Degradable magnesium implant-associated infections by bacterial biofilms induce robust localized and systemic inflammatory reactions in a mouse model

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Abstract

Biomaterial-associated Pseudomonas aeruginosa biofilm infections constitute a cascade of host immune reactions ultimately leading to implant failure. Due to the lack of relevant in vivo biofilm models, the majority of the studies report host immune responses to free-living or planktonic bacteria, while bacteria in clinical situations live more frequently as biofilm communities than as single cells. The present study investigated host immune responses to biomaterial-associated P. aeruginosa biofilms in a clinically relevant mouse model. Previously, we reported metallic magnesium, a prospective biodegradable implant, to be permissive for bacterial biofilm in vivo even though it exhibits antibacterial properties in vitro. Therefore, magnesium was employed as biomaterial to investigate in vivo biofilm formation and associated host immune responses by using two P. aeruginosa strains and two mouse strains. P. aeruginosa formed biofilm on subcutaneously implanted magnesium disks. Non-invasive in vivo imaging indicated transient inflammatory responses at control sites, whereas robust prolonged interferon-β (IFN-β) expression was observed from biofilm in a transgenic animal reporter. Furthermore, immunohistology and electron microscopic results showed that bacterial biofilms were located in 2D immediately on the implant surface and at a short distance in the adjacent tissue. These biofilms were surrounded by inflammatory cells (mainly polymorphonuclear cells) compared to the controls. Interestingly, even though the number of live bacteria in various organs remained below detectable levels, splenomegaly indicated systemic inflammatory processes. Overall, these findings confirmed the resistance of biofilm infections in vivo to potentially antibacterial properties of magnesium degradation products. In vivo imaging and histology indicated the induction of both local and systemic host inflammatory responses to P. aeruginosa biofilms. Even though the innate host immune defenses could not eliminate the local infection for up to two weeks, there was no apparent systemic bacteremia and all the animals investigated survived the infection.

1. Introduction

Applications of medical implants have increased manifold in humans for reasons such as organ loss due to chronic diseases and accidents [1]. Pseudomonas aeruginosa is the principal pathogen responsible for biofilm formation on medical implants [2, 3]. Biofilms are the most precarious infections, since they are thousands of times more resistant to conventional antibiotics and host immune systems [4–6]. Biomaterial-associated biofilm infections are mostly treated by surgical removal of the implant from the infected tissue followed by the administration of antibiotics [7]. Such inevitable surgery together with antibiotic
administration is extremely detrimental to patient health and the economy. Moreover, biofilm infections trigger unrestrained host immune reactions, which can cause immunosuppression, shock, transudation in organs, and defects in coagulation [8–10]. Innate and adaptive immune responses to various pathogens comprise accumulating phagocytic cells and inflammatory cytokines, among others interferons (IFNs) play a critical role [11, 12]. However, the performance of host immune responses is compromised when most of the implant materials require a surgical procedure for their insertion into the body [13, 14]. Initially, open wounds, body liquid-filled gaps between the implant and the surrounding tissue, structural, mechanical, and chemical properties of implants hinder immune cells from invading bacteria [15–19]. Later, bacteria secrete extracellular matrix material (ECM), which prevents phagocytic immune cells such as macrophages and neutrophil granulocytes from engulfing the bacteria. The ECM also limits the diffusion rate of nutrients and metabolites, thereby reducing the bacterial metabolic activities. This impedes the efficacy of the proliferation-dependent antibiotics, thereby reducing the efficacy of various antibiotics [20–22].

To counter biofilm infections and augment the performance of implant materials, various strategies have been introduced. These include, metal alloy implants with antimicrobial properties, antibacterial as well as anti-adhesive nanoparticle coatings, and localized antimicrobial drug delivery systems on the implant surfaces [23, 24]. Metallic magnesium as a biodegradable implant material is being highly investigated for clinical applications due to its in vitro bactericidal corrosion products and antibacterial properties, which can efficiently counter biomaterial-associated biofilm infections [25–28]. Magnesium alloys are presently under intense investigation as degradable, yet sturdy implant materials that could potentially be used for temporary applications to improve bone healing and avoid the long-term side effects associated with permanent metallic implants [29–31]. From our previous investigation as well as from the literature, magnesium and its alloys are reportedly susceptible to P. aeruginosa colonization and subsequent biofilm formation [32–34]. Limited information exists on the capacity of biomaterial-associated P. aeruginosa biofilms to escape the local innate immune defense and spread into the adjacent tissue or even systemically due to the lack of clinically relevant biofilm mouse models [35, 36]. In addition, there is no in vivo information about the role of interferons against biofilm infections. Therefore, we employed in vivo P. aeruginosa biofilm formation on magnesium as a mouse model and report here P. aeruginosa biofilm repercussions directly on the implant interface, in the adjacent tissue, and systemically. We used a non-invasive in vivo imaging system to monitor the in vivo biofilm formation and interferon response to these infections [37]. Side by side, morphological, histological, and electron microscopic analyses were performed to determine biofilm effects on the host.

2. Material and methods

2.1. Preparation of magnesium samples and bacterial cultures

Disc-shaped magnesium samples (99.95% purity) having 5 mm diameter and 2 mm thickness were prepared and processed, as described previously [38]. Bioluminescent and wild type P. aeruginosa strains were streaked on LB agar plates and incubated for 18 h at 37 °C [26, 39]. For liquid cultures, isolates were cultured in BL broth medium and kept at 37 °C with a shaking speed of 150 RPM until the desired OD$_{600}$ = 0.1 was achieved. The bacterial suspension was then immediately placed on crushed wet ice to inhibit further growth.

2.2. Surgical procedures and in vivo infections

Healthy 8 week old female wild type BALB/c mice were purchased (Harlan Winkelmann, Borchen, Germany) and IFN-β-reporter mice (IFN-β$^{+/Δ}$β-lacZ) on the BALB/c background (ifn$^{-1}$β$^{Δ}$β-lacZ) [40] were bred at the Helmholtz Centre for Infection Research, Braunschweig. The mice were kept in individually ventilated cages in a dedicated infection facility with water and food ad libitum. Before surgery, the animals were anesthetized with xylazine (4 mg Kg$^{-1}$) and ketamine (10 mg Kg$^{-1}$) and fur was removed at the site of surgery using a hair trimmer (Aesculap Suhl GmbH, Germany). Under sterile conditions in a laminar flow hood, 0.5 cm long incisions in the skin were made at three sites on the back of the mice using surgical scissors (Fine Science Tools GmbH, Heidelberg, Germany). A subcutaneous pouch was created from the incisions and 99.95% pure magnesium disks with 5 mm diameter and 2 mm thickness were inserted [26]. The wounds were closed by applying simple interrupted sutures (Ethicon Vicryl, Johnson & Johnson Medical GmbH). Immediately after implantation, logarithmically growing cultures of wild type P. aeruginosa (PA01) or bioluminescent P. aeruginosa (PA01 CTX::lux) at OD$_{600}$ = 0.1 were injected subcutaneously onto the surface of the implanted disks [41, 42]. For monitoring the host interferon response, wild type P. aeruginosa (PA01) were injected on subcutaneously implanted magnesium disks in the IFN-β reporter (IFN-β$^{+/Δ}$β-lacZ) BALB/c mice, which allowed tracking of the IFN-β gene induction [40]. D-Luciferin (150 mg Kg$^{-1}$) (Calipers) in phosphate-buffered saline (PBS) was injected intraperitoneally 15 min before examination by in vivo imaging. In vivo luminescence was recorded by using a Xenogen IVIS-200 optical imaging system (Caliper Life Sciences) equipped with an XGI-8 gas anesthesia unit (Caliper Life Sciences, Hopkinton, MA) using 2% isoflurane. The IFN-β-reporter mice were examined daily for a
period of 2 weeks. The BALB/c mice were euthanized on the 8th day post infection and then the magnesium implants with adjacent tissue were removed and fixed in a buffered formalin solution according to standard procedures [32]. Animal experiments were performed with the permission of the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES; Oldenburg, Germany) under approval number 33.42502/07-10.5.

2.3. Field emission scanning electron microscopy (FESEM)
For electron microscopic examination, the explanted magnesium and tissue samples were sputter coated with palladium-gold. The peri-implant tissues were fixed with 5% formaldehyde and 2% glutaraldehyde in HEPES buffer (100 mM HEPES, 90 mM sucrose, 10 mM MgCl₂, 10 mM CaCl₂, pH 6.9) washed twice in TE buffer (10 mM TRIS, 2 mM MEDTA, pH 6.9), dehydrated in a graded series of acetone (10%, 30%, 50%, 70%, 90%, 100%) solutions for 15 min on ice and then dehydrated further in 100% acetone at room temperature. Subsequently, critical-point drying was performed with liquid CO₂ (CPD300, Leica or CPD030 Bal-Tec). The dry tissue samples were sputter coated with palladium-gold (SCD500, Bal-Tec). The samples were examined using a Zeiss Merlin FESEM at an acceleration of 5 kV and by using the Zeiss SmartSEM software version 5.05 (Zeiss, Oberkochen, Germany).

2.4. Transmission electron microscopy (TEM)
The mice bearing infected implants were euthanized with CO₂ followed by cervical dislocation. The peri-implant tissue was fixed in HEPES buffer containing 2.5% glutaraldehyde and 5% formaldehyde. The samples were washed with HEPES buffer and then incubated for one hour at room temperature in 1% aqueous osmium solution. The samples were dehydrated by serial incubation in acetone–water mixtures with increasing acetone concentrations (10%, 30%, 50%, and 70%) on ice, followed by a dehydration step in 70% acetone. Then, the samples were incubated overnight in 70% acetone and 2% uranyl acetate at 4 °C. The following day, the dehydration process was continued in 90% and 100% acetone on ice and eventually in 100% acetone at room temperature. The samples were then embedded in epoxy resin, as previously described [43]. Ultrathin sections from these samples were prepared with a diamond knife, picked up with butvar-coated grids, counterstained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (TEM910, Carl Zeiss, Oberkochen) set to an acceleration voltage of 80 kV. TEM images were digitally recorded at calibrated magnifications with a slow-scan CCD camera (ProScan, 1024 × 1024, Scheuring, Germany) using ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany). Brightness and contrast were adjusted with the help of an Adobe Photoshop version (Adobe).

2.5. Histological procedures
For histological analysis, the animals were sacrificed by CO₂ asphyxiation followed by cervical dislocation. The infected peri-implant tissues were isolated and fixed in 4% formalin for 3 d, as previously described in detail [44]. Briefly, the samples were dehydrated in a series of increasingly concentrated ethanol solutions and then embedded in paraffin. Histological slides of approximately 3 μm thickness were prepared from paraffin-embedded tissue samples. The sections were stained with hematoxylin and eosin (H&E) or periodic acid-schiff (PAS) staining according to standard laboratory procedures. Moreover, immunohistostaining for P. aeruginosa was performed with rabbit-polyclonal anti-Pseudomonas, (Biotrend, AP086) after heat-mediated antigen retrieval. The samples were evaluated histo-pathologically on microscope slides using a light microscope, randomized, and blinded to the experimental groups.

2.6. Tissue homogenization and colony-forming unit (CFU) assay
One week after infection, the mice bearing biofilms were peacefully euthanized with CO₂ followed by cervical dislocation and their visceral cavities were opened. Visceral organs such as the liver, spleen, and gut were removed and their weights were measured. In parallel, the subcutaneously implanted magnesium disks in wounded biofilms and the adjacent tissue mass were isolated. Liver, gut, and peri-implant tissue were then placed in homogenization tubes containing 0.1% (v/v) X-100/PBS Triton solution. These tubes were then fixed in Polytron PT3000 homogenizer (Kinematica) and the homogenization process was carried out for 86 s. The explanted infected magnesium disks were directly submerged in PBS and vigorously vortexed for 5 min at maximum speed to disrupt the attached biofilm mass. The samples were analyzed for bacterial cell density (CFU/ml) by tenfold serial dilution and plating on LB plates at 37 °C for 18 h.

3. Results
3.1. P. aeruginosa biofilm formation on magnesium implants in a mouse model
A major challenge to establishing a biofilm mouse model is the rapid elimination of bacteria by the host immune system. To establish a mouse model, which supports biofilm infections for a prolonged period of time, we implanted subcutaneously three magnesium disks at selected locations in a mouse and then injected bioluminescent P. aeruginosa (PAO1) directly on each of these implants (figure 1, d0). In agreement with our
previous findings, the magnesium implants augmented the survival of the bacteria on their surfaces [32]. Bacteria formed biofilms on the magnesium and persisted for 2 weeks on the surface of these implants (figure 1, d0–d14). This experiment confirmed and reproduced our previous findings that magnesium promotes biofilm formation for an extended period of time. Previously, there was not a single mouse model which allowed biofilm formation on metallic implants with plain surfaces in the presence of an active host immune system. Therefore, we employed our system to investigate the distribution of biofilm infections and associated host immune responses.

3.2. Injected bacteria provoke a local interferon type-I immune response that can be visualized in transgenic mice by in vivo imaging

Type-I interferon response is triggered by various host cells and plays a critical role during the course of viral or bacterial infections. To investigate the interferon response to magnesium-based prolonged P. aeruginosa infections, we employed IFN-β reporter mice. In these transgenic mice, luciferase is expressed together with IFN-β, which is a virally and bacterially induced cytokine [40]. Magnesium implants were placed subcutaneously in the IFN-β reporter mice. The implanted magnesium disks were then infected with wild type P. aeruginosa (PAO1) and the IFN production was observed by in vivo imaging. Side by side, the IFN response measured from subcutaneously implanted sterile magnesium and P. aeruginosa injected into subcutaneous sham surgical pouches without implants served as controls. Immediately after infection, the IFN response was not within detectable limits of the system from the subcutaneous regions in mice carrying infected magnesium, sterile magnesium, or infected tissue (figure 2(A)d0, a–c). After 1 d, robust IFN-β-luciferase activities were detected from the sites that were infected either with P. aeruginosa only or in the presence of magnesium implants (figure 2(A), d1(a–c)). IFN-based luminescence was not observed from the sites with sterile magnesium implants during the entire course of the experiment (figure 2(A), d1b). In vivo expression of IFN-β induced by bacteria administered into the sham surgical pouches was detectable for 2 d and then disappeared. However, robust and prolonged IFN response detectable for 2 weeks was measured from the magnesium infected with P. aeruginosa (figure 2(B), filled circles). Moreover, tissue necrosis was observed from these regions (pictures not shown). These results confirm our previous findings that magnesium implants support biofilm formation by P. aeruginosa, even in reporter mice. Prolonged IFN production and the resulting tissue necrosis confirm that P. aeruginosa biofilms provoke severe localized immune reactions.

3.3. P. aeruginosa biofilms cause intense accumulation of host immune cells

A prolonged IFN response to P. aeruginosa biofilms provided evidence that these infections remain restricted at the site of infection. To confirm and
explore the distribution of biofilms, implants after 1 week of infection were isolated together with adjacent tissue and subjected to electron microscopic analyses. Scanning electron microscopy (SEM) analysis revealed that *P. aeruginosa* encased in slimy material indicative of biofilms were clearly visible on the surface of the implants (figure 3(A); asterisk symbols). In parallel, tissue pockets filled with bacteria were visible into the adjacent tissue (figure 3(B); asterisk symbols). Bacterial intrusions into the tissue indicate the disseminating nature of biofilm infections (figure 3(B)). Interestingly, this process occurred in the presence of large populations of host immune cells (figure 3(C); white arrows). TEM clearly indicated colonies of *P. aeruginosa* embedded deep into the tissue even in the presence of polymorphonuclear leucocytes (PMN) (figures 3(D) and (E); white arrows). Electron transparent regions, a strong indication of biofilms, were clearly visible among the bacterial biofilms (figure 3(D); white arrows). *P. aeruginosa* was observed both intra- and extracellularly in PMN at the site of the biofilm formation (figure 3(F); asterisk symbols). This proves that the process of biofilm formation took place in the presence of active immune cells, which were indeed capable of engulfing the bacteria around the biofilms. Overall, it appeared that bacteria could utilize implant–tissue interfaces to establish biofilms and their distribution was limited to a short distance in the tissue where the spread of the infection was restricted by the host immune cells.

3.4. *P. aeruginosa* biofilms induce strong inflammatory responses in adjacent tissue
Host inflammatory response to bacterial invasion and foreign material is an expected phenomenon [45, 46].

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**Figure 2.** Persistent bacterial infections on implants can be monitored in transgenic mice by *in vivo* imaging of the host interferon response. Magnesium disks surgically implanted into the IFN-β-reporter mice were infected with wild type *P. aeruginosa* (A,a) or kept sterile (A,b). In parallel, bacteria were injected into sham surgical pouches (A,c). (A) IFN-luciferase activity in response to bacterial infections (B, black circles), in response to injected bacteria in the absence of implants (B, empty squares), or in the presence of sterile magnesium implants (B, empty triangles), and background activity (empty circles) are indicated.
In contrast to planktonic cultures, biofilm bacteria persist longer within the host tissue even in the presence of an active immune system. To evaluate the intensity of the inflammatory reaction induced by \(P.\ aeruginosa\) biofilms, tissue sections were isolated from the mice bearing luminescent \(P.\ aeruginosa\) biofilms on magnesium implants after 1 week of infection and then subjected to H&E staining. For comparison, tissue sections from the sham surgical pouches infected with \(P.\ aeruginosa\) and implanted with sterile magnesium were taken as controls. After 1 week, luminescent \(P.\ aeruginosa\) had established biofilms on the magnesium implants. Bacteria injected into a sham surgical pouch in the absence of implants disappeared within a few days. In an overview, the part of the tissue that housed the \(P.\ aeruginosa\) biofilms exhibited a high degree of inflammation with more obvious infiltration of severe neutrophilic inflammatory infiltrates surrounding the bacterially infected implants (figures 4(A) and (B)). Comparatively, the tissue portions that were infected with bacteria in the absence of implants or implanted with sterile magnesium elicited mild foreign-body reactions (figures 4(C)–(F)). These results strongly indicate that \(P.\ aeruginosa\) biofilm interactions with the tissue in the presence of magnesium provoke a high degree of inflammatory response.

### 3.5. Evidence of biofilm constituents in peri-implant tissue

Biofilm matrix is mainly composed of sugar polymers and proteins that can be visualized by PAS staining [47, 48]. Even though electron microscopic analyses clearly revealed \(P.\ aeruginosa\) biofilms in the tissue, PAS staining was applied to further verify \(P.\ aeruginosa\) biofilms in the tissue near the infected magnesium implants. As expected, the PAS positive material indicative of the presence of biofilm matrix was detected in the tissue harboring infected magnesium implants (figures 5(A) and (B), black arrow). However, no PAS positive material could be detected near the infected areas in the absence of implants or in the tissue with sterile magnesium implants (figures 5(C)–(F)). The data also indicated that bacterial implant infections were not solely restricted to the implant surface. Instead, they expanded a short distance into the tissue and were walled off by the host immune cells.
3.6. Histological evidence of biofilm invasion into peri-implant tissue

Previous analyses confirm that tissues near the infected implants are filled with \textit{P. aeruginosa} biofilms. To identify bacteria in the tissue, antibodies targeting \textit{P. aeruginosa} were applied on the tissue sections isolated from the vicinity of the infected magnesium. Interestingly, a distinct region positive for \textit{Pseudomonas} staining was visible in the tissue (figure 6(A), gray area marked by a black arrow). At high magnification, it could be seen that \textit{P. aeruginosa} was dispersed within this particular area of biofilm formation (figure 6(B), black arrow pointing to \textit{P. aeruginosa}). The area positive for pseudomonas staining was surrounded by a high influx of inflammatory cells, which confirms biofilm resistance to the host immune cells (figure 6(B), black arrow). Tissue bearing sterile magnesium or infected with bacteria only was negative for pseudomonas staining (figures 6(C)–(F)). Overall, various tissue-staining techniques confirmed that \textit{P. aeruginosa} biofilms were not limited to the implant surface. Instead, they penetrate into the nearby tissue and trigger the induction of strong immune reactions (table 1).

3.7. Splenomegaly in mice with \textit{P. aeruginosa}-infected magnesium implants

Strong immunogenic and tissue inflammatory reactions associated with the presence of magnesium-based \textit{P. aeruginosa} biofilms convince us that these infections are not localized and can spread into the adjacent tissue. In the next step, the systemic effects of the biofilms were investigated by observing the liver, spleen and gut of mice bearing \textit{P. aeruginosa} biofilms. These organs were then homogenized and the number of bacteria was determined. Bacterial CFUs could not be counted from the liver or spleen, except for the intestine, which seemed normal flora (table 2). The morphology of the intestine and liver of the mice bearing \textit{P. aeruginosa} biofilms was normal and similar to uninfected animals. Surprisingly, the size as well as weight of the spleen isolated from the infected mice had increased compared to the uninfected mice (figure 7(A) compare a with b). These results provide evidence that biomaterial-associated biofilm infections in the host affect systemic organs, particularly the spleen. Importantly, such a significant increase in the size and weight of the spleen is indicative of additional...
complications for patients suffering from biomaterial-associated infections.

4. Discussion

The results in this study confirm and refine previous results in that in vitro antibacterial properties of pure magnesium are not sufficient to prevent bacterial colonization of implants and instead apparently even promote prolonged bacterial survival under in vivo situations. Ours and some other studies have previously reported that in vitro antibacterial properties of magnesium are due to high alkalization. Therefore, one explanation for the absence of in vivo antimicrobial properties could be the absence of high pH in the peri-implant tissue controlled by the efficient body-buffering system [26, 49]. In addition, the development of conditioning film by the blood plasma or wound liquid creates a barrier for the dissemination of magnesium corrosion products, thereby allowing the colonization of bacteria [50, 51]. Since hydrogen gas is a degradation product of magnesium corrosion, it cannot be excluded that the formation of gas pockets on the magnesium surface provides protection to the bacteria from early host cell invasion, and subsequently develop biofilms. Therefore, before clinical applications can be envisioned, it will be necessary to enhance the antibacterial properties of magnesium. Since magnesium alloys can be manufactured that have superior mechanical qualities, it could be envisioned to add antibacterial-acting metal alloys, such as copper or silver [52, 53]. Since in this study no spontaneous infections of the magnesium alloy implants were observed, it may be sufficient to provide temporary protection for a limited time after surgery by antibiotic-releasing coatings until the wound has closed and the implant is protected by a fibrous capsule [54]. By the application of these strategies, the susceptibility of magnesium implants to biofilm infections could be reduced and it can be promoted for clinical applications.

P. aeruginosa is a significant pathogen responsible for implant failure in patients due to its biofilms. One important question about P. aeruginosa biofilms is whether the bacteria are localized on the implant surface, in the interface zone, or evade the tissue. Second, there is limited information about host immune responses to these biofilms. To answer these questions, the major challenge is the lack of clinically

Figure 5. Evidence of biofilm ECM on the infected magnesium implants extending a short range into the peri-implant tissue. The tissue was fixed and subjected to PAS staining for histological examination by light microscopy. PAS positive staining of P. aeruginosa in the peri-implant tissue (A and B black arrow) propagated from the infected magnesium implants, PAS-stained tissue infected with bacteria in the absence of implants (C and D), and tissue bearing sterile magnesium implants (E and F). Scale bars on the left side correspond to 100 μm, scale bars on the right side to 26 μm.
relevant animal models. Previous studies report that bacterial biofilms are mainly restricted on or near the surfaces of implant materials, because the mechanical and structural properties of these materials protect them from being engulfed by the host immune cells \[55–57\]. However, a recent study reported that *S. epidermidis* biofilms penetrated into the nearby tissues after initially growing on the implanted materials, resulting in robust inflammatory immune reaction \[35\]. However, in-depth characterization of biofilm-mediated immune responses was not done in any of these studies. Here, we report for the first time biofilm-mediated immune responses in a highly sensitive reporter mouse expressing type-I interferon luciferase under transcriptional control of the IFN-β promoter \[40\]. Our results indicate robust and persistent type-I

Table 1. Inflammation intensity in tissue adjacent to biofilm-bearing magnesium disks, magnesium only, and infection only.

| Tissue samples                              | H&E staining | PAS staining | *Pseudomonas* staining |
|---------------------------------------------|--------------|--------------|-----------------------|
| Bacterially infected magnesium implants     | 4*           | 3            | 3                     |
| Bacterial infection without implants        | 0            | 0            | 0                     |
| Sterile implants                            | 1            | 0            | 0                     |

Numbers indicate an arbitrary inflammatory score from 0 (no response observed in any of the three samples) to 4 (distinct inflammatory response in all three samples investigated). The data shown were compiled by an extensive analysis by an experienced histologist.

Table 2. Bacterial CFUs obtained from various tissue samples derived from mice carrying subcutaneous *P. aeruginosa*-infected magnesium implants.

| Time post infection | CFUs/magnesium implant | CFUs g\(^{-1}\) peri-implant tissue | CFUs g\(^{-1}\) gut tissue | CFUs g\(^{-1}\) of liver |
|---------------------|-------------------------|------------------------------------|---------------------------|--------------------------|
| 1 week              | 3.60 ± 1.51e9           | 2.77 ± 1.84e9                      | 2.73 ± 1527               | 0                        |
IFN induction from *P. aeruginosa* biofilms compared to the controls. The IFN responses to *P. aeruginosa* biofilms were not immediate and were visible after 24 h of infection. Although molecular mechanisms for delayed IFN response were not investigated, an explanation could be that initially injected planktonic bacteria were not sufficient to stimulate IFN production. However, at later stages, the bacterial number increased by biofilm formation resulted in IFN production. It also confirms that *P. aeruginosa* biofilms are recalcitrant to the immune system even in the presence of the IFN secreting active host cells. In addition, we restricted our research to the finding that strong IFN production is induced in response to *P. aeruginosa* biofilms. However, molecular mechanisms responsible for the activation of IFN in this mouse model need to be investigated. PMNs are powerful biocidal innate immune cells that can attack and occasionally eliminate biomaterial-associated biofilms. The formation of *P. aeruginosa* biofilms in tissue together with PMNs indicates that implanted material provides initial niches for planktonic bacteria, which may otherwise be easily captured and killed by these cells in the absence of magnesium implants. At later stages, bacteria switch to biofilms and are strong enough to cope with immune cells. Consequently, bacterial biofilms from the implant surface penetrate into the nearby tissue and persist there as well. The detection of inflammatory cells, bacteria, and biofilm matrix components in the same tissue endorses that biofilms migrate from the implanted surface to adjacent tissue and are certainly resistant to the host immune system [58–60]. Biofilms in the tissue provide compelling evidence for the proliferating nature of these infections involving strong inflammatory reactions. Such a high influx of immune cells against biofilms could have the involvement of systemic organs, and therefore various body organs of the mice with biofilm-overgrown implants were observed. Interestingly, no systemic organ infiltration by bacteria could be detected by using the approaches described in this study. However, splenomegaly in mice with biofilms was observed. Splenomegaly is reported due to various reasons, but this is the first time that we have observed it in an animal suffering from biofilm infections [61, 62]. It is possible that cytokines or migratory immune cells originating at the infected region could affect the inflammatory spleen response. Increases in the spleen size could nevertheless be indicative of systemic bacterial spread, perhaps at a level too low to detect by the methods applied here or it could be caused by the systemic distribution of inflammatory bacterial degradation products.

The present study validated both localized and systemic inflammatory immune responses to bacterial biofilms established on magnesium implant surfaces. Thus, novel strategies need to be established to control such infections.

5. Conclusion

Persistent bacterial biofilms can form on degradable metallic magnesium implants in a small animal model. Luminescent-labeled bacteria allowed the monitoring of the course of the infection by *in vivo* imaging and the localized host immune response could be visualized by a transgenic mouse model. The resulting splenomegaly may be indicative of a low-level systemic spread of bacteria or bacterial products. In the absence of reliable conventional antibiotic treatments, novel strategies including antibiotic-acting implants may be envisioned to improve the performance of future magnesium-based implants.

**Figure 7.** Prolonged *P. aeruginosa* biofilms on the magnesium implants lead to a significant increase in the size of the spleen. Magnesium implants were infected with growing cultures of *P. aeruginosa* or kept uninfected in individual mice. After 8 d, the mice were euthanized and their organs were removed and imaged with a light microscope (A). Spleen of mice bearing biofilm (A, a) and spleen of control mice (A, b). Changes in length (B) and weight (C) of the spleen isolated from the mice bearing infected or sterile magnesium implants.
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