Chapter 5

How does *Streptococcus pneumoniae* invade the brain?

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Abstract

*Streptococcus pneumoniae* (the pneumococcus) is the major cause of bacterial meningitis. The mechanisms by which pneumococci from the bloodstream penetrate the blood-brain barrier to reach the brain are not fully understood. Receptor-mediated adhesion of the bacteria to the brain endothelium is considered a key event leading to meningitis development. The aim of this review is to discuss recent advances and perspectives related to the interactions of *S. pneumoniae* with the blood-brain barrier during the events leading to meningitis. Altogether, the available data suggest that, by precisely defining the pathways and ligands by which *S. pneumoniae* adheres to specific receptors, it may be possible to interfere with the respective mechanisms and develop strategies to prevent or even cure pneumococcal meningitis.
Streptococcus pneumoniae, the blood-brain barrier, and pneumococcal meningitis

The Gram-positive bacterium Streptococcus pneumoniae (the pneumococcus) is an important commensal resident of the human nasopharynx. Although carriage is usually asymptomatic, *S. pneumoniae* can become invasive and spread from the upper respiratory tract to other organs, leading to serious diseases such as pneumonia, sepsis, or meningitis [1, 2]. *S. pneumoniae* is the major etiological cause of bacterial meningitis, responsible for two-thirds of meningitis cases in Europe and in the USA [3-5].

Bacterial meningitis is a disease with high morbidity and mortality worldwide despite the implementation of several vaccination programs and antimicrobial agents [3-6]. A major route for bacteria to reach the meninges is through the bloodstream [7]. Having reached the blood vessels in the brain, bacteria present in the bloodstream have to cross the blood-brain barrier to enter the brain and cause infection. This view is supported by recent immunofluorescent analyses combined with high-resolution confocal microscopy, where blood-borne *S. pneumoniae* was clearly shown to adhere to the brain vascular endothelium prior to invasion of the brain [8].

The blood-brain barrier is a specialized vasculature system that separates the brain from circulating blood and has critical functions in both protection and nutrient supply of the brain [9-11]. Endothelial cells form the layer that lines the interior surface of the blood vessels [12, 13]. Pathogens can invade the brain only after crossing the endothelial cell layer of the blood-brain barrier and, therefore, they must develop strategies to pass this barrier. The inflammatory features and clinical complications following bacterial meningitis normally observed in humans can be reproduced using animal models. Notably, most *in vivo* models that examine the pathophysiology of bacterial meningitis involve the direct injection of pneumococci into the brain of mice or rats [14-18]. Administration of bacteria directly into the brain bypasses the need for blood-brain barrier translocation. In order to study the interaction of blood-borne *S. pneumoniae* with the brain vascular endothelium *in vivo*, prior to the onset of meningitis, a so-called bacteremia-derived meningitis model was established in recent years [8, 19]. This model, where bacteria are injected into the bloodstream instead of the brain, has allowed the visualization of successive steps from bacteremia to brain invasion. The aim of the present review is to discuss recent advances in our understanding how blood-borne *S. pneumoniae* interacts with the blood-brain barrier endothelium during the events leading up to meningitis, and how to use such knowledge to develop new therapeutic strategies to fight the disease.
Receptor-mediated transcytosis

How bacterial pathogens cross the blood-brain barrier is still subject of debate. Several possible mechanisms have been implicated in this process, such as: 1) destruction of the endothelial cell layers in case of, for example, *Neisseria meningitidis* [20]; 2) traversal of the blood-brain barrier in between the endothelial cells by disruption of tight junctions in case of group A streptococci [21]; and 3) receptor-mediated transcytosis across endothelial and epithelial cell layers in case of *S. pneumoniae* [22, 23]. The view that pneumococci pass the blood-brain barrier via transcytosis rather than pericellularly is supported by experiments, showing a continuous and homogeneous staining of VE-Cadherin in the brain of infected mice [8]. This implies that blood-borne *S. pneumoniae* does not cause a disruption of the endothelial tight junctions.

In this context, it should be noted that bacterial pathogens like pneumococci have the capability to bind to certain receptors on the plasma membrane of epithelial and endothelial cells, and this receptor-mediated binding facilitates bacterial invasion into and translocation over human cell layers. The passage of bacterial pathogens across such layers is a fundamental step for development of invasive diseases and it is necessary for *S. pneumoniae* to cross the blood-brain barrier to develop bacterial meningitis [22, 23].

The platelet-activating factor receptor (PAFR)

The first reported receptor implicated in adhesion to, invasion of, and also transcytosis through endothelial cells is the PAFR. PAFR is a G-protein coupled receptor with seven transmembrane domains and its natural ligand is the platelet-activating factor (PAF). PAF is a mediator in diverse pathologic processes, such as allergy, asthma, septic shock, arterial thrombosis, and inflammation [24-26]. Binding of PAF to the PAFR stimulates numerous signal transduction pathways including phospholipase C, D, A2, mitogen-activated protein kinases (MAPKs), and the phosphatidylinositol-calcium second messenger system [25]. PAFR has been proposed to bind *S. pneumoniae*, thereby facilitating adhesion to, uptake by, and transcytosis through endothelial cells leading to invasive disease [23, 27, 28]. However, while in vitro and in vivo studies indicate that PAFR is involved in the development of invasive pneumococcal disease (IPD), there is so far no unequivocal published evidence that a direct binding between *S. pneumoniae* and PAFR occurs [24]. A recent study suggests that PAFR plays a role in pneumococcal adhesion to endothelial cells, even though pneumococci do not directly bind to PAFR [29]. After immunofluorescent microscopy analysis, colocalization of PAFR and *S. pneumoniae* was not observed in vivo in mouse brain tissue nor in endothelial cells in vitro. Nevertheless, upon blockade of PAFR, adhesion of pneumococci to human endothelial cells was significantly reduced, indicating that PAFR most likely has an indirect role in IPD [29]. When the PAFR was genetically deleted or chemically inhibited, *S. pneumoniae* was still able to adhere to human cells in vitro, and to cause invasive disease in vivo [23, 27].
The laminin receptor (LR)
The LR is an important molecule involved both in cell adhesion to the basement membrane and in signal transduction following this binding event. The LR on endothelial cells was found to interact with neurotropic viruses, including Sindbis virus [30], Dengue virus [31], adeno-associated virus [32], tick-borne encephalitis virus, and Venezuelan equine encephalitis virus [33]. LR was identified to be a common receptor for both *S. pneumoniae* and *N. meningitidis* on the surface of rodent and human brain microvascular endothelial cells [19]. Fluorescent beads coated with the pneumococcal choline-binding protein (Cbp) A [34-36] that were injected intravascularly into mice adhered to the cerebral endothelium. Pretreatment with anti-LR antibody led to an inhibition of the adherence of the CbpA-beads to the endothelium in mice, thus suggesting that the LR interacts with the bacteria facilitating translocation of intracellular pneumococci across the blood-brain barrier endothelium, from the basolateral side of endothelial cells to the brain tissue [19].

The poly immunoglobulin receptor (pIgR)
The plgR transports immunoglobulins across mucosal epithelial barriers (e.g., in the respiratory tract) as a first line of defense to antigens from pathogenic bacteria [37-39]. *S. pneumoniae* has been shown to bind to plgR expressed by human nasopharyngeal epithelial cells and this binding facilitates pneumococcal translocation through the nasopharyngeal epithelium [22]. When human nasopharyngeal epithelial (Detroit) cells were treated with an antibody against plgR prior to pneumococcal challenge, adherence was reduced [22]. This implies that plgR is functionally involved in pneumococcal adhesion to nasopharyngeal epithelium. Furthermore, immunoblotting analyses showed that pneumococcal CbpA binds to human plgR, indicating that CbpA may be required to mediate binding of *S. pneumoniae* to this receptor [22]. PlgR is not only present on the epithelium of the respiratory tract, but can also be found on the plasma membrane of endothelial cells, especially brain endothelial cells [29]. Due to its presence on the blood-brain barrier endothelium, the possible involvement of plgR in pneumococcal adhesion to brain endothelial cells was recently investigated [29]. Notably, immunofluorescent analyses of brain tissue of infected mice showed that most pneumococci adhering to the brain vasculature were colocalized with plgR [29]. Furthermore, by incubating pneumococci with human endothelial cell lysates, it was shown that *S. pneumoniae* can directly bind to plgR [29]. The combined results therefore suggest that plgR is a direct adhesion receptor for *S. pneumoniae* on the blood-brain barrier endothelium.

Even though the main route of brain invasion seems to be the bloodstream, pneumococci may also reach the brain through skull fractures that tear the dura mater, or directly from the nasopharynx. In fact, it was recently reported that *S. pneumoniae* can transmigrate from the nasopharyngeal epithelium to the olfactory nerves that anatomically connect the upper respiratory tract with the central nervous system [40]. Although it was not shown experimentally, an intriguing hypothesis is that the plgR on the nasopharyngeal epithelium...
could mediate the passage of the bacteria from the epithelial cells to the olfactory nerve. Clearly, once this passage has occurred the bacteria can travel along the nerve and reach the brain.

**Platelet endothelial cell adhesion molecule-1 (PECAM-1)**

The PECAM-1 (also known as CD31) is a pan-endothelial protein present in the intercellular junctions of the endothelial cells, that is involved in leukocyte migration, angiogenesis, and integrin activation [41-43]. In particular, the involvement of PECAM-1 in leukocyte-endothelium interaction and leukocyte trans-endothelial migration in the vascular system makes PECAM-1 a key molecule in inflammation and neuroinflammation [43]. Recently, PECAM-1 was implicated in gastrointestinal infection caused by *Salmonella enterica* serovar Typhimurium [44], suggesting an active role of PECAM-1 in host-pathogen interactions. This raised the question whether PECAM-1 might also play a role in host-pneumococcal interactions. Indeed, for the first time it was recently shown that, in brain tissue from intravenously infected mice, most pneumococci did colocalize with PECAM-1 [45]. Blockade of PECAM-1 with a specific antibody in human brain endothelial cells led to a significant reduction of bacterial adhesion, indicating that PECAM-1 is functionally involved in *S. pneumoniae* adhesion to the brain vascular endothelium [45]. Using a biochemical approach, it was demonstrated that *S. pneumoniae* can directly bind PECAM-1 expressed by human endothelial cells [45]. Importantly, PECAM-1 colocalized with plgR on the brain endothelium and, moreover, the two receptors directly interacted with each other as demonstrated by co-immunoprecipitation experiments suggesting the possible formation of a double receptor that facilitates the passage of *S. pneumoniae* across the blood-brain barrier [45] (Figure 1). Nevertheless, to unequivocally demonstrate the joint role of PECAM-1 and plgR in pneumococcal meningitis, *S. pneumoniae* should be intravenously administrated to PECAM-1−/− and plgR−/− mice. In the case of a direct role, as suspected, one would expect that only few bacteria can translocate the blood-brain barrier in the knock-out mice. Although current literature still lacks such experimental evidence, it was previously shown that adhesion of bacteria to the brain vasculature endothelium represents a crucial moment for invasion of meningeal pathogens in the brain. In addition, the observation that blood-borne pneumococci colocalize with both PECAM-1 and plgR *in vivo* strongly indicates that the two receptors jointly facilitate the passage of the bacteria from the bloodstream into the brain [29, 45].

**Cooperation of PAFR, pIgR and PECAM-1 during pneumococcal adhesion to endothelial cells: a new model**

From the current literature [24], it remains unclear what the exact role of PAFR is in IPD. The involvement of PAFR in *S. pneumoniae* adhesion to endothelial cells might well be exerted through the binding of PAF or pneumococcal cell wall components to the receptor and
the subsequent inflammatory consequences of its activation [24]. Inflammation leads to an upregulation of receptors expressed on the plasma membrane of the apical side of the cells [7, 27, 46], and PECAM-1 and pIgR may be among them. In the presence of more pIgR and PECAM-1, *S. pneumoniae* has more sites to adhere to on the endothelial cells. It has been repeatedly shown that blocking PAFR with a specific antibody or antagonist leads to a significant reduction of pneumococcal adhesion to endothelial cells *in vitro* [23, 27, 29]. We therefore hypothesize that, due to PAFR blocking, the upregulation of pIgR-PECAM-1 may be prohibited. Possibly, the presence of pneumococci may trigger the autocrine production of PAF, directly or indirectly, which activates PAFR, leading to an upregulation of pIgR and PECAM-1. This would increase pneumococcal adherence to pIgR and PECAM-1 and, in turn, more adherent bacteria would increase the chances of bacterial invasion into the cells (Figure 1). Whether PAFR activation leads, directly or indirectly, to pIgR-PECAM-1 upregulation still remains an open question. Besides PAF, secreted pneumococcal components, such as pneumolysin [47, 48] and CbpA, could bind to PAFR and activate this receptor [49]. Clearly, this idea still needs to be experimentally validated. Accordingly, an in-depth investigation of secreted and surface-attached pneumococcal proteins, including proteomics and structural analyses, will be required to identify any protein(s) of *S. pneumoniae* that could be recognized and bound by PAFR. Interesting candidate proteins for such studies were recently identified by Pribyl et al. in a proteomics-based survey of the pneumococcal surface-bound and
Humans deficient in receptors PAFR, pIgR, and PECAM-1 have not been identified, but mice survive without them

Humans deficient in PAFR have not been identified. However, PAFR⁻/⁻ mice have been generated [51]. In PAFR⁻/⁻ mice, modifications of known angiogenic factors, such as vascular endothelial growth factor (VEGF), have not been observed, but a sustained rise of the chemokines CXCL2 and CCL2 has been detected, which may compensate for the absence of PAFR as an important molecule in maintaining immunological surveillance [52]. These findings indicate that the absence of PAFR in mice is compatible with health, although it has to be noted that this was observed under controlled conditions. Mice lacking pIgR were of normal size and fertility as well, but displayed increased IgG levels in their sera, suggesting a triggering of systemic immunity [53]. These findings indicate that absence of pIgR is not lethal either, but at the same time it seems that absence of pIgR is sensed as a potential danger by the systemic immune system [38, 53]. PECAM-1 is expressed not only by endothelial cells, but also by platelets, neutrophils, and macrophages, and its signaling pathways might mediate resistance to apoptosis and promote cell survival [41, 42]. Absence of PECAM-1 may therefore lead to several cellular dysfunctions. Interestingly, PECAM-1 was reported not to be required for embryogenesis, fetal maturation, or fertility in mice, and, furthermore, PECAM-1 expression on platelets is not essential for platelet-platelet aggregation [54]. PECAM-1⁻/⁻ animals develop normally and do not show any signs of an abnormal physiology under normal baseline conditions. However, as the animals age they can develop an autoimmune lupus-like syndrome and, when stressed, a variety of abnormal responses have also been noticed. In particular, PECAM-1⁻/⁻ animals exhibited a prolonged bleeding time [55]. Thus, although the absence of the PAFR, pIgR, or PECAM-1 receptors did not cause serious problems in mice during certain circumstances, dysfunctions and abnormalities due to a lack of these receptors cannot be excluded.

Blockade of S. pneumoniae receptors: a new avenue for the prevention and cure of pneumococcal meningitis

Knowledge on the mechanisms by which the pneumococcus interacts with the blood-brain barrier before invading the brain is fundamental in order to develop therapeutic strategies...
to avoid adhesion of *S. pneumoniae* to the blood-brain barrier and, thus, invasion of the pathogen into the central nervous system. Adhesion of pneumococci to the blood-brain barrier is spatiotemporally controlled at different sites throughout the brain, and the local immune system in the brain is activated immediately as soon as bacteria in the bloodstream interact with the blood-brain barrier [8]. The bacteremia-derived meningitis model is used to investigate the interactions of bacteria with the blood-brain barrier, including the events preceding meningitis [8, 19, 29, 45]. To study meningitis itself, intracisternal administration of bacteria was previously used to induce meningitis *in vivo* [14-18]. Using this model, neutrophil influx into the brain was detected only at 30 hours after challenge, indicating that white blood cells can be detected in the brain only at late stages of the disease [14]. A high count of white blood cells in the cerebrospinal fluid (CSF) is often used as a clinical test for diagnosis of meningitis [56]. Moreover, classic symptoms of meningitis, such as rash, neck stiffness, photophobia, severe headache, and impaired consciousness develop late [56-58]. While these symptoms represent the classical picture of suspected meningitis, the disease may be already at an advanced stage in patients displaying them. Thus, it is not really surprising that, at this stage, the risks of brain damage are considerable even if the infection is successfully fought with antibiotics. World Health Organization (WHO) data indicate that, even if the disease is diagnosed early and adequate treatment is started immediately, 5-10% of the patients will die, typically within 24-48 hours after occurrence of the first symptoms. When left untreated, up to 50% of the patients may die. In case of recovery, brain damage, hearing loss, or a learning disability appears in 10-20% of the survivors (http://www.who.int/topics/meningitis/en/).

It is therefore crucial to start interventions before bacteria have invaded the brain in order to optimize the survival chances of patients and to prevent permanent damage. Treatment with antibiotics is the clinically used way to cure meningitis, and routine vaccination against pneumococcal infections with the pneumococcal conjugate vaccine (PCV), which is active against seven to thirteen common capsular serotypes of this pathogen, has significantly reduced the incidence of invasive pneumococcal disease in vaccinated children [59-61]. Knowing that PAFR, plgR, and PECAM-1 mediate the first adhesion of blood-borne *S. pneumoniae* to the vascular endothelium of the blood-brain barrier opens a complementary approach to antibiotics and vaccines for curing or preventing pneumococcal meningitis by developing therapies that interfere with these receptors. This could, for example, be achieved by blocking PAFR, plgR, and PECAM-1 with specific antibodies which would limit the adhesion of pneumococci to the blood-brain barrier and then observing whether the systemic administration of blocking antibodies leads to less severe disease symptoms. Alternatively, blockade of the receptors can be achieved by using either chemical antagonists, such as, for instance, L659989 or WEB2086 previously used to successfully block the PAFR *in vitro* [24], or the pneumococcal proteins that should specifically anchor to the receptors, such as CbpA that was reported to bind to plgR in the respiratory epithelium [22, 62]. Another alternative, but technically more challenging approach, is to prevent binding of *S. pneumoniae* to PAFR, plgR and PECAM-1 on the blood-brain barrier endothelium by blocking the respective ligands...
on the pneumococcal cell surface with engineered soluble derivatives of these receptors. This would have the potential advantage of minimal interference with the physiological functions of these three receptors. Such studies could be aided by targeted infection-imaging approaches, for instance, with positron emission tomography (PET) tracers that bind well to S. pneumoniae [63]. However, while blocking of these pneumococcal receptors could provide protection against meningitis, it will be crucial to develop strategies to avoid possible dysfunctions caused by their blocking before this approach can be implemented in the clinic. Based on literature data, mutated PAFR in mice is not likely to cause serious dysfunctions [51]. However, mutated plgR may interfere with the normal transport of immunoglobulins across cells. It has been shown that treatment with phorbol myristyl acetate (PMA) activates isozymes of protein kinase C (PKC), a downstream protein in the signaling cascade of plgR [64]. This activation stimulates not only plgR transcytosis, but more generally the pathway of a variety of molecules in the transcytotic pathways [64]. Thus, treatment with PMA can stimulate cells to use other transcytotic systems and their receptors, in case immunoglobulin transfer mediated by plgR is altered. In this way transport of immunoglobulins can be maintained through other transcytotic pathways. Regarding the vascular role of PECAM-1, it is important to bear in mind that endothelial cells express a vast range of endothelial adhesion factors (such as VECAM-1 and ICAM-1) that perform similar functions as PECAM-1 [65, 66]. Therefore, is conceivable that such adhesion molecules could complement for PECAM-1’s role in case PECAM-1 is blocked. In principle, it should thus be possible to develop monoclonal antibodies blocking the pneumococcal binding site of the PECAM-1 receptor.

While the blocking of plgR and PECAM-1 showed only a partial reduction of pneumococcal adherence to endothelial cells [45], the blocking of the PAFR, plgR, and PECAM-1 receptors at the same time could be a potential way to completely preclude the adhesion of S. pneumoniae to the blood-brain barrier endothelium and, therefore, prevent completely pneumococcal invasion into the brain. This, however, remains to be assessed experimentally. Clearly, an important advantage of conceivable therapies based on monoclonal antibodies against PAFR, plgR, and PECAM-1 would be that these antibodies need to be administered only as long as pneumococci are detectable in the blood. Once these have been fought successfully with regular antibiotics, the antibody therapy can be stopped, which will limit possible unwanted side effects to a minimum.

Lastly, it has been demonstrated that pneumococci in the blood can reach and adhere to the blood-brain barrier endothelium after which they may invade the brain [8]. Bacteremia is present in 20-30% of cases with pneumococcal pneumonia, which can occur when an infection of the lungs grows out of control [67-69]. Patients with bacteremia and pneumonia can be considered ‘high-risk’ for progression towards brain infection and, for this reason, it could be valuable for them to receive innovative treatments that block pneumococcal receptors. This would prevent pneumococci from adhering to the blood-brain barrier and invading
the brain. Such a treatment would even be of relevance for patients who are hospitalized with meningitis as it would still reduce the numbers of bacteria adhering to the blood-brain barrier and, in combination with antibiotic therapy, both the blood-borne bacteria and the bacteria that have already reached the brain could be eliminated.

**Concluding remarks**

What is the ultimate goal of knowing how *S. pneumoniae* invades the brain? Clearly, if pneumococci cannot adhere to endothelial cells, bacterial invasion will most likely be prevented. Therefore, by precisely defining the pathways by which *S. pneumoniae* adheres to specific receptors, it may be possible to develop drugs that interfere with these mechanisms and to develop strategies to prevent or even cure pneumococcal meningitis, as this still remains a very serious disease with high mortality and morbidity worldwide. Judged by our current understanding of pneumococcal brain invasion, this is a highly ambitious but potentially achievable objective.

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