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Live intramacrophagic *Staphylococcus aureus* as potential responsible for antibiotic therapy 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.

Introduction

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

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

Materials and methods

Based on our previous data[2] on six *S. aureus* clinical and collection strains, we selected the two most representative bacterial strains: one Methicillin-Susceptible *S. aureus* (MSSA) ATCC 27217 and one Methicillin-Resistant *S. aureus* (MRSA) BCB8 isolated from blood cultures. Both strains were fully sensitive to the antibiotics tested (MIC were: daptomycin =

0.125 mg/L for MRSA and 0.25 mg/L MSSA, vancomycin = 1 mg/L for both strains and rifampicin <0.006 mg/L for both strains). Antistaphylococcal agents were provided by drug companies and prepared following label instructions: vancomycin (Sandoz, Levallois-Perret, France), daptomycin (Novartis Pharma SAS, Rueil-Malmaison, France) and rifampicin (Sanofi-Aventis, Paris, France). The *in-vivo* experiments were approved by the French ministry of research review board and have been described elsewhere.[2] Briefly, at least 4 Four-weeks old female Swiss mice (RjOrl/SWISS, Janvier laboratory St Berthevin, France) were used for the experiments. Sterile squares of Dacron® were implanted into a subcutaneous pocket created in the centre of the mice back after general anaesthesia. Two days later, a saline solution containing 10^7 colony forming units (cfu) of *S. aureus* was transcutaneously inoculated onto the graft surface. Antibiotics treatment started two days later. All the antibiotics used were administered at dose regimens resulting in serum concentrations similar to those obtained in humans and through the respective classical routes used in mice model. Mice were randomized into different groups: no treatment (controls); vancomycin group (subcutaneous injection (SC), 110 mg/kg/12 h);[3] daptomycin group (50 mg/kg/24 h, SC);[4] rifampicin group (30 mg/kg/12 h, intraperitoneal);[5] vancomycin-rifampicin group; and daptomycin-rifampicin group. Mice were treated for 48 h and then euthanized following international guidelines. Immediately after the procedure, Dacron® patches were removed and visualized with a confocal laser scanning microscope (Leica TCS SP5 Microsystems, France). Images were acquired using a ×63 oil immersion objective with a numerical aperture of 1.4. For all the experiments, the size of the confocal images was 512 x 512 pixels (either 215 by 215 µm² or 82 by 82 µm²), recorded with a z-step of 1 µm and a 3x zoom. For each biofilm, at least four different regions were analysed and biofilms structures were compared by direct observation. For this purpose, nucleic acids (both bacteria and eukaryotic cells) were stained 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 models[6] that do not allow to observe the interactions between bacteria and host-cells occurring *in-vivo.*[7] Most *in-*

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

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

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

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

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

The limited number of evaluated bacterial strains could be a second weakness of our work. This study follows a previous published work[2] dealing with the antibiotics activity on six different *S. aureus* strains in our mouse model of PVMI. Similar results were obtained for MSSA or MRSA strains and thus, we selected the two more representative bacterial strains to allow more demonstrative differences in the aspect of *in-vivo* biofilm developed onto Dacron® upon antibiotics action.

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

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

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Transparency declarations

Nothing to declare

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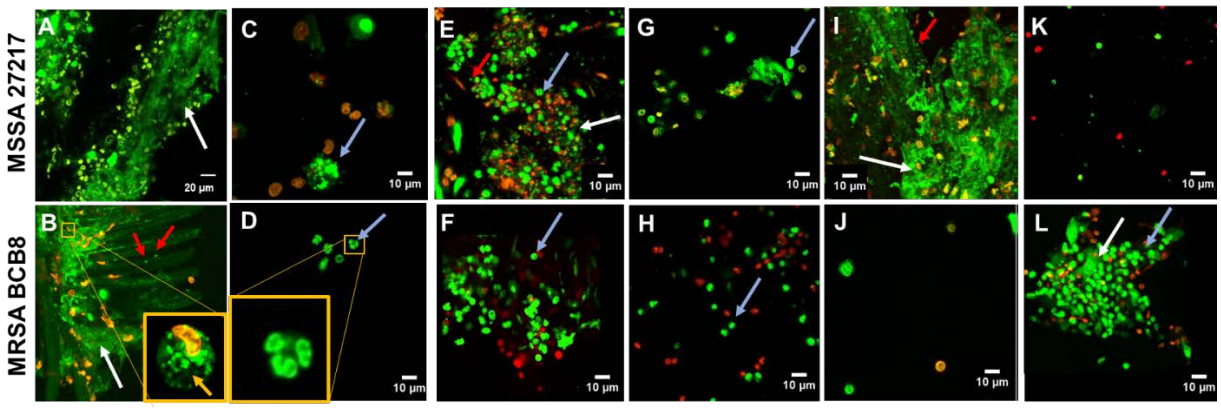

Figure 1: The structures of biofilms developed on Dacron® *in vivo* are antibiotic-dependent. Visualization of Dacron®-related *S. aureus* biofilms depending on antibiotics treatments by confocal laser scanning microscopy. A and B: controls, C and D: daptomycin, E and F: vancomycin, G and H: rifampicin,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

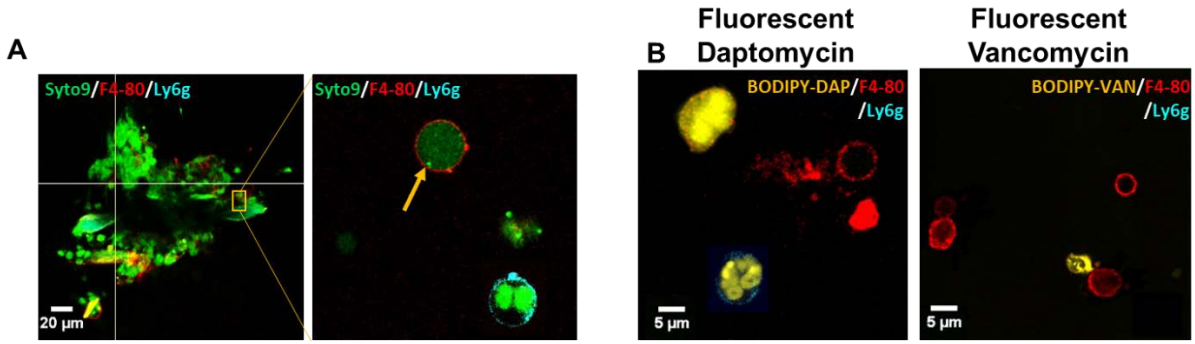

Figure 2: *S. aureus* bacteria are localized in macrophages that daptomycin and vancomycin cannot penetrate.
A: identification of eukaryotic cells. Green staining: Syto9®; Blue staining: Ly6G®(neutrophil polynuclear specific staining); Red staining: F4/80 (macrophages specific staining). Yellow arrow: 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).