Biofilm switch and immune response determinants at early stages of infection

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Abstract

Biofilm development is recognized as a major virulence factor underlying most bacterial chronic infections. When a biofilm community is established, planktonic cells growing in the surroundings of a particular tissue switch to a sessile lifestyle and start producing a biofilm matrix. Initial steps of *in vivo* biofilm development are poorly characterized and difficult to assess experimentally. A great amount of *in vitro* evidence have shown that accumulation of high levels of cyclic di-nucleotides (c-di-NMPs) is the most prevalent hallmark governing the initiation of biofilm development in bacteria. Recent studies also link detection of c-di-NMPs by host cells with the activation of type I interferon immune response against bacterial infections. Here, we discuss c-di-NMP signaling and the host immune response in the context of the initial steps of *in vivo* biofilm development.

Uncertainties in the initiation of biofilm formation in chronic infections

Bacterial infections in the human body are classically divided into acute or chronic infections. This useful clinical classification usually reflects differences in the expression of gene products known as virulence factors as well as in the lifestyle that bacteria causing the infection adopt. Acute infections last a short time and involve single planktonic (free-swimming) bacteria that cause tissue damage through the production of a large amount of virulence factors. They typically cause severe clinical symptoms and can generally be efficiently treated with either one or more antibiotics. In contrast, chronic infections are characteristically associated with bacterial aggregates, commonly referred to as biofilms, where bacteria are physically joined together and encased in a self-produced extracellular matrix made of exopolysaccharides, large surface proteins, fatty acids and DNA. Biofilm-mediated infections persist in spite of antibiotic therapy and the host's innate and adaptive immune responses [1-4]. Although the biofilm is the predominant mode of growth for bacteria in most natural and clinical environments, some bacterial species are more prone to produce chronic infections, suggesting either that they have a high capacity to reach locations suitable for biofilm settlement or that they possess special skills to efficiently switch from a planktonic to a sessile lifestyle on the surface of inert or living tissues. Despite the wealth of knowledge gained over the years on the biofilm formation process and their implication in chronic infections [5,6] very little is known about how the biofilm development process starts on the surface of host tissues and how the host cell responds to this process. In this regard, the animal models currently used to examine *in vivo* biofilm infections, such as device-related endocarditis or placement of a piece of catheter under the skin, provide insights into the factors required for the progression of the infection. However, new methodological strategies, such as direct observation of biofilm development on tissue

surfaces and *in situ* genome-wide transcriptomic profiling, are needed to further explore initial steps governing *in vivo* biofilm development for the progression of a chronic infection. Significant challenges for the implementation of these methods will be the low number of planktonic bacteria from which biofilm infection is supposed to start and also the short period of time occurring between initial bacterial adhesion and the switch to a sessile lifestyle. Thus, taking into consideration the scarce background information, we first provide a brief overview of the main signal transduction pathway, based on cyclic di-nucleotides (c-di-NMPs) secondary messengers, that governs the initial planktonic-to-biofilm switch and then discuss the emerging information about the role of c-di-NMPs as pathogen-associated molecular patterns (PAMPs) triggering a host innate immune response. Subsequently, we raise the possibility that both processes might be connected during the initial steps of biofilm development that precede the establishment of a chronic infection.

Switch to biofilm formation and signal transduction

A first step to trigger the transition between a motile, single-cell state to an adhesive, multicellular state on the surface of a particular tissue is the recognition and transmission of signals that bacteria use to identify it is appropriate to settle down. Several signals have been shown to favor early settlement of bacteria on human tissues including: (i) the presence of inert surfaces (plastics, metals and devitalized bone); (ii) increased amounts of extracellular iron and ferritin that induce a *Pseudomonas aeruginosa* biofilm phenotype in sputum of cystic fibrosis patients; (iii) compromised tissues with elevated levels of glucose in diabetic patients; (iv) imbalances in the commensal microbial communities caused by antibiotic treatments, or age-related deterioration; (v) simultaneous or a previous viral infection; (vi) hydrogen peroxide

produced by neutrophils during the oxidative burst; (vii) the presence of compounds such as indol that has stimulating effects on biofilm formation by many gram-negative bacteria including *Escherichia coli*, *Klebsiella oxytoca*, *Citrobacter kosseri* and *Haemophilus influenza*; extracellular polyamines, calcium and bile salts that modulate biofilm formation in *Vibrio cholerae*, *Yersinia pestis*, *P. putida and S. aureus* [7-12]. Bacteria have two major sensory systems for the recognition of these signals: two-component systems (TCSs) and the c-di-GMP mediated signal transduction network. TCSs are the basic stimulus-response coupling mechanism in bacteria. The best characterized example of a TCS driving the motile/sessile switch is the intricate LadS/RetS/Gac/Rsm signal transduction system of *P. aeruginosa*, where the GacS/GacA TCS represses the expression of the CsrA homolog RsmA, which reciprocally regulates factors involved in acute infection (motility and the type III secretion system) and chronic infection (exopolysaccharides and the type VI secretion system) (for review see [13,14]). However, TCSs are not typically implicated in controlling the switch between planktonic and sessile lifestyles. Instead, they are dedicated to match the production of specific compounds of the biofilm matrix with the environmental conditions. The signal transduction system that has taken on the responsibility to inhibit motility and promote biofilm development in a diverse range of bacterial species is the one that depends on the secondary messenger c-di-GMP. In c-di-GMP signaling, the sensor protein domain reacts to the stimulus by activating an output domain located in the same protein that triggers the synthesis [diguanylate cyclase (DGC), GGDEF domain containing proteins] or degradation [phosphodiesterase (PDE), EAL and HD-GYP domain containing proteins] of c-di-GMP. Then, the resulting c-di-GMP interacts with specific effectors that finally relay the signals to cellular processes [15]. Based on *in vitro* studies carried out with different bacterial species, it is widely

accepted that a high concentration of c-di-GMP enhances biofilm development and represses virulence factor expression whereas low c-di-GMP levels promote bacterial motility and a planktonic lifestyle [16-19]. If this knowledge is extrapolated to what should happen when bacteria start producing a biofilm on the surface of a host tissue, the switch from a planktonic to a biofilm lifestyle would also depend on the accumulation of c-di-GMP through the activation of specific GGDEF domain containing proteins or the inhibition of EAL/HD-GYP domain proteins. Because very often bacteria contain several GGDEF and EAL/HD-GYP domains linked to signal input domains [including PAS, REC, Globin, blue light sensing (BLUF), haemerythrin, GAF, CHASE or MASE domains], it is conceivable that one or more of these proteins sense specific signals on the surface of host tissues, either upon surface contact or as a prerequisite for attachment, to promote increased c-di-GMP accumulation [20]. Unfortunately, to date, there is very little information about the mechanisms that regulate DGC and PDE activities *in vivo* and only few studies have been able to show a role of c-di-GMP during chronic infections. For example, small colony variants of *P. aeruginosa* with elevated c-di-GMP levels have been identified in cystic fibrosis sputum samples [21-23]. Also, Byrd et al. were able to show that *P. aeruginosa* c-di-GMP overproduction mutants had increased persistence as compared to the wild type strain in a chinchilla middle ear infection model [24]. Considering that there is a general agreement that c-di-GMP signaling is determinant for biofilm development, why has the analysis of c-di-GMP contribution to *in vivo* infections and particularly to the initial steps of infection trailed behind? Detection of c-di-GMP is technically very challenging and current methods cannot be applied to measure c-di-GMP during infection. In this regard, the development of a highly sensitive c-di-GMP-dependent reporter system to rapidly monitor the level of the nucleotide in infecting living bacteria would be

extremely useful to investigate the role of this signaling pathway during infection. Besides, since only a few c-di-GMP receptors have been identified, the experimental strategy to characterize the role of c-di-GMP has been based on the analysis of bacterial mutants overloaded with or depleted of c-di-GMP. This is in principal a valid approach that has produced many important insights, but a serious problem related with it is that constant high or low levels of c-di-GMP may affect the activity of several c-di-GMP receptors at the same time and thus have an influence on more than one bacterial process. This scenario may not necessarily resemble a real progression in the establishment of a chronic infection. In support of this prediction, the pioneering work of Pultz et al. [25] has shown differences in the affinities for c-di-GMP of receptor proteins involved in the coordinated inhibition of bacterial motility and increased matrix synthesis during *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) biofilm formation. Such differences in binding affinity were demonstrated to account for a mechanism for the selective activation of c-di-GMP controlled processes. Thus, this model involves the sequential activation of c-di-GMP receptors so that whilst the c-di-GMP concentration is maintained at a low level, bacteria are motile and do not produce biofilm matrix compounds. Upon sensing appropriate environmental conditions, levels of c-di-GMP rise, first activating the receptor responsible for flagella motility repression and later the receptor that stimulates the synthesis of biofilm matrix compounds. This model implies that when a *S.* Typhimurium mutant with high levels of c-di-GMP is used for virulence studies, it is necessary to consider that the resulting phenotypes may be due to both the inhibition of bacterial motility and shielding of cell surface receptors with biofilm matrix compounds, which may not mimic the reality of sequential biofilm formation events *in vivo*.

Despite the broad distribution of c-di-GMP signaling in bacteria, some bacteria lack the enzymes required for c-di-GMP synthesis and instead they use c-di-AMP as a secondary messenger [26-30]. A notable example is *Staphylococcus aureus*, which is one of the bacterial species most frequently associated with biofilm-mediated infections [31,32]. Although our knowledge about c-di-AMP-mediated signaling in bacteria is in its infancy compared to the c-di-GMP network, it is already clear that c-di-AMP controls cell wall properties, as an increase in c-di-AMP levels allows *S. aureus* to grow in the absence of lipoteichoic acid, confers resistance to cell wall active antimicrobials and induces the production of components involved in biofilm formation [28]. Also, it has been shown that secretion of c-di-AMP in *Listeria monocytogenes* is responsible for the induction of an interferon-β (IFN-β) mediated host immune response [29]. However, direct evidences showing that c-di-AMP controls the transition between a planktonic and a sessile lifestyle are still missing.

Considering the prevalence of c-di-GMP signaling in inducing a planktonic/biofilm switch in a broad range of bacteria and that bacteria have invariably linked the synthesis of the biofilm matrix with the accumulation of high levels of c-di-GMP, one has to wonder whether this accumulation has significant relevance during the infection process and if it conditions the interplay between biofilm development and the host cell response. One answer to this question might be that this c-di-GMP accumulation provides an advantage during infection through the deviation of the innate immune response. On the contrary, this accumulation might be detrimental for bacteria because it might allow the immune system to recognize c-di-GMP as a signal to alert on the presence of biofilm-mediated infections. This paradox is discussed below.

Biofilm, innate immune response and c-di-NMPs

Innate immunity is the first line of defense against infection. It recognizes and provides rapid defense against pathogens. The recognition depends on receptors (pattern-recognition receptors, PPRs) that trigger signaling pathways not only to alert the immune system to the presence of infection but also to initiate adaptive immune responses through activation of antigen presenting cells [33]. Even though several classes of PRRs and their ligands are known, PRRs specific for microorganisms within biofilms have not yet been identified. Recent studies have shown that bacterial c-di-NMPs (c-di-GMP and c-di-AMP) act as PAMPs eliciting a host type I interferon (IFN) innate immune response characterized by activation of IFN production (meter aquí el de chlamydia también) [29,34-39]. Thus, bacterial c-di-NMPs produced at high levels during biofilm development could serve as danger signals recognized by the host immune system (Figure 1). Two distinct PPRs able to directly bind cyclic nucleotides and mediate the induction of type I IFN have been described. Binding of c-di-GMP to a dimer of the stimulator of interferon genes (STING) results in the activation of the IRF3 transcription factor, which is required for host transcriptional activation of type I IFNs [40-42]. More recently, DDX41, a DEAD-box helicase, has been shown to directly bind c-di-NMPs and mediate the induction of IFN through a pathway that likely converges upstream of STING [43,44]. c-di-GMP binds STING and DDX41 with a dissociation constant of ~14.54 µM and ~5.65 µM, respectively [43,45]. These dissociation constants are consistent with c-di-GMP concentrations in the bacterial cytoplasm, which have been estimated to be in the sub- to low- micro-molar range [16,46,47]. Moreover, STING dependent type I IFN production can also be strongly induced by endogenous hybrid cyclic GMP-AMP synthesized by mammalian cyclic GMP-AMP synthase (cGAS) in response to the presence of DNA in the cytoplasm [48,49]. It is tempting to

speculate that cGAS might also sense bacterial c-di-NMPs providing a mechanism of signal amplification for a more sensitive innate immune response.

Because both STING and the DDX41 helicase are cytoplasmic proteins, it is assumed that these proteins recognize and respond to DNA and c-di-NMPs derived from microorganisms that enter the cytosol. In particular, it is possible that STING and DDX41 could directly detect c-di-NMPs produced by intracellular biofilm communities. Compelling evidence indicates that in addition to extracellular biofilms, some bacteria can form biofilm-like polysaccharide-containing structures inside the host epithelial cells named intracellular bacterial communities (Figure 1B). These structures were firstly described in uropatogenic *Escherichia coli* (UPEC), the predominant causative agent of urinary tract infections (UTIs) but they have also been recognized for other bacterial pathogens such as *Klebsiella pneumoniae* [50], *P. aeruginosa* [51] and *S. aureus* [52,53]. Because intracellular biofilms are not confined to a membrane, c-di-GMP and/or c-di-AMP, if secreted, could freely diffuse in the cytoplasm of infected cells and bind to cytoplasmic cell host receptors. Furthermore, a recent study has provided evidence that STING is able to detect c-di-AMP synthesized by *Chlamydia trachomatis* even though *Chlamydia* is confined to a membrane-bound vacuole. This raises the possibility that STING can directly survey membrane-bound pathogen-containing vacuoles for leaking microbe-specific metabolites to mount type I IFN responses required to control microbial infections [54]. On the other hand, several lines of evidences suggest that extracellular c-di-NMPs can also elicit an immune response. Studies for evaluating the immunostimulatory and immunomodulatory properties of c-di-AMP and c-di-GMP suggest that these external molecules could be able to activate IFN acting via up-to-now uncharacterized surface receptors or nucleotide transporters [55-59]. Furthermore, c-di-GMP treatment reduces, in a dose-dependent manner,

bacterial colonization by biofilm-forming *S. aureus* strains in a mouse model of mastitis infection [57]. The concentration of c-di-GMP used in this study (50 nM) is close to the physiological upper limit for freely diffusible c-di-GMP in the bacterial cytoplasm [60]. Independently of whether c-di-NMPs need to be present in the cytosol or can activate the innate immune system from the outside to the inside of the cell, they need to be released from bacteria at sufficient levels to activate host receptors. In the case of *Listeria monocytogenes*, genetic and biochemical studies have identified two major multidrug resistance (MDR) efflux pumps, MdrM and MdrT, as necessary and sufficient for secretion of c-di-AMP and induction of the cytosolic surveillance pathway (CSP) in host immune cells [29,61]. Bioinformatic analysis identified the widespread presence of homologs of the MdrM and MdrT efflux pumps in bacteria, including pathogenic *staphylococci*, *streptococci* and *mycobacteria*, strongly suggesting that these bacteria could also secrete c-di-AMP. With respect to c-di-GMP, there is no evidence suggesting the secretion of this nucleotide outside bacteria. However, one could imagine that the interaction with the host cell may trigger c-di-GMP secretion during infection.

In summary, there is convincing support for the idea that c-di-NMPs are detected by the innate immune system as a novel PAMP type. However, a deeper insight into the mechanisms of c-di-NMPs detection by the host cell is needed to conclusively establish that accumulation of c-di-NMPs during the biofilm switch alerts the innate immune system of the presence of a bacterial infection.

Does c-di-NMPs dependent activation of type I IFN production play a role against biofilm-mediated infections?

The answer to this question is unclear because conflicting reports about the role of type I IFN during bacterial infection have been published [62-64]. In general, production of

type I IFN has been reported to be protective against extracellular bacteria. For instance, stimulation of type I IFN production by *S. aureus* promotes an effective host defense and also treatment of cutaneous infections with exogenous IFN-β promotes bacterial clearance [65]. Similarly, *Streptococcus pyogenes* elicits type I IFN production and inhibition of its production increases the susceptibility of the host to streptococcal diseases [66,67]. The production of type I IFN is also required for resistance against encapsulated *Escherichia coli* strains in a sepsis model of mice infection [68]. In contrast, it is well known that the expression of type I IFN enhances the susceptibility to infection by *L. monocytogenes* [69-71]. An interesting explanation for the mechanism by which type I IFN production could be detrimental during *L. monocytogenes* infection has been provided in a recent study with *S.* Typhimurium [72]. Using transgenic mice deficient in the receptor for type I IFNs, Robinson *et al.* showed that *S.* Typhimurium uses type I IFN signaling to kill macrophages through the induction of a specialized pathway for programmed necrosis, referred to as necroptosis. In regards to c-di-NMPs signaling, it is not difficult to imagine a scenario where local accumulation of c-di-NMPs surrounding the bacterial biofilm could elicit type I IFN production, which would protect biofilm bacteria by inducing the death of recruited macrophages (Figure 2).

However, because these studies have used acute infection models, it is necessary, before drawing any conclusion about the role of type I IFN against biofilm infections, to consider that soon after the switch to a sessile lifestyle, bacteria start producing the biofilm matrix. The extracellular matrix is made of proteinaceous compounds and/or exopolysaccharides and provides a mechanical stability and protection from host defenses. One of the most common matrix exopolysaccharides secreted by a wide range of bacteria is a polymer of β-1,6-N-acetyl-D-glucosamine called PGA or PIA/PNAG [31,73,74]. This exopolysaccharide provides protection against phagocytosis by steric

hindrance and charge repulsion which reduces antibody and complement deposition on the bacterial cell surface [75-79]. A more detailed analysis of the mechanisms that account for the innate immune evasion during *S. aureus* biofilm infection strongly suggests an active reprogramming of the innate immune response [80,81]. The macrophages recruited at the host biofilm interface are polarized towards an alternative activated M2 phenotype that possesses anti-inflammatory properties and shows reduced induction of nitric oxide synthase concomitant with robust arginase-1 expression. In addition, a rapid necrosis of those macrophages that invaded deep into the biofilm was described. Interestingly, that these biofilms induced macrophage dysfunction might provide a potential explanation as to why biofilm infections persist in an immunocompetent host. Because classical TLR2 (cell enveloped compounds ligand) and TLR9 (DNA ligand) recognition pathways apparently do not dictate biofilm mediated immune response deviation [80], a possibility exists that signaling mediated by c-di-NMPs might be related to the above described phenotypes. Future research using biofilm infection models and genetically modified bacteria in which the synthesis of c-di-NMPs exclusively occurred at the infection site would be useful to gain insight into biofilm mediated chronic infections.

Concluding remarks and future directions

Despite the wealth of knowledge gained over the years about bacterial biofilm formation and its involvement in the development of chronic infections, there are still major unresolved questions about how the biofilm formation process starts on the surface of host tissues (Box 1). A good example of the current uncertainty is the role that c-di-NMPs and the innate immune system play during *in vivo* infection. A widely accepted truth in the bacterial biofilm field is the crucial role that c-di-NMPs play to

control the transition between a free-swimming and a sessile lifestyle. At the same time, several groups dedicated to identifying PRRs have recently discovered that c-di-NMPs are recognized by the innate immune system leading to the induction of type I IFNs. Considering that activation of biofilm development coincides with the accumulation of highest levels of c-di-NMPs in all the bacteria tested to date, it seems reasonable to assume that the innate immune system could recognize c-di-NMPs as a signal of the presence of an incipient bacterial biofilm. To investigate this hypothesis, future challenges will be to develop methods to measure the accumulation of c-di-NMPs in biofilm infected tissues and explore the mechanisms through which the innate immune system recognizes extracellular c-di-NMPs.

¿por qué has quitado el resto?

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Box 1. Outstanding questions

1. Do c-di-NMPs play a role during early steps of bacterial biofilm formation that precede the establishment of a chronic infection?
2. Are there biological markers of early biofilm formation process *in vivo*?
3. Does the interaction with the host cells induce the accumulation of c-di-NMPs? If that is so, which are the signals in the host that induce the accumulation of c-di-NMPs in bacteria?
4. Does the innate immune system recognize the incipient biofilm development through the detection of c-di-NMPs?
5. Is cGAS able to sense bacterial c-di-NMPs in order to amplify the activation of type I IFN production?
6. Does biofilm matrix composition confer different protection against type I IFN-mediated immune response?
7. Do c-di-NMPs or biofilm matrix components condition macrophage polarization for the subsequent immune response?
8. Does c-di-NMPs and DDX41/STING represent new targets for combating chronic infections?

Figure legends

Figure 1.

Stimulation of type I IFN production through DDX41 helicase and STING activation by c-di-NMPs during bacterial biofilm infections. Environmental signals favor the initiation of a biofilm by transitioning from a planktonic to a surface attached mode of growth. After adhesion, bacteria proliferate and synthesize a biofilm matrix, establishing an extracellular biofilm on the cell surface (A). In some situations, bacteria invade the host cell to proliferate within the cytoplasm developing an intracellular biofilm (B). c-di-NMPs either produced by intracellular bacteria or internalized via uncharacterized host cell surface proteins, interact with the DDX41 helicase and/or STING. Movement of STING to a perinuclear punctuate structure is necessary for the recruitment and activation of the kinase (TBK1) in order to phosphorylate the interferon regulatory factor (IRF3) to induce production of type I IFNs (IFNs). Abbreviations: ER, endoplasmic reticulum.

Figure 2.

The bacterial cyclic di-nucleotides paradox.

The drawing shows a working model about the double function that c-di-NMPs could play during the first stages of biofilm related infections. On one hand, cyclic di-nucleotide second messengers stimulate the synthesis of the biofilm matrix compounds. On the other, the host cells have evolved a pathway to sense c-di-NMPs that might act as biofilm specific PAMPs. As a consequence, type I IFN is produced priming a local innate immune response. The biofilm matrix shields bacteria by constituting a steric barrier against phagocytosis, hampering deposition of antibodies and protecting against

antimicrobial defensins. In addition, biofilms might induce a rapid macrophage death and macrophage reprogramming towards a M2 phenotype characterized by a poorly microbicidal activity. Abbreviations: NK, natural killer cells; macrophages; Th1, Th1 lymphocytes.

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