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*Review Article*

# Host Defence against Bacterial Biofilms: "Mission Impossible"?

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Bacteria living as biofilms have been recognised as the ultimate cause of persistent and destructive inflammatory processes. Biofilm formation is a well-organised, genetically-driven process, which is well characterised for numerous bacteria species. In contrast, the host response to bacterial biofilms is less well analysed, and there is the general believe that bacteria in biofilms escape recognition or eradication by the immune defence. In this review the host response to bacterial biofilms is discussed with particular focus on the role of neutrophils because these phagocytic cells are the first to infiltrate areas of bacterial infection, and because neutrophils are equipped with a wide arsenal of bactericidal and toxic entities. I come to the conclusion that bacterial biofilms are not inherently protected against the attack by neutrophils, but that control of biofilm formation is possible depending on a timely and sufficient host response.

## 1. Introduction

Since the seminal work by Bill Costerton and the effort by many more scientists, bacterial biofilms have emerged as a novel pathogenic principle, particularly of opportunists causing persistent and in part destructive inflammatory process in man and in animals [1–7]. Over the years it became increasingly clear that bacteria (and also fungi) form ordered, well-organised communities, embedded in a slimy material that is produced by the bacteria. Because of the slime, biofilms are occasionally even visible to the naked eye (example in Figure 1).

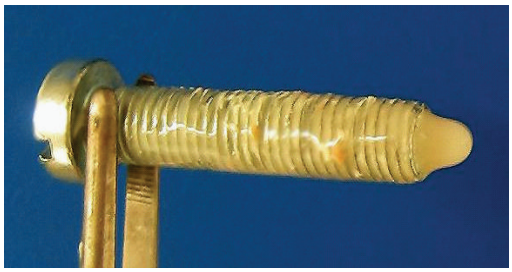
Biofilms were actually first described for water-dwelling bacteria in their natural environment and later on recognised as problem-causing "biofouling" agents in water-dependent industries, such as paper mills, or on boats [8]. There is a general agreement that living in biofilm was of advantage for the bacteria particularly when nutrition was scarce and the environmental conditions were unfavourable [9–11].

Scarce nutrition, however, is most likely not the reason that drives biofilm formation in the human body. There, the host's defence mechanisms might exert an evolutionary force that drives opportunists to acquire pathogenic factors or other protective means. Mucosal biofilms, for example, are colonising the colon. As "commensals" they are minding their own business and do not interfere with host functions. Moreover, they apparently escape detection and do not elicit an adverse response [12, 13]. Biofilms at other sites were recognised as the ultimate cause of persistent and destructive infections and inflammatory processes. Because inflammation indicates activation of an immune response, the latter observation leads to the questions how the immune system recognises and reacts to biofilm infections. This paper will focus on possible interactions between bacterial biofilms and innate host defence mechanisms, particularly the interaction of phagocytic cells with biofilms.

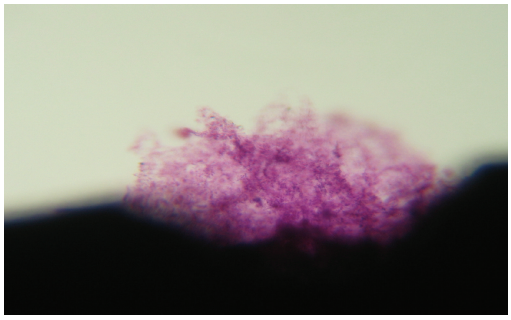
There are excellent reviews on biofilm formation in the literature (e.g., [14–16]) and on a very informative website of the Center of Biofilm Engineering (http://www.biofilm.montana.edu/) for further reference; therefore I will touch the issue of biofilm formation only briefly and only as far as it is required for the general understanding.

## 2. Bacterial Biofilms: No More Lone Rangers?

Examples for biofilms are shown in Figure 1. Even without a microscope the slimy mass can be recognised, which in this particular case consists of *Staphylococcus aureus*, surrounded by the slime, scientifically referred to as extracellular polymer



(A)



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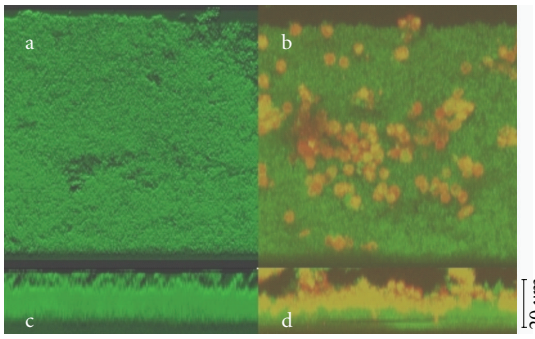


(C)

Figure 1: Biofilms (all *S. aureus*) in their various appearances are shown. (A) A screw recovered from an infected implant and cultivated for 2 days is shown. (B) An in vitro generated biofilm, stained purple, on a slightly nicked Kürschner wire (figure taken with permission from; Wagner et al. 2011 [48]). (C) A biofilm was cultivated on a cover slide; (a) and (b) show the top view, and (c) and (d) the side view. To the biofilm, PMN were added (b and d) stained orange (figure taken with permission from [205]).

substances (EPS, in the older literature also called extracellular matrix).

Biofilm formation is the result of a genetically driven process that alters the behaviour of the individual bacterium. It starts with attachment of bacteria to a surface (or to each other). Bacteria lose their motility and acquire—among others—the ability to produce and release materials for the EPS. The process is controlled by the so-called quorum-sensing molecules, which are produced and released by the bacteria and hence are also known as "autoinducers" [17, 18]. The very basic process of biofilm formation appears to be similar for all bacteria, on the molecular level; however, great differences exist. Biofilms have distinct, species- and occasionally even strain-specific properties. The composition of the EPS differs greatly among the species and might even vary depending on environmental and culture conditions. Moreover, different species use different quorum-sensing molecules, whereas some are shared with others (those of *P. aeruginosa* with other Gram-negative bacteria).

An almost philosophical question is what drives bacteria into forming a biofilm. In natural surroundings it could be shared food resources, protection from predators and unfavourable conditions, and also an easier exchange of genomic material [19–21]. Very possibly, living in a biofilm is not the exception, but the preferred life-style of bacteria and single, free-floating bacteria could only be an experimental artefact generated by culture. If so, our perception of host-pathogen interaction has to be reconsidered, as have our techniques to isolate and propagate bacteria for diagnostics purposes, or to determine susceptibility to antibiotics and biocides. The latter is extremely relevant, because bacteria in biofilms acquire a relative resistance towards antibiotics and biocides [22–25]. Moreover, bacteria living as biofilm *in vivo* might escape detection by standard microbial diagnostic methods, giving false-negative results [26–28].

## 3. Bacterial Biofilms as Pathogen

As pointed out above, especially bacteria that are considered "opportunists" become pathogenic when organised as biofilm. Well-studied examples are *Pseudomonas aeruginosa*, and *P. cepacia* which are known as pathogens of immunocompromised or immunodeficient patients [29, 30]. Biofilms of *P. aeruginosa* and *Burkholderia cepacia* contribute to chronic infections in patients with cystic fibrosis [31–35], or nonhealing wounds [36, 37]. Other chronic infections could be attributed to biofilms, for example, otitis media [38], periodontal disease [39, 40], rhinosinusitis [41], and skin infections [42, 43]. Biofilms might consist predominantly of one bacteria species, but others of mixed populations [44] (reviewed in [45]).

Especially well studied with regard to biofilm formation are the so-called device-associated infections. Artificial surfaces, particularly indwelling catheters or tubing, may be colonised by bacteria, particularly by *P. aeruginosa* or staphylococci species and spread from there [46, 47]. One explanation is that the bacteria preferentially adhere to artificial surfaces—as opposed to inner surfaces covered with epithelial cells [48, 49]. A rather intriguing explanation is that epithelial cells might actively prevent biofilm formation, for example, by inactivating the autoinducers participating in biofilm formation [50, 51]. In line with an active role of epithelial cells is the observation that patients with impaired epithelial function (patients with cystic fibrosis or ciliary dyskinesia syndrome) have a high risk to develop chronic bacterial infection [52–55].

## 4. Implant-Associated Osteomyelitis: A Prototype of Biofilm Infection

To analyse the immune response to biofilm infection, our group has focussed on the so-called implant-associated osteomyelitis, a persistent bacterial infection caused by

formation of bacterial biofilms on endoprostheses or on osteosynthesis materials, such as screws, plates, or nails. Infection of implanted material is a major complication of orthopaedic and trauma surgery, which as last and devastating consequence could result in functional impairments of the extremity and even in their loss (reviewed in [56–59]). Depending on the circumstances and individual risk factors of the patient, the incidence of infection ranges from 1 to 6 infection per 1000, which, considering the ever increasing number of patients requiring endoprostheses, amounts to many patients and increasing costs [60, 61] and explains the clinical and scientific interest in this condition [62].

Implant-associated osteomyelitis is particularly well suited for studying the local immune response, because the infected implant has to be removed, the infected site becomes accessible: infiltrating immunocompetent cells can be recovered and characterised *ex vivo*, and also tissue can be obtained for further analysis (an example is shown in Figure 2). Moreover, besides the infection, the patients do not suffer from diseases that might affect the immune system.

## 5. The Local and Systemic Immune Response in Patients with Implant-Associated Osteomyelitis

Fever and increase of C-reactive protein concentrations and of leukocyte count in the peripheral blood are established parameters for infection. In implant-associated osteomyelitis, those systemic reactions do not occur regularly; in approximately 100 patients we observed over the last years, only 20% presented with enhanced leukocyte count and 40% with enhanced C-reactive protein concentrations. The local reaction, however, was impressive: in all patients leukocytes were found at the infected site, predominantly PMN (50 to 70%), to a lesser degree T-lymphocytes (5 to 20%), NK-cells (5%), and a few monocytes. The cells were highly activated. Neutrophils upregulated adhesion proteins (CD11b, CD18), Fc-receptors MHC class II molecules [63], or the chemokine receptors CXCR6 [64]. Also functionally, the PMN were altered: enhanced production of oxygen radicals was seen, and a reduced chemotactic activity [65].

The infiltration of T cells was somewhat unexpected, because at least according to the common immunological point of view, T cells are not directly involved in the defence against bacteria. In the local wound lavage of our patients, however, T cells were apparently activated, particularly CD8+ cells, and some also expressed pattern recognition receptors, which to some degree explains how these cells recognise bacteria [66, 67].

Analysis of tissue samples from patients with implant-associated osteomyelitis confirmed the findings: in areas of bone degradation, PMN were abundant, some lymphocytes and monocytes were seen, as were osteoclasts, the latter, as expected, particularly next to the bone and in resorption lacunae (example in Figure 3). A correlation of neutrophil density with the number of osteoclasts was calculated in a study comprising patients with osteomyelitis, supporting the notion that the infection and the ensuing proinflammatory microenvironment may promote osteoclastogenesis and bone resorption [68].

Infiltration of proinflammatory cells, particularly of PMN, was also seen in other biofilm infections. Particularly well studied is the *P. aeruginosa* infection in patients with cystic fibrosis or with chronic, nonhealing wounds [36]. Infiltration of neutrophils indicates that the immune system has reacted to the infection in an appropriate manner, leading to the question how the infiltrated cells will now interact with the biofilm.

## 6. Bacteria and Bacterial Biofilms from the Viewpoint of the Host

The host defence discriminates between pathogens and commensals, so colonisation with bacteria does not necessarily elicit an immune response. With our intestinal "microflora" and our skin germs we live in mutual acquiescence. There is even evidence that those bacteria modulate and shape our immune system from early childhood throughout the whole life [69–72]. The bacteria are tolerated because they reside in privileged areas of immune tolerance. This compartmentalization allows a local, but not systemic immune response. The epithelial cell layer and the cytokine micromilieu are essential to maintain compartmentalization. Changes in the composition of the human commensal bacterial microflora could enhance the susceptibility to allergic diseases [72]. Disruption of the epithelial barrier or invasion of bacteria by other means will perturb the friendly coexistence and will activate a host response.

Invading bacteria encounter an intricate and complex host defence system. Different cells, numerous cellular receptors, signalling pathways, and effector molecules have been identified, which recognise potential dangerous invaders and execute their elimination. This diversity is needed because different bacteria species also differ with regard to their susceptibility towards the host defence (there are numerous excellent reviews that can be used for reference; e.g., [73–75] to name a few).

With an intact immune response, many infectious agents are eliminated without being noticed by the host; we only know from immunocompromised or immunodeficient hosts how frequent infections really are.

*6.1. PMN as "First Line Defence".* I focus here on PMN, which are usually the first cells to arrive at a site of bacterial infection and hence are often referred to as "first line defence." That PMN are crucial for the host defence against bacteria is supported by the fact that lack of PMN (neutropenia) or congenital defects of PMN result in an enhanced risk to develop life-threatening infection (reviewed in [76–78]).

Prerequisite for the host defence is the ability of PMN to sense a localised infection, to emigrate from blood vessels, and to migrate actively through the tissue to the site of infection. This process is very well controlled and has been unravelled over the years [79–81]. Numerous factors have been identified that can attract neutrophils, notably cytokines (reviewed in [82–84]). Of interest, also bacteria-derived

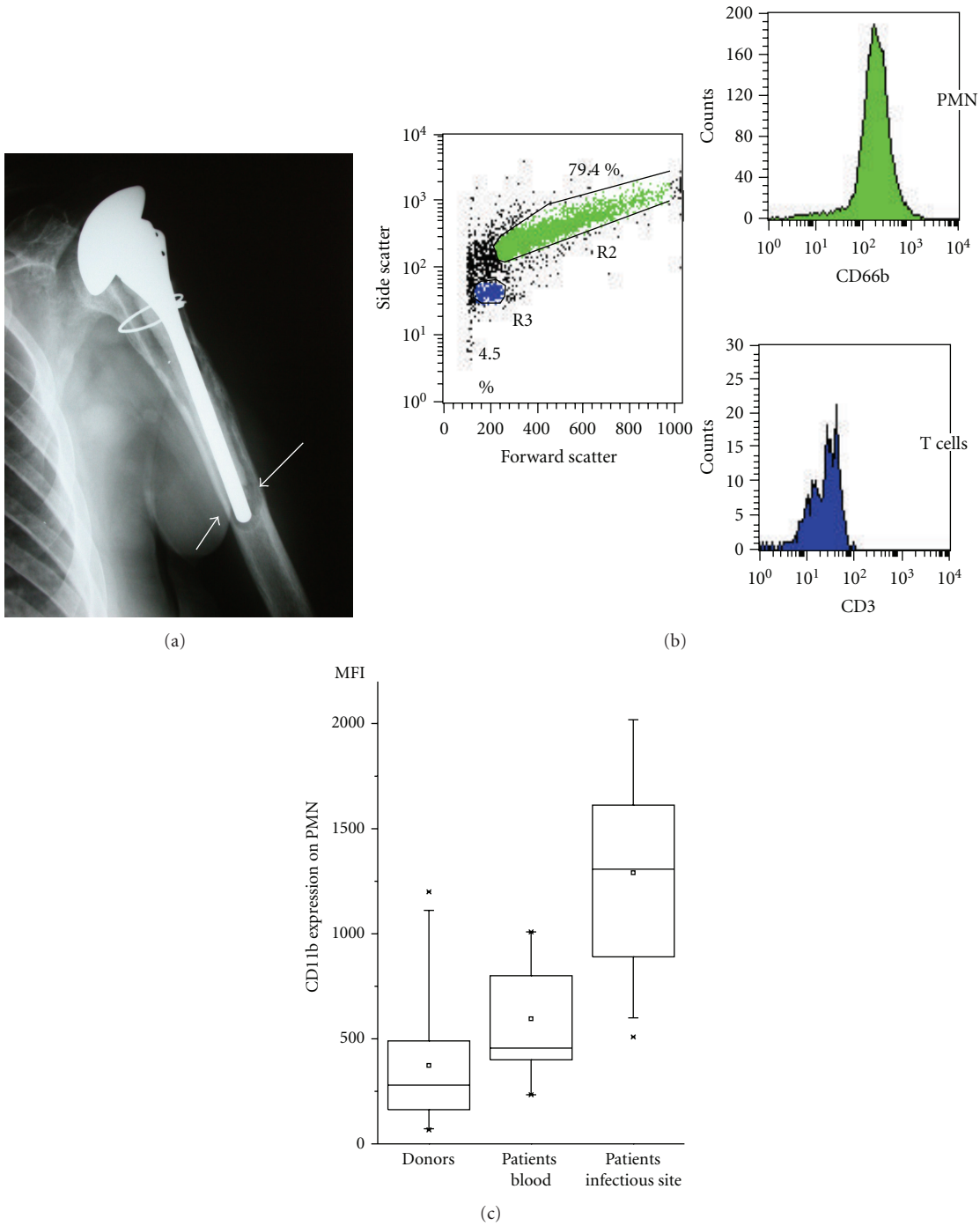


Figure 2: Experimental data from patients with implant-associated osteomyelitis are shown: (a) the X-ray shows a shoulder prosthesis and next to it—marked by the arrows—areas of bone resorption (image courtesy of Dr. C. Wagner, Klinik für Unfall-Wiederherstellungs-Hand-und Plastische Chirurgie, Klinikum Ingolstadt). (b) When the infected implant is removed, infiltrating cells were harvested by use of sterile saline (lavage). These cells are then ex vivo analysed by cytofluorometry. The forward-side scatter (left panel) shows two major cell populations, which were identified as PMN (CD66b positive) and T cells (CD3 positive). (c) The infiltrated PMN were activated as seen by upregulation of CD11b; for comparison cells of the peripheral blood of the patients and of healthy donors are shown (the box- and whiskers-blot shows the summary of 40 patients and 10 healthy donors).

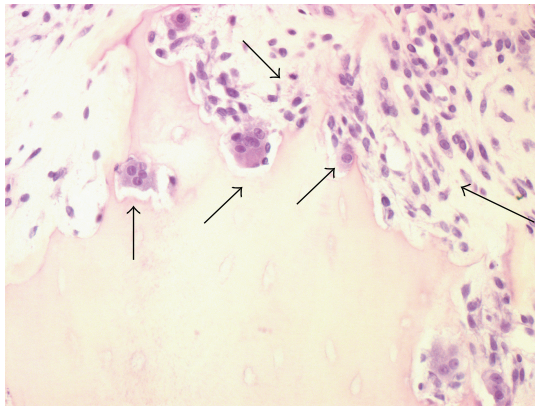

Figure 3: From the site of an infected implant, a biopsy was taken. Following haematoxylin staining, the eroded bone with prominent resorption lacunae appears pink. Next to it, osteoclasts are seen (giant cells with numerous nuclei, marked by thick arrows) and the tissue is infiltrated by PMN (thin arrows) (figure courtesy of Dr. M. M. Gaida, Institute of Pathology, University of Heidelberg).

products might serve as chemoattractants. The most prominent example is f-Met-Leu-Phe, a tri-peptide that was generated according to naturally occurring formyl peptides [85–87]. We and others also described a chemotactic activity for N-(3-oxy dodecanoyl) homoserine lactone, a quorum sensing molecules derived from *P. aeruginosa* [88–91] (see the following).

Once arrived at the infected site, PMN take up bacteria by phagocytosis. Bacteria may be killed via generation of reactive oxygen species (ROS). The relevance of ROS as bactericidal principle is shown by genetic defects within the cascade leading to ROS generation (chronic granulomatous disease, CGD). The patients suffer from often severe and recurrent bacterial and fungal infection and rely on life-long treatment with antibiotics, if causal therapy is not possible [78, 92, 93].

PMN contain also a number of other cytotoxic or bactericidal substances, which might act intracellularly or extracellularly (reviewed in [94, 95]). The significance for the host defence of many of these entities is not yet apparent. Presumably, they have more or less specialised functions or targets, which remain to be identified. In the context of biofilm infection, lactoferrin is of special interest, because aside from its bactericidal activity, it also prevents biofilm formation as shown for staphylococci species [96, 97] (see the following).

As local defence, there is also an arsenal of the so-called cationic antimicrobial peptides (CAMPs) including defensins, cathelicidins and thrombocidins, which are partly contained in neutrophils, but also in epithelial cells and others [98, 99]. Their bactericidal potential is limited due to countermeasures of the bacteria [100]; with regard to biofilm clearance, for the cathelicidin LL-37, an inhibitory effect was described (see the following).

*6.2. How PMN Recognise Bacteria.* PMN are considered as "non-specific" or "innate" effector cells, meaning that they do not recognise bacteria in an antigen-specific manner and that they do not generate memory responses. Rather, PMN sense evolutionary conserved surface molecules that are shared by many bacteria species. Numerous receptors recognising those conserved structures as "foreign and potentially dangerous" and therefore referred to as pathogen-associated molecular patterns or microbe-associated molecular patterns (PAMPs or MAMPs) have been identified. Among those are the so-called Toll-like receptors, which are found on all immunocompetent cells. In humans, the family comprises 9 receptors, which differ with regard to their target structures, which include lipopolysaccharides, lipoteichoic acid, peptidoglycan, flagellin, and others (reviewed in [101–103]). Toll-like receptors appeared early in evolution and were probably the first receptors to distinguish between "self" and "microbe" [104, 105]. Aside from Toll-like receptors, also other pattern recognition receptors are known. CD14, for example, recognises lipopolysaccharides [106, 107], the so-called scavenger receptors bind other microbial constituents, such as complex carbohydrates [73, 108, 109]. PMN also recognise bacterial DNA. This is relevant when considering the host defence against biofilms, because DNA is part of the extracellular biofilm substance and is required for efficient biofilm formation [110–112]. Toll-like receptor 9 was described as a DNA receptor [113], but PMN react to bacterial DNA also independently of TLR9 [114–116].

So it appears that PMN and also the other phagocytic cells express a wide array of receptors which by themselves or in combination with each other sense bacteria and may then elicit an adequate response [117]. Adding to the complexity is the fact that for many bacteria species clearance from the circulation and efficient phagocytosis and killing depends on "opsonisation," that is, on coating of bacteria with antibody and complement (complement C3b/C3bi) [118–120]. Receptors for immunoglobulin G (CD16, CD32, and following activation also CD64) mediate phagocytosis and intracellular killing together with the complement receptors (CR1, CR3). There is an abundance of literature on the dependence and efficiency of phagocytosis induced by Fc-receptors, complement receptors or a combination thereof and also a combination of Fc-receptor with other receptors [74, 121–125], and, as expected, escape mechanisms of the target bacteria [126–128]. An important caveat is that the majority of data describing phagocytosis by PMN or other phagocytic cells are derived from experiments using planktonic, that is, "free-swimming," bacteria. As I will explain in more detail in the following, the situation might be quite different when biofilms are considered.

*6.3. How PMN Interact with Biofilms.* Neutrophils (and other phagocytic cells as well) are often compared to single cell organisms such as *Dictyostelium discoideum*, because migration and phagocytosis are redolent of chasing and trapping of a prey [129–131]. Although the analogy is arguable on a molecular level, neutrophils might be regarded as predators and—to carry the analogy further—bacteria as prey. Observing the interaction of PMN with a staphylococci biofilm emphasizes this impression (see video clips in supplementary material available online at doi:10.5402/2012/853123).

Also seen from the video clip is that the PMN attack the bacteria and the extracellular substance/EPS, the slime, as well. The EPS is not a massive impermeable wall, but rather a hydrogel-like structure, composed of exopolysaccharides and proteins, dead bacteria, bacterial DNA, and enzymes. Composition and structure of the EPS varies widely among the bacteria species, and even within one species, there are strain-specific properties. For *P. aeruginosa*, for example, mucoid strains have been isolated from patients with cystic fibrosis, which produce alginate as a major EPS constituent, whereas strains of other origin do not (for review see [132–134]). So when considering the interaction of neutrophils with biofilms or EPS, respectively, the findings need to be interpreted with caution as they might not be true for biofilms of other species and not even for biofilms from the same species, because also culture conditions affect biofilm properties. Culture dishes versus polystyrene used for catheters or metals which are used for implants can make a major difference, particularly with regard to the initial uptake of the bacteria. Also the application of the *in vitro* findings to the *in vivo* situation or from the animal model to human disease is rather challenging, because the "models" usually do not reflect exactly the naturally occurring disease; moreover, the experimental animals do not react necessarily similar to human. Mice, for example, have less neutrophils compared to humans (on average only 20% of the leukocytes are PMN), so it is quite possible that the quality of the initial, innate response involves more monocytes and hence differs from the human situation.

The majority of data on the interaction of neutrophils (or other phagocytic cells) with biofilms are derived from studying *P. aeruginosa* or staphylococci biofilms, the former because it is one of the major infectious agents of hospital-acquired infection, particularly of patients in intense care units who require indwelling catheters and tubing, and also because of its presence and role in cystic fibrosis; the latter as a major cause of implant-associated infection.

Our group is focussed on PMN, but I will occasionally also refer to data obtained with peripheral blood leukocytes or monocytes/macrophages. Monocytes share some functional characteristics with neutrophils but differ in other regards.

*6.4. PMN and P. aeruginosa Biofilms.* When analysing the interaction of neutrophils with *P. aeruginosa* biofilms generated *in vitro*, it was observed that neutrophils settled on biofilms, and they, however, did not move around and exhibited little or no bactericidal activity [135]. Apparently, *P. aeruginosa* biofilms downmodulated leukocyte functions [136–138]. Subsequent experiments attributed the inhibitory capacity to components of the EPS, particularly to alginate, a high molecular weight, acetylated polymer composed of nonrepetitive monomers of $\beta$-1,4 linked L-guluronic and D-mannuronic acids. In vitro, alginate inhibited phagocytosis [139] and directed migration of PMN [140]. Together, these data point to a role of EPS components as a defence mechanism against immunocompetent cells.

Data derived from patients with cystic fibrosis confirm that notion: initially, the lung of the patients is colonised by nonmucoid strains. When the disease progresses, mucoid phenotypes emerge, which produce alginate, which in turn is linked to structural changes of the biofilm and results in a worsening of the clinical prognosis [32, 141, 142]. Thus, although alginate is apparently not required for biofilm formation in the first place, it enhances the resistance towards the host defence [139].

Rhamnolipids were identified as further extracellular components with the potential to fend off the leukocyte attack. Rhamnolipids are amphiphilic molecules composed of rhamnose and hydrophobic fatty acid moieties and are also known as the heat-stable hemolysin of *P. aeruginosa* [143].

Rhamnolipids are produced by *P. aeruginosa* upon biofilm formation. The synthesis is controlled by quorum-sensing molecules [144, 145]. Their apparent physiological function is the maintenance of the ordered structure of the biofilm, particularly of the fluid-filled channels [146]. Early data by Shryock et al. (1984) and Kharazmi et al. (1989) described an activation of neutrophils or macrophages by low doses of rhamnolipids and lytic, necrotic cell death in higher concentrations [147, 148]. Eventually, the cytotoxic potential of rhamnolipids was linked to the pathogenicity of *P. aeruginosa* biofilms: rhamnolipids could actively fend off the neutrophils, leading to persistence of bacteria; moreover, lysed neutrophils may release their content of proteolytic enzymes, which may cause tissue damage, and hence progression of the inflammatory response [149–151]. Elastase derived from neutrophils was considered a major pathogenic agent [152–155], a presumption supported by the fact that elastase and cellular debris of neutrophils are found in the sputum or the bronchial lavage of patients with cystic fibroses [155–157].

An interesting aspect is that the bacteria react actively to the neutrophil attack. In response to PMN, the rhamnolipid synthesis is upregulated, which means that *P. aeruginosa* recognises PMN [158]. How this type of interkingdom signalling works is not yet known. Possibly, cytokines derived from the infiltrating neutrophils are recognised by the bacteria. That this is possible in principle had been shown for cytokines such as tumour necrosis factor $\alpha$, interleukin 1 and 6, respectively, which do promote bacteria growth [159–161]. Moreover, interferon-gamma has been implied [162], but because interferon gamma production by neutrophils has been shown only in mice, other candidates have to be considered in humans.

In summary, *P. aeruginosa* biofilms have various means to counterattack the immune defence, which—at least in part—explains their persistence.

## 7. Neutrophil-Derived Mediators with the Potential to Prevent Biofilm Formation

*7.1. Lactoferrin.* So the question arises, whether or not the host has any means to destroy or prevent the generation of *P. aeruginosa* biofilms. As with many other infectious agents, I think, the success of the host defence is a matter of timing. Established biofilms might be difficult to attack; there is, however, evidence that prevention is possible. Lactoferrin,

which is stored preformed in neutrophils but is also present in numerous external secretions, is able to prevent biofilm formation. The effect was attributed to the ion-chelating capacity of lactoferrin, which, however, might not be the only mechanism [97, 163, 164]. Decreased levels of lactoferrin might predispose to biofilm infections, as suggested for patients with chronic rhinosinusitis [165]. In line with these data, decreased lactoferrin concentrations were also observed in patients with cystic fibrosis. An enhanced cleavage of lactoferrin by the protease cathepsin B was described, which resulted in loss of bactericidal and antibiofilm activity [166, 167]. Because cathepsin B release can be induced by elastase, it is possible that the lactoferrin effect might be abolished in situations where elastase prevails [168]. Nevertheless, the fact that lactoferrin inhibits biofilm formation prompted the question for its therapeutic use, for example, in chronic wounds, where it might be especially useful, because its antibiofilm action is not limited to *P. aeruginosa* [169–173].

*7.2. Cathelicidin: Human Cationic Antimicrobial Protein 18 (LL-37).* LL-37 is the C-terminal part of antimicrobial protein (hCAP18), which is mainly expressed by neutrophils and epithelial cells, but is also found in body fluids. Numerous functions have been ascribed to LL-37, including induction of chemotaxis, angiogenesis, or chemokine secretion. LL-37 is an antimicrobial peptide, that is produced in response to infection, for example, by mycobacteria [174, 175]. LL-37, however, also inhibits biofilm formation by decreasing the adherence of the bacteria, by stimulating their motility, and by downregulating quorum-sensing-dependent genes, required for biofilm formation [176]. An antimicrobial effect of LL-37 was shown in a rabbit model of *P. aeruginosa* infection [177], and because the inhibitory effect is not limited to *P. aeruginosa*, it is attractive to assess its use for therapy of chronic wounds and other biofilm infections [178–181].

## 8. PMN and Pseudomonas Quorum-Sensing Molecules

A further interesting observation regarding the interaction of *P. aeruginosa* and cells of the host defence is the fact that immunocompetent cells recognise quorum-sensing molecules. As described above, quorum-sensing molecules are produced by bacteria as autoinducers and participate in biofilm formation. *P. aeruginosa* produce, among others, N-acetyl homoserine lactones, which also interact with mammalian cells. For N-(3-deoxy-dodecanoyl) homoserine lactone (AHL-12), an immunomodulatory function was described, particularly inhibition of the T-cell activation and induction of apoptosis [182–185]. This so-called "interkingdom signalling" was interpreted as another means of bacteria to evade host defence mechanisms [186, 187]. Data by Vikstrom's group and ours, however, suggest that AHL-12 might activate the local host defence by stimulating phagocytic cells: enhancement of phagocytosis, upregulation of pertinent surface receptors, and induction of chemotaxis were shown [88–91, 188]. How AHL-12 interacts with the mammalian cells is still under investigation. Free diffusion into the cells, binding to an intracellular transcription factor or to a surface receptor, has been proposed [88, 89, 91, 189–192], as has been the activation of Rac1, of Map kinases, and an independence of the Toll-like receptor activation cascade [89, 91, 192]. Another, not yet answered question is whether or not these *in vitro* findings are relevant to the infection *in vivo* (reviewed in [193]).

## 9. PMN and Staphylococci Biofilms

Staphylococci have developed numerous active and passive means to evade host defence mechanisms (reviewed in [127, 194–196]), and biofilm formation is just one of those. Depending on the experimental system, biofilm formation might not be "superior" to the other strategies [197–199], which explains to some extent the apparent inconsistency of data and interpretations in the literature.

Host defence against staphylococci biofilms is mainly studied with *S. aureus* and *S. epidermidis*, because these bacteria are frequently isolated from patients with osteomyelitis (and implant-associated osteomyelitis) and are thought to be the "ultimate cause" of this chronic and destructive inflammation [2, 200–204].

*In vitro* data indicate that *S. aureus* biofilms are not inherently protected against the attack by neutrophils or macrophages. Phagocytosis and generation of oxygen radicals was seen, as was clearance of biofilm and release from the neutrophils of DNA (see also the video clip and Figure 4) [96, 205–208]. When tested under comparable conditions, *S. epidermidis* biofilms appeared to be less sensitive towards the neutrophil attack, but still clearance of biofilm and phagocytosis was seen [207].

Of note, in contrast to the situation with planktonic bacteria, phagocytosis of *S. aureus* biofilms occurred also in the absence of opsonisation with antibody and complement; killing, however, required the additional signal provided by IgG [208, 209]. For *S. epidermidis*, even coating with complement appears to be required, and preventing complement deposition on the biofilm was suggested as a means to protect the bacteria [210].

The observation that PMN and other leukocytes adhere to staphylococci biofilms also in the absence of opsonising antibodies suggested that the cells recognise biofilm constituents. Because leukocytes express numerous receptors for microbial patterns, it was obvious to assess their participation in leukocyte binding. So far, these experiments did not yield conclusive results. Using an *in vivo* mouse model of catheter infection, participation of the toll-like receptors TLR2 (binding among others to lipoteichoic acid) and TLR9 (binding to bacterial DNA) could be ruled out [211].

Our group is trying to identify molecules within the extracellular substances of staphylococci biofilms that can interact with neutrophils. Entities inducing degranulation of PMN including up regulation of CD11b and release of lactoferrin were found; their further characterisation is still underway [212].

Whereas phagocytosis and killing of staphylococci is possible in principle, the effector functions might not be very efficient. In vivo experiments in a mouse catheter model indicated that the macrophages were functionally

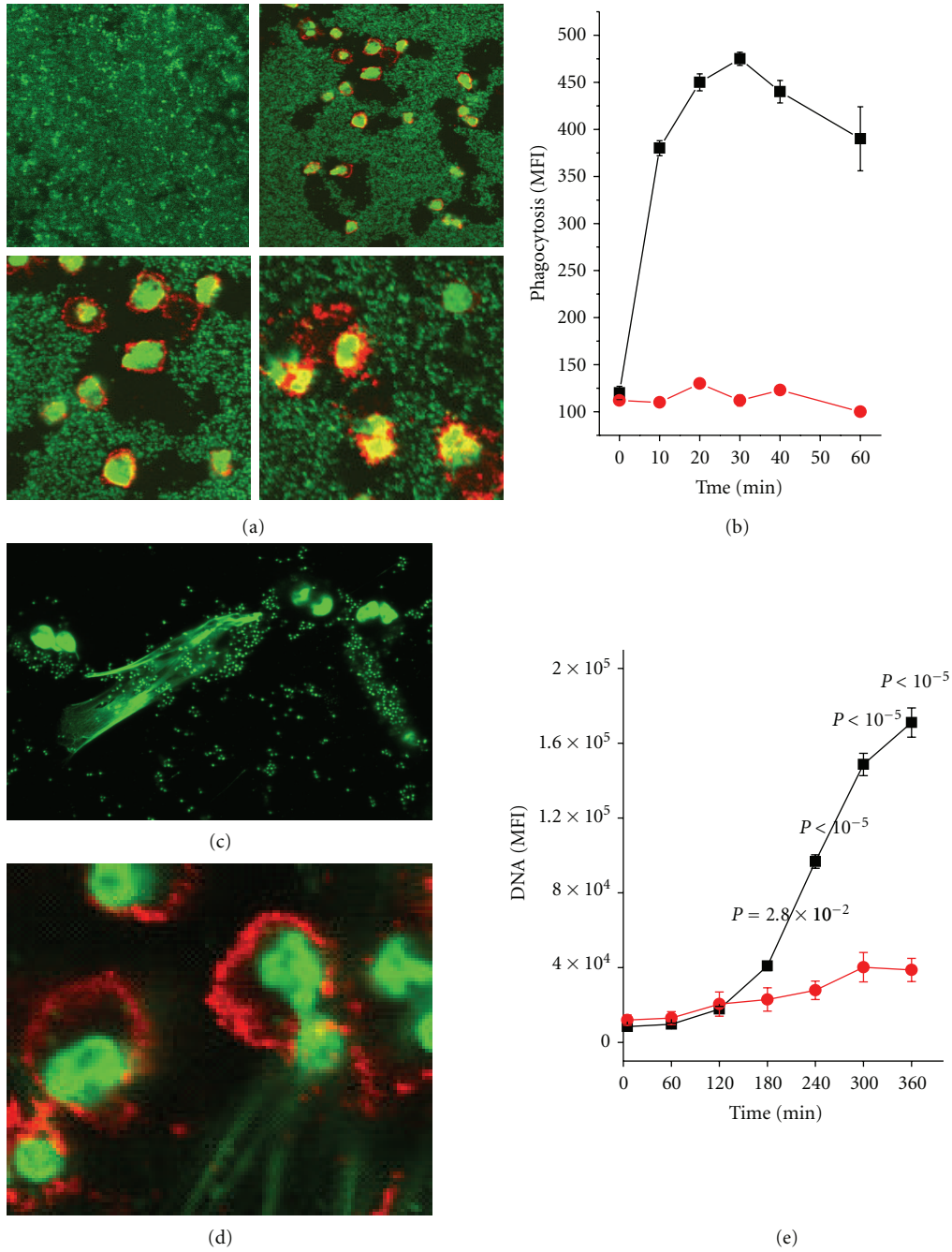


Figure 4: Phagocytosis of *S. aureus* biofilms: (a) to establish *S. aureus* biofilm (stained in green) (upper left), PMN (red) (upper right) and the reaction were observed over time. By 30 min, biofilm-depleted areas were seen. The images on the lower panel show a zoom after 30 and 45 min, respectively. (b) On the left, a quantitative analysis is shown. In these experiments, the PMN were recovered from the biofilm and green fluorescence taken up the PMN were used a measure for phagocytosis (mean fluorescence intensity, MFI) (details of these experiments and the original figures are taken with permission from [96]. After prolonged incubation of PMN with biofilms, release of DNA was seen. (c) shows prominent DNA strands with bacteria, and in (d) a PMN in the process of releasing DNA is shown. (e) shows the quantitative analysis (experimental details in [96]).

impaired [211]. Previous data by others suggested that the "slime" contains material interfering with the reactive oxygen species, the major cytotoxic entities of PMN and of other phagocytic cells as well [213–216]. The entity within the slime has not yet been identified in these experiments nor has its mode of action.

In subsequent experiments, Vuong and colleagues showed that staphylococci produced an extracellular polysaccharide, the so-called "polysaccharide intercellular adhesin" (PIA) [217]. PIA is crucial for biofilm formation and—as shown in experimental animals—is involved in catheter infection and protects against major components of human

innate host defence [218, 219]. An epidemiological study linked the genes responsible for PIA production to infection derived from indwelling medical devices [220], supporting the notion that biofilm formation, but may be also antihost properties, is required for infection to occur and to persist.

As pointed out above, an important issue when studying biofilm formation is the initial attachment of the bacteria to a surface. Particularly the quality of the surface decides the time course of the attachment. *S. aureus*, for example, attach more rapidly to metals used for implants and prosthesis compared to conventional plastic culture dishes [48, 221], and biofilm formation occurs also more rapidly on metals, especially of rough surfaces. Therefore, also the susceptibility of the biofilm towards the attack by neutrophils might vary with the underlying material, which to some extent also might explain diverging results.

Interaction of host defence and biofilms is also observed at the level of the quorum-sensing system, which regulates biofilm formation and other virulence factors as well. In *S. epidermidis*, the agr quorum-sensing system was identified and a peptide signalling molecule (phenol-soluble modulin, PSM) [222, 223]. Apparently, agr or rather agr-controlled events affect the host interactions: agr mutant strains of *S. epidermidis* were not able to induce cytokine production or chemotaxis of neutrophils [224] and were susceptible to antimicrobial peptides and oxygen radicals [225]. Essentially similar observations were made for *S. aureus*, suggesting that the quorum-sensing operon might participate in host defence mechanisms other than biofilm formation [226].

## 10. Consequences of a Failed Immune Defence against Biofilms

Infiltration of neutrophils and other immunocompetent cells is a hallmark for biofilm infection and is especially well analysed in cystic fibrosis and implant-associated osteomyelitis (see above). The infection, however, persists, and destructive inflammatory processes are seen, for example, bone degradation in implant-associated osteomyelitis. Very likely, destruction is caused by infiltrating leukocytes, because in the failed attempt to kill bacteria, they might release their bactericidal and cytotoxic entities into the surroundings causing extended tissue damage. This so-called "frustrated phagocytosis" or "failed attempt" could then degrade tissue and generate a proinflammatory environment that attracts more leukocytes, inflammation progresses [227, 228] and eventually results in generation of bone-degrading osteoclasts [229–231]. Inflammation will also proceed, because necrotic or lysed leukocytes are not cleared from the inflamed site. Clearance of neutrophils, that have exerted their bactericidal action, however, is a prerequisite for limiting an inflammatory process in time and spatial manner, whereas the failure to clear spent neutrophils is thought to promote inflammation [117, 232–234]. Clearance of spent neutrophils, however, occurs when following phagocytosis the cells become apoptotic and recognised and taken up by macrophages without spilling their content. In case of biofilm infections, neutrophils might even promote biofilm formation, which again would aggravate the inflammatory response [235, 236].

## 11. Why Do Biofilm Infection Occur and Is the Host Defence Indeed a "Mission Impossible"?

Basically, neutrophils have the potential to prevent biofilm formation, recognise, phagocytose, and clear bacterial biofilms nevertheless persistent infections occur. This does not necessarily mean that biofilm formation is never prevented and that biofilms are never eradicated, because an efficient host defence might occur unnoticed, and the frequency of infections, particularly by opportunists, becomes only apparent, when patients with immunodeficiencies are considered. A hint that biofilm infections can be controlled comes from analysing osteosynthesis materials, routinely removed from patients with bone fractures. By advanced techniques, bacteria could be recovered from these materials, despite the fact that the patients showed no sign of infection [237].

Assuming that neutrophils can clear biofilms the question arises why infections persist. I think the paradigma "too-little-too-late, applies especially well to biofilm infections on implants, because artificial surfaces are prone to be colonised by bacteria. Gristina et al. called that "race for the surface" [49] and suggested that early colonisation of an appropriate surface would accelerate biofilm formation and that thereby opportunists would acquire virulence factors. Since that time, numerous studies confirmed that artificial surface promotes biofilm formation thereby giving the bacteria a clear advantage over the host defence [238, 239], reviewed in [58, 59], and explains why patients with orthopaedic implants may have a high risk to develop an infection and why in experimentally induced infections less bacteria are required in animals with inlaying metal [239]. The same reasoning applies to patients with infection due to indwelling catheters, because the surface of these devices is also readily colonised by bacteria, infections occur and may spread. Since catheters are used frequently in critically ill patients, an enhanced susceptibility towards infection is also possible [240–243]. In addition to artificial surfaces, also a compromised epithelium could favour biofilm formation, because the initial colonisation by bacteria cannot be prevented. In addition to conditions that favour the adherence of and hence the initial colonisation by bacteria, also predisposing factors of the host have to be considered. Underlying diseases might compromise an effective immune response, for example, diabetes, cancer, or immune-mediated disease, but also exogenous factors like obesity or smoking [244–246]. Aside from these acquired impairments of the immune response also genetically determined "primary" immunodeficiencies have to be considered. According to most textbooks, immunodeficiencies are rare, life-threatening conditions, caused by a gene defect that affects expression of central receptors, enzymes, signalling molecules, or the differentiation of immunocompetent cells. Those defects are usually monogenic and inherited in either x-linked or autosomal manner [247, 248]. In recent years, however, it became increasingly

clear, that immunodeficiencies might be not that rare. Probably each individual suffers from some immunodeficiency or other, which might predispose to infection by a single, defined agent in otherwise healthy patients. Because these defects are not necessarily associated with an easy-to-detect phenotype and might not reside in haematopoietic cells, they will escape the routine detection [249–251].

In conclusion, I would say that the defence against biofilms is not a "mission impossible" but that it might occur regularly and very possibly by means of early intervention. Only when the bacteria get a "head-start" and win the "race for the surface," biofilms may form and bacteria may escape eradication; when the immune cells are abundant and early enough biofilm formation will be controlled mission accomplished.

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