A novel mechanism of manipulation of the host immune response by *Porphyromonas gingivalis*: The role of PPAD and fimbriae in TLR2-dependent signaling.

Abstract

The keystone oral pathogen, *Porphyromonas gingivalis*, is a gram-negative, anaerobic bacterium implicated in the development and progression of periodontitis, chronic inflammation of tooth-supporting tissues. It is worth to highlight that periodontitis is the most common bacteria-driven inflammatory disease in humans. Furthermore, many systemic diseases, such as arthritis, atherosclerosis, and Alzheimer's disease are also correlated with periodontitis and the prevalence of *P. gingivalis*. *P. gingivalis* produces a wide array of virulence factors, including lipopolysaccharide (LPS), gingipains, outer membrane vesicles (OMVs), fimbriae, capsules, and peptidyl arginine deiminase (PPAD). This enables this bacterium to masterfully manipulate host immune responses as it uses them to alter the environment and host-induced signals to support its fitness. *P. gingivalis* achieves this by promoting inflammatory conditions which provide nutrients for this fastidious asaccharolytic bacterium, at the same time providing protection from killing by the host immune system.

PPAD is a unique virulence factor in the Procaryota kingdom since it is only encoded and expressed by *P. gingivalis*. This enzyme converts C-terminal Arg residues into citrulline in bacterium- and host-derived proteins and peptides. Previous results from our lab demonstrated that PPAD mutant strains are unable to stimulate the proinflammatory response and activate the prostaglandin E2 (PGE2, proinflammatory factor causing bone resorption in periodontitis) synthesis pathway in primary human gingival fibroblasts (PHGFs). Different pattern recognition receptors (PRRs) are responsible for *P. gingivalis* recognition by host cells, but many reports demonstrated the predominant role of Toll-like receptor-2 (TLR2) in this process. Therefore, the main aims of my thesis were the investigation of the role of citrullination in TLR2-dependent host cell signaling, examination of its mechanism, and identification of citrullinated *P. gingivalis* proteins crucial in this process.

During this study we have determined that TLR2 activation by *P. gingivalis* is strain-specific and dependent on citrullination. Of note, *P. gingivalis* fimbriae are one of the most important TLR2 ligands in the context of periodontitis. Fimbriae, bacterial cell surface appendages, are among the vital virulence factors of *P. gingivalis* as they are engaged in

bacterial adhesion to surfaces, host cells, and various bacterial species facilitating biofilm formation. In our recently published paper, we have shown that fimbriae are apparently modified by PPAD and that citrullinated fimbriae enhance TLR2 activation. We also found that the lack of PPAD reduced proinflammatory responses induced by *P. gingivalis* fimbriae in PHGFs. Among *P. gingivalis* laboratory strains and clinical isolates we can distinguish 6 types of fimbriae (I, Ib, II, III, IV, V) depending on the sequence of the *fimA* gene coding the major fimbriae subunit. In this project, 10 clinical strains obtained from periodontitis patients were tested for their ability to stimulate the proinflammatory response in host cells as well as their biochemical properties such as PPAD activity, fimbriae expression, and the *fimA* genotype. Interestingly, most of them were weak TLR2 agonists, possibly due to the expression mostly of the type II FimA in contrast to the laboratory strain ATCC33277, which encodes the type I FimA and is a strong inflammation inducer.

The logical step forward to characterize the mechanism of TLR2 activation by *P. gingivalis* fimbriae was the identification of PPAD modification site within the fimbriae structure. To confirm the occurrence of the modification induced by PPAD we tried to rescue the cell response to fimbriae from the PPAD-deficient *P. gingivalis* mutant by addition of purified PPAD. However, this attempt failed, so we assumed that citrullination occurs only during fimbriae assembly. Because fimbriae polymeric shaft assembly requires Arg46-Ser peptide bond hydrolysis of FimA lipoprotein by arginine-specific gingipains (RgpA or RgpB) to release mature FimA, we assumed that this Arg at the C-terminus of a peptide anchored into the outer membrane is citrullinated by PPAD and serves as the signal for TLR2. To verify this assumption we tested *P. gingivalis* mutants producing FimA with Arg46 of FimA substituted by other residues and found that all Arg46 mutants exerted a similar level of TLR2 activation as the wild-type strain thus excluding PPAD-modified FimA as a ligand for TLR2. Therefore, we focused on accessory fimbriae subunits FimCDE that constitute the tip of the fimbriae shaft composed of FimA. Our results obtained from the reporter cell line clearly showed that *P. gingivalis* mutants lacking accessory fimbriae subunits are significantly weaker TLR2 agonists than the wild-type strain. For this reason, we presumed that accessory fimbriae subunits undergo modification by PPAD and are crucial for TLR2-dependent host cell signaling. This was confirmed in experiments using accessory fimbriae subunits deficient *P. gingivalis* strains and isolated fimbriae containing FimCDE co-purified with different variants of FimA. Together our results unambiguously indicate that PPAD modified accessory fimbrial submits but not FimA induces the proinflammatory response of PHGFs via the TLR2 signaling pathway.

The final part of this thesis is focused on the verification that the response of immune cells is citrullination dependent. Macrophages are important immune cells during *P. gingivalis* infection, they respond quickly to bacteria, phagocytose them, secrete cytokines, present antigens to T cells, and play a critical role in tissue homeostasis. Because of that, we used human monocyte-derived macrophages as a model. Our data indicate that citrullinated fimbriae enhance macrophage proinflammatory responses like an expression of the PGE2 synthesis pathway components and cytokines genes, as well as cytokine secretion. Finally, we have revealed that PPAD and all tested fimbriae mutants exhibited much lower survival rates in macrophages, possibly due to the dampening of Akt activation and downstream reactive oxygen species (ROS) production.

In conclusion, this thesis constitutes an important study showing for the first time the complex impact of *P. gingivalis* citrullionome on host cells. Our research highlights the significance of bacterial protein citrullination in the context of *P. gingivalis* virulence and identifies the new mechanism by which *P. gingivalis* can subvert host immune responses. In the future, these observations may contribute to the development of new treatment methods for periodontitis and other comorbidities associated with this bacterium.