at 361 nm; the fluorescence emission was collected in the range 400-450 nm). Macrophages were stained with F4/80® macrophage-specific antibody (Alexa Fluor® 647 conjugate BM8), excited with a Neon laser at 633 nm; the fluorescence emission was collected in the range 650-750 nm. BODIPY-FL®-daptomycin (kindly provided by Cubist Pharmaceuticals) and BODIPY-FL®-vancomycin (from Sigma) were excited at 488 nm and the fluorescence emission was collected in the range 500-550 nm.

Results

Typical structures of *in vivo* MSSA and MRSA-infected Dacron® patches were illustrated in Figure 1. As expected for an infection site, high amounts of bacteria and immune cells were trapped in a dense and thick reticular extracellular matrix (Figure 1, A, B). We can also note that there are only few amounts of damaged cells after Dacron removal from mice. When antibiotics were applied, the observed structures were antibiotic- and strain-dependent. In the case of antibiotics inefficiency, the visualization of Dacron® patches was very similar to the controls (Figure 1, E,F,I,L). By contrast, the material surface displayed lightened structures with only scattered immune cells when antibiotics were efficient (Figure 1, C,D,G,H,J,K). In more details, for MRSA BCB8, vancomycin did not show any significant effect on biofilms compared to the control samples by contrast to rifampicin and daptomycin. For MSSA 27217, the effect of daptomycin as well as vancomycin was more limited, yielding bacterial

structures close to the control ones while rifampicin was the most active. The combination of rifampicin to vancomycin appeared as very efficient in the overall structure lightening, which was not the case for the daptomycin-rifampicin combination. These results all correlate with bactericidal activity measurements (Table S1).²

Additional observations were obtained with the fluorescent staining of immune cells and antibiotics, highlighting that: (i) the eukaryotic cells on the infection site were essentially polynuclears and macrophages (Figure 2A), (ii) live *S. aureus* bacteria were found inside both live and dead macrophages (Figure 2B) but not in polynuclear cells, and (iii) BODIPY-vancomycin and – daptomycin were visualized in polynuclear cells but none of them penetrated macrophages load with live *S. aureus* bacteria (Figure 2C).

Discussion

The visualization of antibiotics within biofilm-associated bacteria may contribute to our understanding of the differential effects of antistaphylococcal agents on material-associated *S. aureus* infections and that was the purpose of the present study. Dacron[®] implants where biofilms grown in alive mice were visualized by confocal fluorescence microscopy immediately after mice were euthanized. This procedure allowed: i) to observe *in situ* live bacteria interactions with the prosthetic vascular material but also with the host cells (polynuclear cells and macrophages), widely present on the infection site, ii) to correlate the biofilms structures with antibacterial activity of antibiotics (daptomycin, vancomycin and their association with rifampicin), iii) to highlight live *S. aureus* bacteria inside the macrophages present at the biofilm sites while antibiotics do not penetrate these immune cells.

These *in vivo* results are different from the ones found in *in-vitro* biofilm models⁶ that do not allow to observe the interactions between bacteria and host-cells occurring *in-vivo*.⁷ Most *in-*

1 vivo models rely on bacterial counts within infected materials, but do not include imaging
2 techniques visualizing the direct effect of antibiotics on biofilms developed *in-vivo*.⁸ The *S.*
3 *aureus* PVMI mouse model presented herein may provide original data in this field.

4 Our results do not reveal significant change in the biofilms structures from one *S. aureus*
5 isolate to another but a differential effect of antistaphylococcal agents. We confirm the
6 dramatic efficacy of rifampicin by comparison with other treatments,^{8, 9, 10} probably due to its
7 well-known great intracellular penetration and activity. Unexpectedly, daptomycin, often
8 referred as one of the most active antistaphylococcal agent on biofilm,^{11 12} can yield to very
9 limited effects. The antibiotic was active on MRSA BCB8-related biofilm onto Dacron[®] but
10 not for MSSA 27217 biofilm. These results support previous findings showing that there is no
11 difference between vancomycin and daptomycin activities *in-vitro*¹³ or *in-vivo*.¹⁴ We do not
12 have a definitive explanation for such differences of daptomycin efficacy according to the
13 staphylococcal strain but we already reported in an *in-vitro* model¹⁵ that tolerance toward this
14 molecule may be related to a physiological change involving structural modifications of the
15 membrane, a strain-dependent process.

16 Previous literature findings have reported the capability of *S. aureus* to survive within
17 osteoblasts in the context of bone and joint infections, resulting in persistent and relapsing
18 infections.¹⁶ We visualize here the same possible capacity to survive inside host cells (i.e.,
19 macrophages) during *S. aureus* PVMIs. An important consequence of such process is that
20 intramacrophagic *S. aureus* are able to escape the phagolysosome, leading to free replication
21 in the cytoplasm. This can trigger cell death mechanisms from its host cell, multiply actively
22 and disseminate, but also activate anti-apoptotic programs to persist hidden in intracellular
23 position and induce chronic or relapsing infections.¹ In addition, intramacrophagic position
24 could represent a shelter for *S. aureus* against antibiotics: the less able to penetrate host
25 eukaryotic cells they are, the less efficient they could be.

1 Thus, we hypothesize that the persistence of *S. aureus* inside macrophage during PVMIs
2 could be an explanation to the relative inefficacy of antibiotics without surgery during these
3 infections, and to the high risk of relapse when the infected material is not removed. This
4 could also explain why antibiotic efficacy could be different according to the strain involved,
5 since the capability of *S. aureus* to invade mammalian cells could vary from one strain to
6 another.¹⁷

7 This study presents some limitations. First, the site of vascular prosthetic material
8 implantation. For evident technical reasons, it was impossible to implant our Dacron[®] along
9 the vascular system. Some authors already described extra-anatomic animal model, with
10 implantation of vascular material in subcutaneous position, to evaluate different prophylactic
11 procedure to prevent PVMIs.¹⁸ Nevertheless, in a clinical setting, most PVMIs occur from the
12 wound or from an adjacent infectious focus and not through hematogenous route.¹⁹ Therefore,
13 the infection process usually starts along the external part of the vascular prosthesis, not the
14 endoluminal layer. In this context, our model reproduces this natural history of infection and
15 may be reasonably used to evaluate different antibiotic regimens for PVMIs treatment.

16 The limited number of evaluated bacterial strains could be a second weakness of our work.
17 This study follows a previous published work² dealing with the antibiotics activity on six
18 different *S. aureus* strains in our mouse model of PVMI. Similar results were obtained for
19 MSSA or MRSA strains and thus, we selected the two more representative bacterial strains to
20 allow more demonstrative differences in the aspect of *in-vivo* biofilm developed onto Dacron[®]
21 upon antibiotics action.

22 In conclusion, this *in-vivo* mouse model of *S. aureus* PVMIs allows the direct observation of
23 the impact of major antistaphylococcal agents on Dacron[®]-related biofilm. We visualized
24 intramacrophagic *S. aureus* onto the biomaterial and we hypothesize that intramacrophagic *S.*
25 *aureus* could be also present during PVMIs in clinical settings and may explain why bacteria

1 may persist, and relapse, even after prolonged and appropriate antibacterial therapy. More
2 studies are needed, but we can postulate that the use of antibiotics active against biofilm-
3 embedded and intracellular bacteria such as rifampicin could be a very good option in PVMIs.

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16 **Transparency declarations**

17 Nothing to declare

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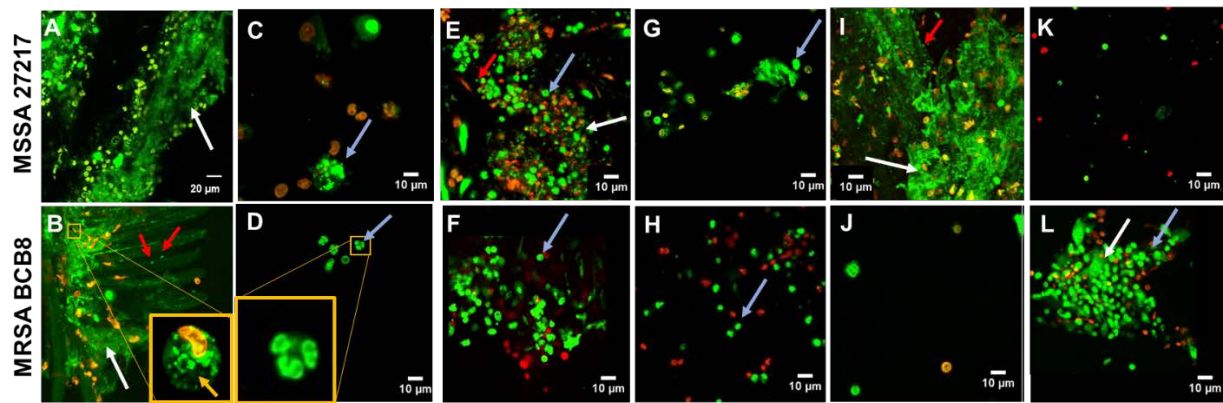


Figure 1: The structures of biofilms developed on Dacron® *in vivo* are antibiotic-dependent. Visualization of Dacron®-related *S. aureus* biofilms depending on antibiotics treatments by confocal laser scanning microscopy. A and B: controls, C and D: daptomycin, E and F: vancomycin, G and H: rifampicin, I and J: daptomycin-rifampicin, K and L: vancomycin-rifampicin.

Green staining: Syto9® (live cells), red staining: Propidium Iodide (membrane-damaged cells). White arrows: reticular structures corresponding to the extracellular matrix. Red arrows: single bacteria included within this structure. Blue arrows: eukaryotic cells. Yellow arrows: intracellular *S. aureus*.

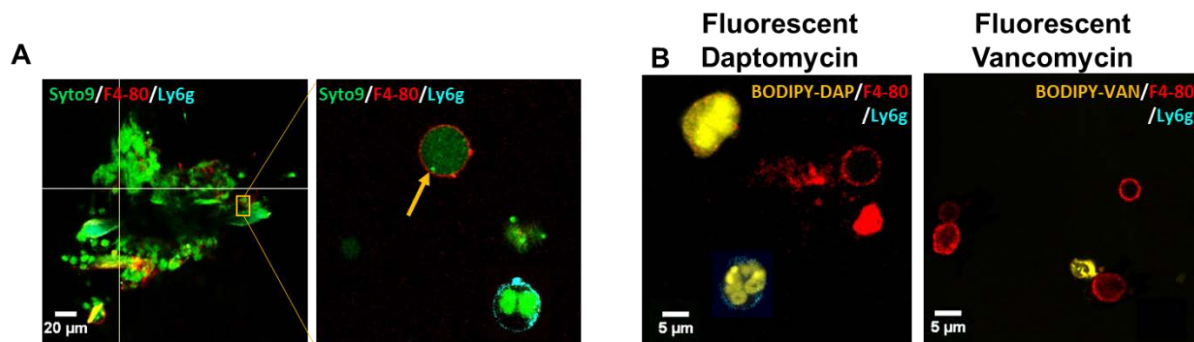


Figure 2: *S. aureus* bacteria are localized in macrophages that daptomycin and vancomycin cannot penetrate.

A: identification of eukaryotic cells. Green staining: Syto9®; Blue staining: Ly6G® (neutrophil polynuclear specific staining); Red staining: F4/80 (macrophages specific staining). Yellow arrow: intramacrophagic live *S. aureus* (observed on biofilms treated or not with vancomycin and daptomycin).

B: antibiotic staining. Yellow staining: BODIPY-FL-vancomycin or -daptomycin; Blue staining: Ly6G® (neutrophil polynuclear specific staining); Red staining: F4/80 (macrophages specific staining).