Evidence for inoculum size and gas interfaces as critical factors in bacterial biofilm formation on magnesium implants in an animal model

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Abstract

Infections of medical implants caused by bacterial biofilms are a major clinical problem. Bacterial colonization is predicted to be prevented by alkaline magnesium surfaces. However, in experimental animal studies, magnesium implants prolonged infections. The reason for this peculiarity likely lies within the –still largely hypothetical– mechanism by which infection arises. Investigating subcutaneous magnesium implants infected with bioluminescent *Pseudomonas aeruginosa* via *in vivo* imaging, we found that the rate of implant infections was critically dependent on a surprisingly high quantity of injected bacteria. At high inocula, bacteria were antibiotic-refractory immediately after infection. High cell densities are known to limit nutrient availability, restricting proliferation and trigger quorum sensing which could both contribute to the rapid initial resistance. We propose that gas bubbles such as those formed during magnesium corrosion, can then act as interfaces that support biofilm formation and permit long-term survival. This model could provide an explanation for the apparent ineffectiveness of innovative contact-dependent bactericidal implant surfaces in patients. In addition, the model points toward air bubbles in tissue, either by inclusion during surgery or by spontaneous gas bubble formation later on, could constitute a key risk factor for clinical implant infections.

Keywords: *Pseudomonas aeruginosa*; bacterial biofilm; magnesium alloy implant; bioluminescence; animal model; *in vivo* imaging; antibiotic resistance; gas interfaces

1. Introduction

Biomaterial-associated infections are a major risk factor for the failure of implanted medical devices [1, 2]. Approximately 5% of implanted internal fractured bone fixation devices and 30% open fracture devices have been reported to become infected, with treatment costs up to over \$100,000 per patient [3, 4].

Little is known about the early stages of such infections. During open surgery, infections could arise by accidental contaminations especially in a non-sterile environment. After surgery, infections have been proposed to be due to microbial dissemination from small injuries via the blood circulation [5]. Moreover, in the absence of host tissue adhesion to medical implant surfaces for instance due to gas accumulation in the case of magnesium alloy implants, bacteria have less competition by the adhering host cells to settle on exposed surfaces [6-8]. It was therefore proposed that exposed implant surfaces were prone to bacterial colonization by single bacteria which is consistent with a popular model for environmental bacterial biofilm formation, such as in aqueous environments under flow conditions. In this view, individual planktonic bacteria are thought to adhere to implant surfaces in competition with host tissue cells. Subsequent bacterial proliferation would lead to micro-colony formation and colonization of the implant surface [9]. Eventually, at high bacterial densities, secreted signaling molecules trigger a process called quorum sensing that facilitates biofilm formation [7]. Biofilms are characterized by a protective viscous matrix consisting of secreted extracellular polysaccharides, nucleic acids and numerous other components [10]. Due to the protective matrix in combination with nutrient limitation, slow proliferation and reduced metabolism bacteria embedded in biofilms become highly recalcitrant to various insults, such as the host immune defenses and antibiotics [11, 12]. Over the

time, additional bacterial adaptations may contribute to bacterial persistence in patients. This includes phenotypically diverse subpopulations and the acquisition of multi-antibiotic resistance [13]. Often the only efficacious therapeutic option is the surgical implant removal. Subsequently, prolonged antibiotic treatment is required to completely eradicate the infection before a fresh implant can be inserted [14, 15].

The causes and mechanisms that lead to implant infections have not yet been elucidated in detail. A deeper understanding would answer long-standing questions in basic research and could facilitate establishing novel preventative or therapeutic strategies such as anti-infective implants [16]. However, present *in vitro* models are limited and cannot mimic specific essential clinical features such as bacteria-host tissue interactions or host immune defenses. In general, animal experiments are demanding due to ethical considerations, costs, time requirement and reproducibility issues, depending on how closely the clinical situation is to be reproduced [17, 18]. For these reasons genetically well characterized small animal models are frequently favored as a first approach in basic research. Conventionally, bacterial infections are quantified by determining the number of colony forming units (CFU). However, for each determination animals must be sacrificed and results are associated with a high variability for multiple reasons, such as variable courses of individual infections or difficulties to completely collect and separate bacteria [19-21]. Alternatively, genetically engineered bioluminescent pathogens or immune cells in synergy with *in vivo* imaging techniques are preferred to minimize animal use, costs and experimental variation by allowing non-invasive monitoring of individual infections over the time [22, 23].

To examine the effect of the bacterial inoculum size on implant infection kinetics, bioluminescent *P. aeruginosa* bacteria were employed. *P. aeruginosa* are notorious for

infections in the respiratory tract of cystic fibrosis patients and also for medical implant infections [24-27]. Luminescently labeled bacteria allowed non-invasive monitoring of the infection over the time. Ciprofloxacin was used as an efficacious antibiotic. As a DNA topoisomerase inhibitor, it acts bactericidal by leading to DNA strand breaks [28]. Furthermore, magnesium served as an experimental implant material. Magnesium alloys are presently investigated as novel degradable bone repair materials to circumvent surgical removal and long-term side effects [29-32]. Interestingly, in cell culture assays, metallic magnesium acts bactericidal due to alkalization by generating hydroxide as a corrosion product [17]. Unexpectedly, in comparison to conventional implant materials, magnesium implants were found to be even more susceptible to infections by diverse bacterial species [18, 33, 34]. In the presence of magnesium implants, bacterial infections were more persistent and antibiotic treatment resistant and this model system could therefore serve to identify risk factors for clinical implant infections [18].

In this study, the role of the inoculum size in the establishment of persistent magnesium implant infections was evaluated. In addition, infected implants were examined in more detail for conspicuous features by electron microscopy. The data shows that the frequency of persistent infections was dependent on a very high inoculum size. Interestingly, injected suspended bacteria could initially survive antibiotic treatment even before forming a more highly resistant biofilm. The study focuses on demonstrating the basic feasibility of establishing lasting infections in response to large inocula on magnesium implants and to develop an explanatory working model.

2. Materials and Methods

2.1. Cultivation of *P. aeruginosa*

The current study was performed by employing a *Pseudomonas aeruginosa* strain which was made bioluminescent by the insertion of entire lux operon encoding sequence into bacterial genomic DNA under control of the quorum sensing sensitive promoter pqsA as previously described (PAO1::*luxCDABE*) [35-37]. The bacterial strain has previously been used in our laboratory for monitoring implant infections [18]. The bacteria were seeded on LB agar plates and incubated overnight at 37°C. Single colonies from the plates were cultured in LB broth (shaking speed of 100 RPM) at 37°C. Cultures were grown to an optical density of 0.1 measured at a wavelength of 600 nm. Subsequently, cultures were adjusted with LB broth to colony forming units of 10⁵ CFU of *P. aeruginosa* in LB medium (High infection dose), 10⁴ CFU of *P. aeruginosa* in LB medium (Medium infection dose) and 10³ CFU of *P. aeruginosa* in LB medium (Low infection dose) and temporarily stored on ice until use. For *in vitro* antibacterial assay, overnight cultured luminescent *P. aeruginosa* on LB agar plates were grown in LB medium and adjusted to 10⁵ CFU/ml. Ciprofloxacin (Fluka Chemie GmbH, Deisenhofen, Germany) was twofold serially diluted in a 96-well plate (Thermo Scientific, Schwerte, Germany) from a starting concentration of 25 µg/ml and then bacteria were immediately added into each concentration. The plate was incubated at 37°C (shaking speed of 150 RPM) and bacterial luminescence was determined at 0, 2, 4 and 6 hours by luminescence plate reader (Infinite 200 PRO, TECAN, Männedorf, Switzerland).

2.2. *Surgical procedures and implant infection in mice*

Animal experiments were conducted on 8 weeks old female BALB/c mice (Harlan Winkelmann, Borcheln, Germany) under the permission number (33.42502/07-10.5) from Lower Saxony State Office for Consumer Protection and Food Safety (LAVES; Oldenburg, Germany). For each *in vivo* experiment, a minimum of three animals were

used. For surgical implantation procedure, mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Next, the backs of the mice were shaved with a hair trimmer (Aesculap Suhl GmbH, Suhl, Germany). Mice were then shifted under sterile bench and in each mouse, three surgical incisions were made (each 1 cm) to generate subcutaneous pouches. Sterile magnesium implants (Institute of Materials Science, Leibniz University Hannover) 5 mm in diameter and 2 mm in thickness were inserted into these pouches and the wounds were closed with simple interrupted sutures (Ethicon Vicryl, Johnson & Johnson Medical GmbH). The employed magnesium samples had a purity of 99.94% and they generated gas pockets after subcutaneous implantation in mice [18, 38]. Immediately after implantation, 5 microliters of each of the 10^5 (high infection), 10^4 (medium infection) and 10^3 (low infection) CFU of *P. aeruginosa* were locally injected on the top of the implants in respective animals. For bioluminescence detection, animals were anesthetized with 2% isoflurane in XGI-8 gas anesthesia unit (Caliper Life Sciences, Hopkinton, MA) and imaged on a daily basis by using a Xenogen IVIS-200 optical imaging system (Caliper Life Sciences). For luminescence data analysis, Xenogen Living image software Version 2.6 (Caliper Life Sciences) was used. For systemic antibiotic treatment, ciprofloxacin (Fluka Chemie GmbH, Deisenhofen, Germany) at concentrations of 8 mg/kg and 24 mg/kg body weight was intravenously injected on daily basis into respective animals.

2.3. *Histological evaluation*

Peri-implant tissues from infected or sterile implants were extracted after 13 days of infection. Samples were treated as previously described [38]. Briefly, tissue samples were fixed in 4% formalin for 3 days. The samples were dehydrated by incubation in solutions with increasing concentrations of ethanol in water, followed by incubation in

xylene overnight and then embedded in paraffin. Embedded samples were cut (3 μ m sections), mounted on slides and stained with hematoxylin and eosin (H&E). H&E stained slides were then photographed at 40-fold magnification with a Zeiss Axioscope 40 microscope equipped with a Zeiss AxioCam Mrc digital camera (Zeiss, Germany).

2.4. *Electron microscopic procedures*

For electron microscopy, samples were treated as described previously [34]. Briefly, peri-implant tissue samples were first fixed by incubation for 1 hour on ice in 2.5% glutaraldehyde and 5% formaldehyde in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Samples were then washed twice in TE buffer and dehydrated in a graded series of acetone solutions for 15 minutes on ice. Followed by dehydration in 100% acetone, a critical-point drying was done in liquid CO₂. The dried samples were then sputter coated and examined using a Zeiss Merlin Field Emission Scanning Electron Microscopy (FESEM). For transmission electron microscopy, peri-implant tissue samples were fixed in HEPES buffer containing 2.5% glutaraldehyde and 5 % formaldehyde. Followed by a washing with HEPES buffer, samples were incubated for 1 hour at room temperature in 1% aqueous osmium solution. Further, samples were dehydrated by serial incubation in increasing concentrations (*i.e.*, 10%, 30%, and 50%) of acetone on ice. Samples were then incubated overnight in 70% acetone overnight. Further dehydration was done in 90%, and 100 % acetone. After complete dehydration, samples were embedded in epoxy resin and cut into ultrathin sections. Finally, samples were examined in a TEM910 transmission electron microscope (Carl Zeiss, Oberkochen) at an acceleration voltage of 80 kV. Acquired images were digitally recorded at magnifications (ProScan, 1024 x 1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany).

2.5. Determination of CFUs on implants

For the CFU assay, disc implants were explanted after 13 days with care taken to include bacteria-containing puss around the implant. Bacteria were then removed from the implant and resuspended in ice-cold phosphate buffer saline (PBS) by vigorous vortexing for 10 minutes at maximum speed. Subsequently, the suspensions were serially diluted and spread on solid LB nutrient agar containing petri dishes. After overnight incubation at 37°C, the resulting colonies were counted.

2.6. Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics Version 25. Briefly, non-parametric Mann-Whitney U test for comparison between two groups and Kruskal-Wallis H test for comparison between more than two groups were applied. Significant values were adjusted by the Bonferroni correction for multiple tests.

3. Results

3.1. Cultured *P. aeruginosa* bacteria are sensitive to ciprofloxacin

Ciprofloxacin can have bactericidal and bacteriostatic activities [39]. To allow *in vivo* monitoring, a bioluminescent derivative of the laboratory strain *P. aeruginosa* (PAO1) containing the lux CDABE operon under control of pqsA promoter integrated in the genome was employed. To evaluate the temporal response of the luminosity to the antibiotic ciprofloxacin, the strain was cultured in the presence of a series of comparatively high ciprofloxacin concentrations deemed necessary for the envisioned biofilm treatment.

Using this assay antibacterial activity of ciprofloxacin was observed at concentrations starting from 0.39 µg/ml or higher (Fig. 1). The luminosity data indicated that even at

very high drug concentrations during the first 2 hours the antibiotic acted mainly cytostatic, whereas after 4 hours, ciprofloxacin concentrations over 1.56 $\mu\text{g/ml}$ indicated a decrease of the luminosity and cytotoxic effects. After 6 hours incubation, cytotoxic effects were observed even in the presence of lower drug concentrations. Therefore, the setup appeared useful to detect and distinguish cytostatic and cytotoxic activity within a timeframe deemed suitable for *in vivo* monitoring.

Fig. 1. Antibacterial activity of ciprofloxacin in suspension cultures of *P. aeruginosa*.

Ciprofloxacin at the concentrations indicated was added to logarithmically growing cultures of the bioluminescent *P. aeruginosa* strain PAO1::*luxCDABE* (10^5 CFU/ml). After the incubation periods indicated in hours, the light intensity was recorded by luminescence plate reader 200 PRO (bars). (-): standard LB medium. Pairwise comparisons of the ciprofloxacin treated cells and untreated cells with Mann-Whitney U test indicated statistically significant differences in bacterial luminescence ($p=0.050$) at all time points 2h and 6h prior to Bonferroni adjustment. (n=3). The error bars indicate standard deviations.

3.2. Correlation of *in vivo* bioluminescence imaging with CFU assays

The colony-forming units (CFU) assay is an established technique for the assessment of viable bacteria and for the evaluation of antibiotic efficacy [40, 41]. Nevertheless, *in vivo* imaging is preferred due to the advantage of non-invasively monitoring individual infections over time [42]. However, multiple factors interfere with imaging. These include light absorption by the implant, variations in the intervening tissue thickness, color, fur density and also reduced bacterial metabolic activity. The strain contains a bacterial density-dependent *pqsA*::*luxCDABE* reporter gene expression cassette and is expected to be particularly active at high bacterial concentrations such as in biofilms. In our experience the light emission is dependent on metabolic activity and it is therefore not expected to be linearly dependent on the bacterial cell density. To determine if the luminescence intensity correlated with the CFU assay, magnesium discs were subcutaneously implanted in mice and immediately infected by local injection of bacterial suspensions. Initially, comparatively high inocula were used to permit immediate verification of the bacteria by *in vivo* imaging. The lower detection limit of freshly injected bacteria by *in vivo* imaging was 10^9 (Fig. 2). The median MIC of ciprofloxacin against *P.*

aeruginosa in vitro was reported to be 0.5 mg/ml and *in vivo* 0.5 mg/kg resulted in 100% survival of infected mice when applied parenterally [43]. Since implant-associated bacterial infections in the clinic are notoriously resistant to antibiotic treatment, 16x and 48x overdoses were applied such that we expected that bacteria could survive solely in a biofilm mode. Interestingly, bacterial activity increased over the time despite antibiotic treatment (Fig. 2A-C). In fact, after thirteen days, bacterial activity was even lower in the absence of antibiotics. Moreover, tissue above these implants appeared necrotic, presumably due to cytotoxic side effects of destructive but eventually efficacious immune responses (Fig. 2A, arrow and data not shown). In contrast, even though the bioluminescence data and CFU counts indicated robust bacterial activity, the skin of ciprofloxacin treated mice looked healthy. Apparently, the antibiotic treatment did not suffice to eradicate the infection; nevertheless, it may have helped to reduce bacterial invasion and subsequent damage of the surrounding tissue. In comparison, the *in vivo* imaging approach was less sensitive than CFU determination. The data correlated under the diverse experimental conditions used with an approximately logarithmic relationship (Fig. 2D). In conclusion, luminosity was a useful parameter for monitoring bacterial activity.

Fig. 2. Correlation of CFU assay data and *in vivo* luminosity from infected implants. (A), bioluminescent images of animals immediately after implantation (d0), infection and ciprofloxacin administration (left) and 13 days after infection (d13). (B), CFU counts from infected implants in the absence (-) and presence of daily intravenous ciprofloxacin injections as indicated. (C), bioluminescent signal from infected implants in untreated (-) and ciprofloxacin treated mice. (D), Overall correlation of CFU and luminescence data shown in (B) and (C). The curve shown was calculated by the standard settings for the exponential curve fit algorithm of the Excel program (Microsoft). Inocula were chosen to allow a reliable confirmation by *in vivo* imaging. Statistical analysis by employing Kruskal-Wallis H test showed no statistically significant difference in either

colony forming units counts (CFU) or bacterial luminescence between untreated implants and ciprofloxacin treated implants. The n for each data point is 3 and the error bars represent the standard deviations for colony forming units (B) and bacterial radiance (C).

3.3. *Inoculum dependent persistence and antibiotic resistance of bacterial implant infections*

To evaluate the time-course of infections *in vivo*, subcutaneously implanted magnesium discs were locally infected with various amounts of luminescent bacteria. The resulting luminosity was monitored by *in vivo* imaging. Antibiotic susceptibility was investigated by daily intravenous injections of ciprofloxacin in either low (8 mg/kg) or high concentrations (24 mg/kg body weight), respectively. Immediately after inoculation, luminescence could be detected only from the highest inocula, whereas infections with lesser amounts of bacteria could not be visually confirmed (Fig. 3A, d0). In contrast to untreated controls, bacterial luminescence decreased after 6 hours in ciprofloxacin treated mice, confirming the *in vivo* efficacy of the antibiotic (Fig. 3B, d0.25). Nevertheless, bacterial luminescence from infected implants reappeared 2 days after infection even in antibiotic treated mice. This shows that a part of the bacterial population survived the immediate antibiotic treatment as a suspension even without having been given the necessary time span to adhere to implant surfaces or to form a biofilm (Fig. 3A, d1-d3). Subsequently, bacterial bioluminescence appeared on all implants infected with high inocula in the control mice, in the absence or presence of antibiotic (Fig. 3A, d4-d12). After day 7, the luminescent area from high inocula showed irregular extensions that were located outside of the implanted disc (Fig. 3A, d7-d12, white arrows). This finding was in accordance with previous electron microscopic observations, suggesting that the bacteria could invade the peri-implant tissue to a limited degree [18]. The

bioluminescence from infected implants was lower in mice treated with the highest antibiotic doses (Fig. 3A, 24 mg/kg). Therefore, the administration of high ciprofloxacin concentration was at least partially efficacious in controlling biofilms even from high bacterial inocula. On day 14, bacterial bioluminescence decreased considerably on high inocula infected implants without ciprofloxacin treatments (Fig. 3A; d14, white arrow and Fig. 3B). This correlated with visible tissue necrosis of peri-implant skin that may have been caused by high amounts of secreted bacterial toxins or by cytotoxic host immune responses. Interestingly, antibiotic treatment prevented toxic side effects even though the infection per se could not be eliminated. Infections with medium or low inoculums did not result in increases in the luminescent area and therefore appeared more susceptible to ciprofloxacin administrations (Fig. 3C and D). As in the previous experiments, during the first day bioluminescence from implants infected with low bacterial inocula was below the detection limit and could not be observed (Fig. 3C and D). However, from day 2 on, the luminescence became noticeable (Fig. 3A, d2). Even in the absence of antibiotics, a luminescent signal from implants infected with low inocula appeared from only one out of three infected implants, suggesting that the very high inocula used were relevant to allow bacterial proliferation and to establish persistent infections (Fig. 3D).

The survival and proliferation of viable bacteria in the presence of antibiotics was determined (Fig. 3B and C). The luminescence gradually decreased in response to the ciprofloxacin treatment in a dose dependent manner (Fig. 3B). However, the luminosity from implants with high inocula still remained higher compared to implants infected with lower inocula, confirming the importance of unexpectedly high initial bacterial numbers to establish persistent implant infections (Fig. 3B and C). Until day 7, highest bacterial

activities were observed from infected implants without antibiotic treatment (Fig. 3B). From day 9 on, ciprofloxacin administration resulted in reduced bacterial activity (Fig. 3C). From low inocula in the presence of ciprofloxacin no bacterial luminescence could be detected and even in the absence of antibiotics a signal was observed solely from one out of three infected implants (Fig. 3D). It is well known that larger inocula increase infection rates. However, considering present models that propose implant infections initiated by individual bacteria and the fact that the bacteria could multiply, there is so far no explanation as to why such high inocula are required to establish a detectable persistent infection. We previously observed that subcutaneously injected luminescent *P. aeruginosa* in the absence of implants disappeared quickly upon ciprofloxacin treatments [18]. However, the data in Figure 4 show that after infection of magnesium implants with a high bacterial dose there was no reduction of bacterial luminescence, suggesting that the dense bacterial population was in an antibiotic refractory state before biofilm formation could occur. Overall, these results were in accord with the notion that a very high inoculum size was an important parameter in determining the establishment of persistent magnesium implants infections and antibiotic resistance.

Fig. 3. Infectious dose of *P. aeruginosa* determines antibiotic resistance and persistence on implants. Magnesium discs in mice were subcutaneously implanted either sterile (N) or infected after implantation by locally injecting high (H; 10^5 CFU), medium (M;

10⁴ CFU) or low (L; 10³ CFU) inocula of bioluminescent *P. aeruginosa* suspensions, respectively. (A), the luminescence intensity is represented by false colors in the images as indicated by the scale bar on the lower right side. Abbreviations: (-), infected implants without antibiotic treatment; (8 mg/kg), infected implants with daily administrations of 8 mg/kg ciprofloxacin intravenously; (24 mg/kg) daily intravenous administration of 24 mg ciprofloxacin per kg body weight. (B-D), the graphs represent average radiance over the time after inoculation from implants infected with high, medium or low inocula (Y-axis). Error bars indicate standard deviations. A Kruskal-Wallis H test showed that there was no statistically significant difference in bacterial luminescence between infected implants without ciprofloxacin treatments (-), infected implants with daily administrations of ciprofloxacin (n=3 for each type of infection).

3.4. Antibiotics can efficaciously diminish systemic inflammatory effects but not local implant infections by P. aeruginosa

The primary response to infections is the rapid recruitment of innate immune cells from the blood circulation by diffusible inflammatory mediators. These cells play an essential role in locally containing infections [44]. Histological analyses confirmed the accumulation of inflammatory immune cells in infected peri-implant tissue compared to tissue next to sterile implants (Fig. 4A and B). Similarly, scanning electron microscopy revealed dense bacterial populations and individual host immune cells at infected sites (Fig. 4C and D). The high bacterial density is supportive of biofilm formation while the presence of host immune cells reflects the existence of a host immune response to the infection. The high antibiotic concentrations used were expected not to permit the survival of individual bacteria. This would prevent the escape from the protective environment of a biofilm since in the absence of biofilm formation infectious bacteria have been shown to be sensitive to 10 mg/kg ciprofloxacin [45, 46]. Unfortunately, we

were not successful to reliably determine the bacterial load of various body organs. However, there were several indications of disseminating bacteria or inflammatory bacterial remnants (Fig. 4E to F). Transmission electron microscopy of infected peri-implant tissue revealed phagocytic cells such as polymorphonuclear leukocytes or monocytes, some of which in the process of engulfing bacteria (Fig. 4E). Immune cells appeared to accumulate mainly in the periphery of the infected area. Nevertheless, in agreement with our previous findings, splenomegaly in mice carrying implants with high bacterial inocula could have been due to the systemic spread of bacteria (Fig. 4F, left to right) [34]. Spleens isolated from ciprofloxacin treated mice with infected implants had an intermediate phenotype, morphology and weight (Fig. 4F and G). Therefore, even though systemic antibiotic administration could not eradicate implant-associated infections, its positive effects in reducing local and systemic inflammatory responses strengthened the notion that the spread of bacteria could be reduced.

Fig. 4. Antibiotic therapy reduces systemic effects of implant infections. Two weeks after implant infection, tissues adjacent to implants were extracted, fixed, sectioned and subjected to Hematoxylin and Eosin staining. (A), peri-implant tissue adjacent to a sterile implant. (B), peri-implant tissue near the infected implant. (C), bacterial biofilms in the peri-implant tissue (asterisks). (D), host immune cells (arrows) near implant surfaces. (E), polymorphonuclear leukocytes (PMNs) denoted by white arrows engulfing bacteria

(asterisks). Histological images were taken using a 40x light microscope objective on hematoxylin and eosin (H&E) stained tissue sections. White arrows are pointing the host inflammatory cells. Labels are as follows: (c) connective tissue; (m) skeletal muscle. (F), Spleens were isolated from mice implanted with sterile magnesium (N) or implants infected with 10^5 CFU of *P. aeruginosa* without (-) or with daily intravenous injections of 8 mg Ciprofloxacin/kg body weight or 24 mg Ciprofloxacin/kg body weight. Photographs and weighing of spleens was done immediately after their isolation from mice (mean values $\pm$ standard deviation from 3 replicates). Statistically significant differences in spleen weights ($n=3$) were determined using Kruskal-Wallis H test, $\chi^2(2) = 10.4$, $p = 0.016$, with a mean rank weight score of 2 for sterile magnesium group (N), 11 for infected group without ciprofloxacin treatment (-), 8 for infected group receiving 8 mg/Kg body weight ciprofloxacin, and 5 for infected group receiving 24 mg/Kg body weight ciprofloxacin. Pairwise comparisons of the groups were performed with 2-sided Mann-Whitney U test. Significance values have been adjusted by the Bonferroni correction for multiple tests. Significant difference in spleen weights between sterile control group (N) and infected group (-) was observed ($p = 0.13$). Moreover, prior to Bonferroni adjustment comparisons between sterile control group (N) and infected group receiving 8 mg/Kg ciprofloxacin as well as between infected group (-) and infected group receiving 24 mg/Kg ciprofloxacin gave both $p = 0.042$. The result is significant at $*p < 0.05$, at $\#p < 0.05$ prior to Bonferroni adjustments.

4. Discussion

4.1. Characteristics of magnesium implant-associated infections

The results confirmed that above the sensitivity threshold, *in vivo* imaging data is useful and correlates with the number of viable bacteria on infected subcutaneous implants in mice. *In vivo* imaging of individual implants over the time showed that bacteria could establish persistent and antibiotic resistant infections in the presence of magnesium

implants. This is remarkable in several aspects. First and foremost, the bacteria could survive immediate antibiotic treatments even though it is presently thought that biofilm formation is essential for bacteria to become antibiotic treatment resistant [12]. Dense bacterial populations in subcutaneous pocket containing a biomaterial are likely to be able to evade antibiotic therapy, as this is the clinical scenario of human implant infections. Further, there is growing appreciation that non-specific antibiotic effects may promote disease by creating favorable niches for opportunistic pathogens. Biofilms have been reported to increase in the presence of ciprofloxacin potentially due to the inhibition of swimming and swarming [47]. Biofilm formation is thought to be a stepwise process requiring a surface to initiate bacterial assembly and a certain time span to mature. However, the freshly injected suspended bacteria survive immediate antibiotic treatment; it therefore appears unlikely that the observed resistance could be due to biofilm formation. Alternatively, since the efficacy of many antibiotics is dependent on proliferation or on metabolic activity, bacteria become less sensitive to antibiotics at high cell densities when nutrients become limiting [48]. Therefore, even without forming a bona fide biofilm, the high infection dose used is most likely required to overwhelm the host immune defenses and to acquire a degree of antibiotic resistance sufficient for survival of at least a critical fraction of the bacteria [49].

After a temporary reduction of bacterial activity, persistent infections could be established in the presence of magnesium containing implants only. This has not been observed when using conventional implant materials (titanium and porous glass) in the same experimental setup, suggesting that unique properties of magnesium or magnesium corrosion products are responsible [18, 37]. Persistent implant infections in general and magnesium implant infections in particular have been demonstrated to

involve bacterial biofilms [34, 50]. Adhesion of bacteria to implant surfaces is presently assumed to be a critical initial step towards the formation of biofilms [7]. However, after implantation, exposed magnesium implants exhibit an initially high corrosion rate and the reaction generates a bactericidal alkaline interface [51, 52]. In addition, corrosion-dependent hydrogen production by implanted magnesium discs has been shown to be sufficient to generate visible subcutaneous gas-filled pouches that could further antagonize adhesion [30, 51, 53, 54]. Since conventional implant materials like titanium alloys did not support persistent biofilms, magnesium implant surfaces would have to be even more hospitable than conventional implant materials, which we consider as an unlikely scenario [18, 34]. Presumably, bacteria observed on infected magnesium implants by electron microscopy are therefore nonviable and were by chance incorporated in the developing magnesium corrosion layer (Fig. 5). For these reasons, it appears likely that freshly implanted magnesium surfaces do not promote bacterial adhesion.

It has so far not been investigated by what mechanism bacterial persistence could be stimulated by magnesium alloys. A key factor that distinguishes magnesium alloys from other implant materials investigated is the generation of corrosion products, hydroxide that has been shown to act bactericidal, magnesium ions and hydrogen [55]. Even though the high pH is quickly neutralized in the tissue, hydroxide is continuously produced at the immediate material interface and therefore is not expected to result in a hospitable surface for bacterial attachment (unpublished observations) [56]. Magnesium is essential for all living organisms. However, magnesium is highly abundant in the human body and mostly iron ions rather than magnesium ions have been found to be limiting for pathogenic bacteria [57]. While increased magnesium ion concentrations may

contribute to the observed antibiotic resistance, it appears less likely that this effect could suffice to initiate biofilm formation. Lastly, hydrogen has been shown to accumulate in tissue near magnesium alloy implants resulting in macroscopic bubbles [29]. Even though hydrogen is not known to support pathogenic bacteria, its evolution initially causes tissue cavities. Hydrogen is also known to diffuse rapidly and the entrapped gas rapidly equilibrates with gases carried by the circulation blood [58, 59]. Interfaces play an important, if not essential role in bacterial biofilm formation. If magnesium implant surfaces can promote persistent biofilm formation without offering an adhesive solid surface, we propose here that instead gas interfaces, either with tissue or wound liquid are decisive in promoting biofilm formation on magnesium implants. Palpable gas bubbles appeared in the un-coated magnesium implants which correlated with areas where luminescent bacteria were detected (Fig. 5A and B). However, by applying phosphate coating the generation of gas cavities and bacterial luminescence was not detected [38]. Moreover, by applying direct ciprofloxacin coating on magnesium, bacterial luminescence could not be detected (Fig. 5A and B). Later on, micro-cavities were detected in the periimplant tissue 2 weeks later (Fig. 5 C and D). We speculate that these cavities were caused by a decreased but still continuing hydrogen evolution. Gas-liquid interfaces are well known to be favorable sites for environmental biofilm formation on water [60, 61]. Similarly, exposed surface burn wound infections are known to be at a high risk for infections [62]. For these reasons it appears probable that even internal unprotected gas-raw tissue or wound liquid interfaces would similarly provide a niche for bacterial biofilm formation. Even 2 weeks after implantation, microbubbles were apparent on infected magnesium implants, suggesting that gas

interfaces could not only be responsible for the establishment of biofilms on magnesium implants but also for sustaining the infection later on (Fig. 5C).

Fig. 5. Gas cavities in tissue adjacent to magnesium implants. (A), Mice with phosphate coated magnesium (a and b), ciprofloxacin (250 μg) coated magnesium (c), and un-coated magnesium (d) at the indicated time points in days (d0-d12). (B), superficial mice image shows gas bubble and infection around un-coated magnesium (d). There are no signs of visible gas cavities and bacterial biofilms around phosphate coated magnesium implants (a-c). (C), After 2 weeks, the mice were sacrificed and tissue adjacent to implants was removed, fixed and images were taken with a scanning reflection electron microscope. Micro gas cavities in the infected peri-implant tissues and (D), details of a cavity in peri-implant tissue with bacteria (arrows).

4.2. Implications for anti-infective strategies and aspects of clinical relevance

A key feature of the above model is that bacteria do not need to attach to implant surfaces as a mandatory step to form biofilms. As previously discussed, this would render anti-adhesive or contact dependent bactericidal implant surface coating strategies futile [50].

So far, besides the notion that tissue adhesion is of importance to prevent implant infections by avoiding susceptible exposed implant surfaces, there is no evidence what the detailed mechanisms are that lead to infections late after surgery. The model proposes that gas interfaces in the periphery of implants promote the survival of infectious bacteria (Fig. 6). The model states that gas interfaces are an important risk factor for bacterial infections. Clinically, tissue-air interfaces at risk occur inevitably during open surgery. After wound closure, infection prone foam or air bubbles could remain entrapped in the implant periphery. Even later on, in particular for movable parts like joints or heart implants, mechanical stress could lead to spontaneous gas bubble formation that represents an ongoing risk for opportunistic infections. For these reasons, not only reducing wound-tissue exposure during surgery but also minimizing chances for gas bubble inclusion or formation after surgery could reduce implant infection risks in the clinic.

Fig. 6. Model of clinical and experimental implant infection enhancement by gas interfaces. (A), Consecutive stages of biofilm formation on gas-tissue or gas-wound liquid interfaces are indicated from left to right. An implant (grey square) is shown imbedded in tissue (red) surrounded by decreasing amounts of wound liquid (orange). (1) As an initial step either magnesium corrosion-dependent, accidental intraoperative or mechanical stress induced gas bubbles (white ovals) are proposed to promote the survival and proliferation of opportunistic bacteria (brown). This first step could not be reproduced in the mouse model, possibly due to differences between the human and the murine immune system, the use of less pathogenic bacterial strains or due to other unknown experimental parameters. (2) With time, a dense population of bacteria forms at the interface between gas bubble and either wound liquid or tissue. Bacterial inflammatory mediators attract phagocytic immune cells (yellow) from the blood circulation. The dense bacterial population could experimentally be reproduced by local injection. Likewise, magnesium implants generate hydrogen bubbles as a corrosion product, with a decreasing tendency due to the formation of a protective corrosion layer (light gray). Hydrogen then equilibrates with gases bound in the tissue and transported by the circulating blood. The interface thereby becomes attractive for invading bacteria. (3) Further proliferation leads to maximal cell densities, extracellular matrix

accumulation and biofilm formation associated with increased antibiotic resistance. Peripheral bacterial cells can efficaciously be attacked or engulfed by phagocytic cells. (4) Secreted bacterial proteases allow bacterial invasion into the surrounding tissue. (5a) Cytotoxic immune responses together with bacterial toxins lead to tissue necrosis and expulsion of the infected implant. (5b) In the presence of antibiotics individual bacteria escaping from the biofilm are eradicated, the infection and the immune response are locally contained. The model is a modification of a previous version that did not include gas bubbles [63]. Not drawn to scale.

5. Conclusions

In the current study, we found a critical role of initial infectious dose in modulating early events of *P. aeruginosa* biofilm formation on subcutaneous magnesium implants and bacterial antibiotic resistance. Gas interfaces are proposed to be responsible for enhancing magnesium alloy implant infections and to represent a risk factor for clinical implant infections in general. Consequently, it would be of primary importance to minimize time and wound surface air exposure of tissue during surgery. With respect to implants, the accidental generation and maintenance of gas bubbles should be minimized. To this end tight tissue adhesion to implant surfaces by smooth, host cell adhesion-friendly surfaces and minimizing mechanical tissue stress could diminish critical gas bubble formation. Conversely, movable implant parts, pores or concave surfaces that could hinder tissue adhesion or prolong the existence of gas bubbles should be avoided whenever possible. In addition, the model predicts that novel implant surfaces that antagonize bacterial adhesion or innovative contact-dependent bactericidal surfaces will be ineffective *in vivo*. Since *P. aeruginosa*-related infections are critical problem in the clinic, the present study indicates a highly reproducible potential of *P.*

aeruginosa to make biofilms on magnesium implants. We believe this finding could be employed to investigate molecular mechanisms underlying clinical infectious biofilm formation and to investigate the efficacy of direly needed prospective anti-biofilm agents.

Author contributions

conceptualization, PPM and MIR; methodology, MIR; validation, PPM., and MIR; formal analysis, PPM; MIR; and SPS; investigation, PPM and MIR; resources, PPM and MS; data curation, MIR, SPS and AIT.; writing—original draft preparation, PPM and MIR.; writing—review and editing, PPM, MS, MIR, SPS and AIT; visualization, PPM and MIR; supervision, PPM and MS; project administration, MS and PPM.; funding acquisition, PPM.

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Competing interests

The authors declare no conflicting interests.

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