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Complement Evasion Strategies of Human Pathogens

The evolutionary arms race

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Abstract

Although the complement system, a pivotal component of innate immunty, is centrally involved in host defense against pathogens, its overactivation or deregulation may excessively amplify inflammation and contribute to immunopathology. Periodontitis, an oral infection-driven chronic inflammatory disease, has been linked with complement disruption by periodontal bacteria, resulting in inflammation and pathogenesis. The mechanisms of this disruption have partially been revealed, yet in this thesis we investigated novel periodontal pathogens and/or complement evasion pathways in periodontitis. Furthermore, complement inhibition by *S. aureus* is a key step in its infection, but the impact of its proteases on complement, highlighted in our studies, has not been well characterized before.

We focused on a major but relatively poorly studied periodontal bacterium *T. forsythia*. We showed that its two novel peptidases contribute to its complement resistance by cleaving several key complement components. Interestingly, both of the proteases were able to cleave C5 to release biologically active anaphylatoxins C5a, activity of which has been largely implicated in pathogenesis of periodontitis.

Further, we showed that another important periopathogen, *Prevotella intermedia*, acquires resistance towards complement by binding complement inhibitor found in human serum, factor I, and its two major cofactors C4b-binding protein (C4BP) and Factor H. This mechanism contributes to complement resistance of this bacterium.

We also found that major periopathogens have the ability to bind to the membrane-bound complement inhibitor CD46. Strikingly, even though this molecule improved initial attachment of bacteria to the epithelial cells, the final outcome was not beneficial for the bacteria – in cells without CD46 they persisted much longer without getting cleared, indicating activation of certain killing mechanisms upon CD46 stimulation. The phenomenon may be related to autophagy, which may be affected by CD46 as shown previously.

Recent studies implicated an involvement of a novel gram-positive *Filifactor alocis* in the pathogenesis of the periodontal disease. Taking into account the key role of complement deregulation by periodontal bacteria, we focused on *F. alocis* capacity to manipulate this system. We pinpointed different strategies, such as production of proteases or non-productive binding of C3, employed by this bacterium.

Finally, *Staphylococcus aureus*, is known for its impressive repertoire of complement inhibitors. We tested a panel of major proteases of *S. aureus* and identified their substrates within complement cascades. We also showed that two of the proteases are able to release C5a, similarly to proteases of periodontal bacteria.

Taken together, we explored the knowledge about complement manipulation by various common human pathogens. We identified both unique as wells as common strategies of bacterial complement evasion. Exploring virulence mechanisms shared by different pathogens can give rationales for developing effective therapies for infectious diseases.

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Complement Evasion Strategies of Human Pathogens

The evolutionary arms race

Monika Jusko

LUND
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Cover image:

Complement protein complexes: C1 complex (left), MBL-MASPs (middle), Membrane attack complex (right)

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"Out of life's school of war: what does not destroy me,
makes me stronger."
F. Nietzsche

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List of Papers

The following articles are part of this thesis work and will be referred to in the text with their respective roman numerals:

I. **Jusko M**, Potempa J, Karim AY, Ksiazek M, Riesbeck K, Garred P, Eick S, Blom AM (2012) A metalloproteinase karilysin present in the majority of *Tannerella forsythia* isolates inhibits all pathways of the complement system. *J Immunol. 2012 Mar 1; 188(5):2338-49.*

II. **Jusko M**, Potempa J, Kantyka T, Bielecka E, Miller HK, Kalinska M, Dubin G, Garred P, Shaw LN, Blom AM (2013) Staphylococcal proteases aid in evasion of the human complement system. *J Innate Immun. 2014;6(1):31-46.*

III. Malm S, **Jusko M**, Eick S, Potempa J, Riesbeck K, Blom AM (2012) Acquisition of complement inhibitor serine protease factor I and its cofactors C4b-binding protein and factor H by *Prevotella intermedia. PLoS One. 2012; 7(4): e34852.*

IV. **Jusko M**, Potempa J, Ksiazek M, Mizgalska D, Riesbeck K, Garred P, Eick S, Blom AM A metalloproteinase mirolysin of *Tannerella forsythia* inhibits all pathways of the complement system.

V. **Jusko M**, Potempa J, Malm S, Riesbeck K, Eick S, Blom AM Membrane-bound complement inhibitor CD46 guards gingival epithelial cells against periodontal bacteria invasion.

VI. **Jusko M**, Miedziak B, Riesbeck K, Eick S, Potempa J, Blom AM Novel periopathogen *Filifactor alocis* employs multiple strategies to evade the complement system.

Paper I was reprinted with the permission of The American Association of Immunologists, and Paper II – with the permission of Karger Publishers, Basel, Switzerland.

Abbreviations

APC	Antigen presenting cell
BCR	B-cell receptor
CR	Complement receptor
DC	Dendritic cell
C4BP	C4b binding protein
C5aR	C5a receptor
CRD	Carbohydrate recognition domain
DAF	Decay accelerating factor (CD55)
FDC	Follicular dendritic cell
GCF	Gingival crevicular fluid
IC	Immune complex
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
LPS	Lipopolysaccharide
MASP	MBL-associated serine protease
MAC	Membrane attack complex
MCP	Membrane cofactor protein (CD46)
MBL	Mannose binding lectin
NLR	NOD-like receptor
NK	Natural killer cell
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
TCR	T-cell receptor
TLR	Toll-like receptor

Introduction

The first forms of life to appear on Earth about 4 billion years ago were ancestors of modern bacteria, and for about 3 billion years our planet was exclusively populated by microscopic organisms, *Archaea* and *Bacteria* [1]. According to the theory of endosymbiosis, eukaryotes arose from the engulfment of a primitive prokaryotic cell by a larger host cell, ancestor of eukaryotic cells, which was itself possibly related to *Archaea* [2]. Therefore, it is not questionable that bacteria played a vital role in evolution of all organisms. At the same time they never stopped evolving themselves, constantly occupying new niches and giving rise to novel strains and species with unique features. It is estimated that there are 5×10^{30} bacteria on Earth with total biomass exceeding that of all plants and animals. They populate every piece of soil, thrive in waters, penetrate rocks and do not flinch from occupying volcanos or cold seeps. Last year researchers reported microbial activity in the Mariana Trench in the central west Pacific, representing the deepest oceanic site on Earth [3]. Given that extreme adaptive potential, it shouldn't be surprising that bacteria found numerous comfortable niches in other organisms including humans. The human body is constantly exposed to microorganisms, and more than that - there are about ten times more bacterial cells in human body as there are human cells. Most, but not all, microorganisms composing this normal flora are benign (non-pathogenic), which does not mean passive. All of them interact with their hosts in a various beneficial and occasionally harmful ways. They are found in all those regions of the human body that constitute barriers with the environment, such as the skin, oral cavity, intestinal tract, and urogenital tract. Normally bacteria cannot be found in the organs, or in the blood, lymph, or nervous system – their appearance in any of these locations indicates serious infectious disease. The nature of the relationships between bacteria and the host depends on two constantly changing factors: the virulence of the bacteria and the resistance of the host. The changes in these two factors are an extensive field of research, with thousands studies published every year, unraveling complex ways, in which human body deals with microbes every day, as well as diverse and astonishing mechanisms of virulence of human pathogens. Unfortunately bacteria

are constantly and rapidly evolving, leaving us at best one step ahead in this arms race and in a desperate need for a development of novel therapies for infectious diseases. This underscores the importance of the studies focusing on the virulence mechanisms of human pathogens, such as those exemplified in this thesis work.

Scope of the studies included in this thesis:

This thesis work focuses on the relationships between bacteria and the host immunity in health and disease. As an introduction to my articles, I will summarize most of the knowledge I gained while studying complex interplay of few human bacterial pathogens with immune defenses. I hope to give a broad and clear picture of what happens in human body when it deals with different pathogens. As this task seems to be rather ambitious, I will need to focus on some things, while only mentioning the others, yet I hope it will still be quite a good background for the articles that follow. The common denominator of all the studies presented here is that all of them stumble across the complement system. Given its essential role as the first arm of defense, precisely tuned to detect the presence of invaders, and as the central effector system, bridging the innate with the adaptive immune responses, it is not surprising that all bacteria entering our body hold it on gunpoint. Therefore, in the first large part of this thesis I will summarize how this system works and why it is so vital for our immunity. You will find out how pathogens are detected, marked for removal and killed. You will also see how complement is bridged with other elements of our immunity.

In the second part we will move on to specific examples of complement-bacteria interactions. Here you will find out how bacteria try to manipulate complement activation and why this system has a double-edged sword nature during infections. The major part of this thesis is devoted to bacteria of the human oral microbiome, involved in periodontal disease. Unlike other human commensal microbiomes, the oral one will cause a disease in majority of people during their lifetime. Therefore, I will summarize different aspects of this common and fascinating disease. You will also find more details in papers I and III-VI, which show the following bacterial players and their survival tactics with complement in focus:

Porphyromans gingivalis (**paper V**), classical periopathogen, one of the most well studied species, recently elevated to the rank of 'the keystone pathogen' of oral bacterial community, as demonstrated in few elegant studies. Lots have been said about it, but we will take a closer look how it interacts with host regulator of complement, CD46 found on oral epithelium, and what triggers are pulled as a result.

Tannerella forsythia (**paper I and IV**), a modest and slightly neglected partner in crime of *P. gingivalis*, correlates equally well with periodontal disease. Its fastidious growth requirements made many scientists discouraged, resulting in a lot lower press record. Yet, we will defend its honor as a major periopathogen by demonstrating that it is well equipped with factors (proteases) inhibiting innate immunity system (complement and phagocytes) and contributing substantially to the immunopathogenesis of the diseased plaque.

Prevotella intermedia (**paper III**), minor sister of the other periopathogens, yet trying hard to keep up a decent position in the family. It is known to produce proteases targeting complement, however here we will focus on its ability to steal host complement inhibitors in order to disguise itself in the protective coat.

Filifactor alocis (**paper VI**), compared to aforementioned periopathogens, is found in very low abundance in oral microbial community. This novel Gram-positive rod is repeatedly identified in periodontal lesions using DNA-based methods. Therefore it has been suggested to be a marker for periodontal deterioration. Knowing that innate immunity, in particular complement, is strongly implicated in periodontitis, we will explore the potential of *F. alocis* to trigger and evade this system.

In another paper (**II**) included in this thesis, we will also take a closer look on a human pathogen, ***Staphylococcus aureus***, who similarly to the pathogens above, is rather an opportunist in nature. However, compared to slow but persistent joint action of oral bacteria, this one sits quietly on the host skin or in the nasal cavity, and patiently waits for an occasion to rapidly demonstrate his deadly power just by himself. In **paper II** we will look on the panel of staphylococcal proteolytic enzymes, and show how they contribute to its astonishing potential to evade host immunity.

Our immune system

Innate and adaptive immunity

The innate immunity is evolutionary ancient, fast responding system. In the majority of animals the natural barriers (skin, mucosa) and the innate immunity are substantial to provide full defense against invaders. In vertebrates however, additional adaptive immunity has evolved, to complement the innate defense mechanisms. Both innate and adaptive immunity distinguish between self and non-self, but they differ in the mechanism of this recognition. The innate immunity utilizes genetically fixed receptors and secreted proteins that recognize common features of whole groups of pathogens, and a subsequent scenario of activation is the same, every time certain pathogen is encountered. The adaptive immunity utilizes somatic gene arrangements to generate extremely specific receptors and antibodies. The first time new pathogen comes in the body, this adaptive response is delayed, as it takes a couple of days for full development of protection, and during that time only innate immunity controls the infection. Next time however, the body is well prepared for a repeated challenge, with already formed specific antibodies and memory cells – the adaptive immune response is much faster and greatly enhanced. Yet, initiation and subsequent direction of adaptive response still depends on the innate one, so adaptive immunity can not provide full protection on its own, like the innate does in many simpler organisms. This is manifested by the fact that individuals lacking certain components of the innate immunity are highly susceptible to some infections, despite intact adaptive immune system. Let's then see more in detail what constitutes different arms of our innate immunity and how they work together to keep us healthy.

Physical barriers

An average person is covered by about two square meters of skin, that when intact, protects us against bacteria, viruses and parasites. We are however linen on the

inside with much larger area of mucous membranes (covering the lining of the mouth, nose, and eyelids and the whole digestive, respiratory and reproductive tracts), which needs to be defended as well – in total about 400 square meters. The constitutive innate immune mechanisms of the skin and mucosal epithelia consist of commensal microorganisms that occupy niches suitable for bacterial growth, and cell layers that provide physical barrier and secrete chemical barrier components – antimicrobial agents. All internal mucous epithelia produce viscous fluid called mucus, rich in glycoproteins (mucins), preventing microorganisms from adhering to the epithelium or making them be expelled out (like in lungs). Other secreted antimicrobials include enzymes, such as lysosyme in tears (which can digest bacterial cell walls) and antimicrobial peptides, such as defensins, cathelicidins, and histatins (which can directly lyse bacteria). In addition, the cells in both skin and mucous epithelium express pattern recognition receptors (PRRs), which allow discriminating between harmful microbial pathogens and "self", providing an integral key feature of immune system.

TLRs and NODs/NLRs

Aforementioned PRRs include three families of molecules. Toll-like receptors (TLRs) recognize their ligands on either the extracellular surfaces or within cell membrane invaginations, endosomes [4] (Fig. 1). NOD-like receptors (NLRs) are intracellular, cytoplasmic sensors of pathogens [5] (Fig. 1), similarly to the third group, retinoid acid–inducible gene–1 (RIG-1)-like receptors, cytosolic helicases that primarily sense viruses [6]. Historically, TLRs were the first to be discovered in 1997, with a finding of toll protein in a fruit fly *Drosophila melanogaster* [7]. This knowledge was further expanded in 2000, when intracellular system of pathogen recognition was discovered, based on receptors resembling disease resistance proteins in plants [8, 9].

All PRRs recognize conserved molecular structures consistently found on pathogens (pathogen associated molecular patterns, PAMPs), and provide constant surveillance of the extracellular and intracellular environments. They are very economical, as each PRR can detect a whole group of pathogens by recognizing their essential component that is not so easily mutated, such as lipopolisaccharide (LPS) of Gram-negative bacteria or single-stranded RNA of many viruses. This way our body does not need to make specific receptors for each

type of different microorganisms, since most of them are well covered in quite limited repertoire of innate PRRs. To date, 10 human TLRs have been described, recognizing distinct types of microbial structures. In the family of NLRs, nearly 20 members have been identified so far [10]. Both families are mainly expressed on epithelial barriers, as well as phagocytic cells, patrolling all tissues, macrophages, neutrophils and dendritic cells (DCs).

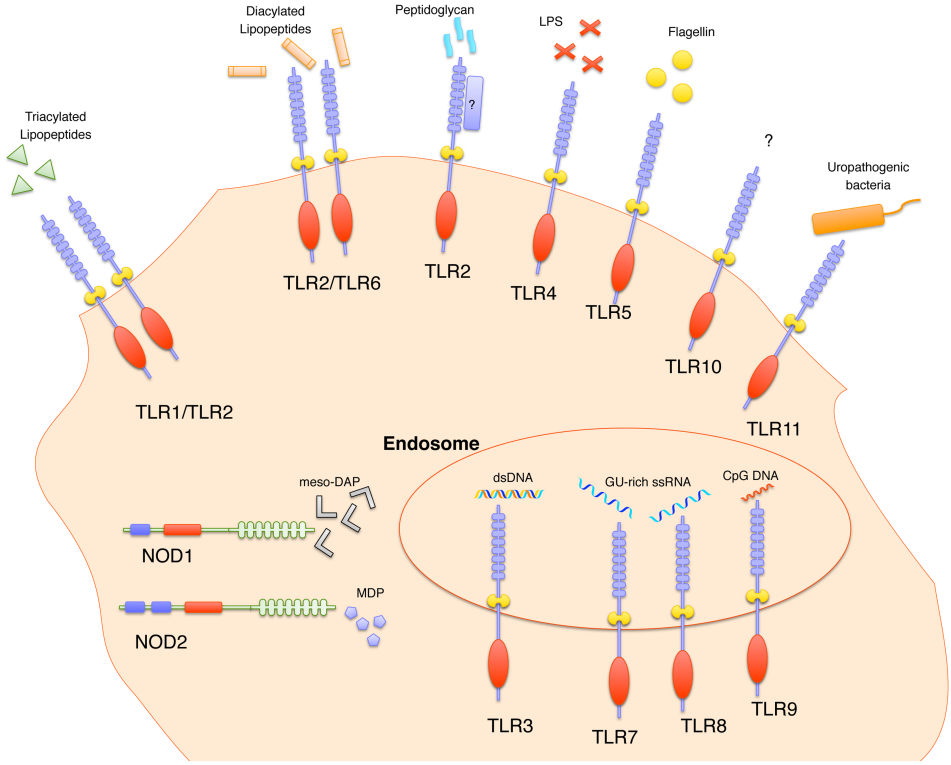

Fig.1. TLRs and NLRs structures and their known ligands. TLRs detect extracellular pathogens or those engulfed within endosomes, while the NLR family members sense the invaders in the cytosol. Leucine-rich repeat motifs (marked in purple in TLR and green in NLR structure) recognize various PAMPs. LPS, lipopolysaccharide; meso-DAP: meso-diaminopimelic acid; MDP: muramyldipeptide. This schematic drawing was prepared using the Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

They have a dual role of recognizing the invader and activating fast innate immune response against it, as well as alarming the adaptive immunity [11]. Leucine-rich repeat motifs, present in both TLRs and NLRs, are believed to be a scaffold for recognition of various PAMPs. Upon pathogen recognition, different adaptor

proteins bind to the TLRs cytoplasmic domains or NLRs N-terminal effector domains (exposed after activation) and facilitate propagation of the signals to downstream kinases and transcription factors. This ultimately leads to the induction of immunoregulatory genes (that activate or suppress the innate immune and inflammatory response, such as cytokines), and likely regulation of cell death and survival [9]. Distinct localization of different TLRs and NLRs on and in the cells and their conjugation with different adaptor molecules ensures that both membrane and cytoplasm of the cells are well guarded and the cell responds adequately to any microbial stimuli. To make the system even more precise, there is also substantial level of crosstalk between these two families and their signal transduction pathways [8].

Complement system and its activation pathways

A number of essential PRRs do not remain associated with the cell that produces them, but circulate in blood and body fluids, in which the cells are immersed. Among them, the complement system proteins, synthesized mainly in the liver and present at high concentrations in blood and all body fluids, are the most essential components of our innate immunity. The complement system is very old and conservative defense mechanism - even sea urchins, which evolved about 700 million years ago, have one [12]. Human fetus begins to synthesize complement components as early as 8 week after conception [13]. Every pathogen that breached epithelial barriers and initial antimicrobial defenses encounters complement and has a hard time to escape. In the absence of infection, complement proteins circulate in an inactive form, however as soon as they detect pathogen (carrying certain PAMPs), they become activated. This happens very fast, as complement proteins are present at very high concentrations, and in addition they are organized in cascades. The recognition of pathogen by initial complement complexes sets off a proteolytic cascade in which one activated complement protein triggers the activation of the next complement protein in the sequence. The activation of complement can be initiated via three distinct pathways termed classical, lectin and alternative, depending on the molecular stimuli (Fig. 2). Importantly, complement is a key player in many pathological processes as it recognizes altered self (e.g. apoptotic and necrotic cells, reperfusion injuries,

transplants). We will focus however on the pathogen recognition and the following effector mechanisms leading to its removal.

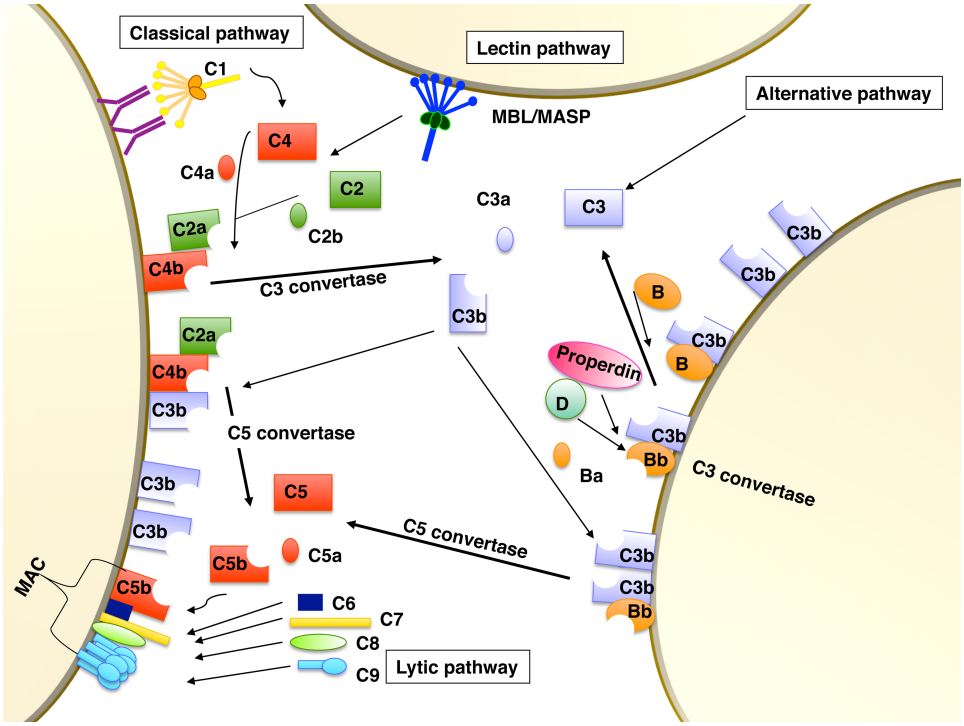

Fig.2. Complement activation pathways. Complement can be activated via three distinct pathways termed classical, lectin and alternative, depending on the molecular stimuli (see more details in the text).

The Alternative Pathway

The alternative pathway of complement activation is the oldest one, as it first appeared in the ancient invertebrates, sea urchins. These primitive species have been demonstrated two possess two essential complement components, C3 and factor B [14, 15]. The more evolved species, tunicates, possess components of the lectin pathway [16], whereas the classical one appeared the latest, with the emergence of immunoglobulins (Ig) in the cartilaginous fish[17]. To date, more than 50 complement proteins have been described. But still the crucial one is C3 that circulates in the blood in an inactive form, with all the binding sites hidden until

the molecule becomes activated [18]. In particular, it contains very active thioester bond that becomes exposed upon activation and can attach to either of the two chemical groups: amino or hydroxyl groups [19]. Cells as well as microbes are made of proteins and carbohydrates, both rich in the aforementioned chemical groups and therefore providing great target surfaces for activated C3. C3 is a very abundant protein in serum, reaching between 1 to 1.5 mg/mL. In the body there is a constant low level of spontaneous C3 activation due to the hydrolysis of the thioester bond in the native molecule – as a result hydrolyzed C3, C3(H_2O) is formed (tick-over theory) [20]. This 'steady state' activation facilitates fast propagation of complement cascade upon pathogen invasion. However not only microbial, but any surface that is not protected, will be targeted by complement. Therefore our organism has a whole set of complement regulators and inhibitors, which quickly inactivate bound C3 and prevent further propagation of complement cascade. This situation changes when C3(H_2O) binds to a pathogen surface, where it is stabilized, and associates with another human complement protein, serine protease factor B, in the presence of Mg^{2+} ions. The latter, after binding to C3(H_2O) becomes accessible to another serine protease factor D, which cuts off small part Ba, yielding C3(H_2O)Bb complex [21]. This is where the danger begins for a pathogen, as C3(H_2O)Bb is an initial alternative pathway convertase, that starts to cleave more C3 molecules into small C3a and large C3b fragment containing metastable thioester group. Most of the fluid-phase C3b as well as C3(H_2O) becomes inactivated by plasma serine protease factor I, in the presence of cofactors available in plasma or on the cell surfaces [22]. However, the C3b molecules bound to the bacterium recruit more factor B, and together, stabilized by properdin, they form more alternative pathway convertases, C3bBb, capable of cleaving more C3 molecules [23] (Fig. 2). This is how the most important function of alternative pathway is fulfilled – massive amplification of the complement activation [24]. The vast majority of C3b molecules bound to the activating surface are generated by the alternative pathway convertase, even if initially the activation was started by the classical or lectin pathways [25]. In addition, nascent C3b molecules initiate positive feedback loop reaction, whereby each new C3b can act as a nucleus for another C3 convertase, or can bind to already formed convertases and give rise to the complex of C3bBb3b - C5 convertase of changed specificity toward an abundant serum protein C5. C5 is turned over similarly to C3, into a small (C5a) and large (C5b) fragment, initiating self-assembly of MAC, membrane attack complex (see Fig. 2 and Complement effector mechanisms). The alternative pathway is not only

initiated spontaneously, by C3 hydrolysis, but also parallel model of activation has been proposed, in which properdin was shown to recognize microbial surfaces directly and mediate convertase formation [26, 27].

The Classical and Lectin Pathways

The antibody-triggered pathway of complement activation was discovered first (hence named classical), in the late 1800s with the recognition that killing of bacteria by specific antibodies required an additional, nonspecific thermolabile factor present in serum [28-30]. Activation of this pathway is primarily dependent on immunoglobulins (IgM and IgG) present in immune complexes (IC; antibody-antigen, i.e. antibody bound to an antigen from a pathogen surface) [31, 32]. Immunoglobulins are considered to be mainly part of the adaptive immunity and they allow an organism to remember and recognize a pathogen that it has previously encountered. Exceptions from these are "natural" IgM antibodies expressed by specific peritoneal B1 cells, which bind to lipid and carbohydrate antigens present on microbes. These are usually of low affinity but compensate by being excellent activators of complement. Furthermore, the classical pathway is not only activated by antibodies, and therefore it does not dependent on our adaptive immunity system, but certainly links both immunity arms together. In addition to antibodies, classical pathway recognition component (C1q) can also recognize a great variety of structurally different target molecules, such as pentraxin-3, C-reactive protein [33], bacterial porins, e.g. OmpK36 on *Klebsiella pneumoniae* [34], mycoplasma, parasites, certain viruses (including HIV), lipopolisaccharides, apoptotic cells, as well as several proteins (including β-amyloid), carbohydrates, lipids, polyanions, and other compounds. The majority of C1q-binding sites on different structures contain charged motives and the interactions are ionic in nature [35].

C1q is a hexameric protein formed by six monomers composed of A-, B- and C-chains – these three chains polymerize together to form a triple-helical structure similar to collagen [36]. Six of these structures assemble with their N-terminal tails to form a molecule with one thick 'collagen-like stalk' and six globular heads protruding toward the C-termini, which appears in electron microscopy like a bunch of tulips [37] (Fig.3). C1q molecule associates in a Ca^{2+}-dependent manner with a tetramer of two serine proteases C1s and C1r (C1s-C1r-C1s-C1r), staying in their inactive zymogen form until C1 complex activation [38]. The C1q heads

contain charge-patterns responsible for the binding of the molecule subunits to a target surface. This binding leads to a conformational change in the complex, which activates the serine protease C1r that cleaves C1s (Fig. 2) [39]. Once activated, one serine protease C1s molecule cleaves many C4 components into C4a and C4b (amplification loop) [40]. The cleavage of C4 results in exposing of a thioester bond (so far buried in C4, similarly like in case of C3) on C4b structure, and C4b can then attach covalently to microbial surfaces via their surface hydroxyl or amino groups [41]. C2 is another serine protease cleaved by C1s, and the C2a fragment binds to surface-bound C4b [40]. The C4b2a complex constitutes the classical pathway C3 convertase, which promotes cleavage of C3 into C3a and C3b. C3b later joins with C4b2a (the C3 convertase) to yield classical C5 convertase (C4b2a3b complex).

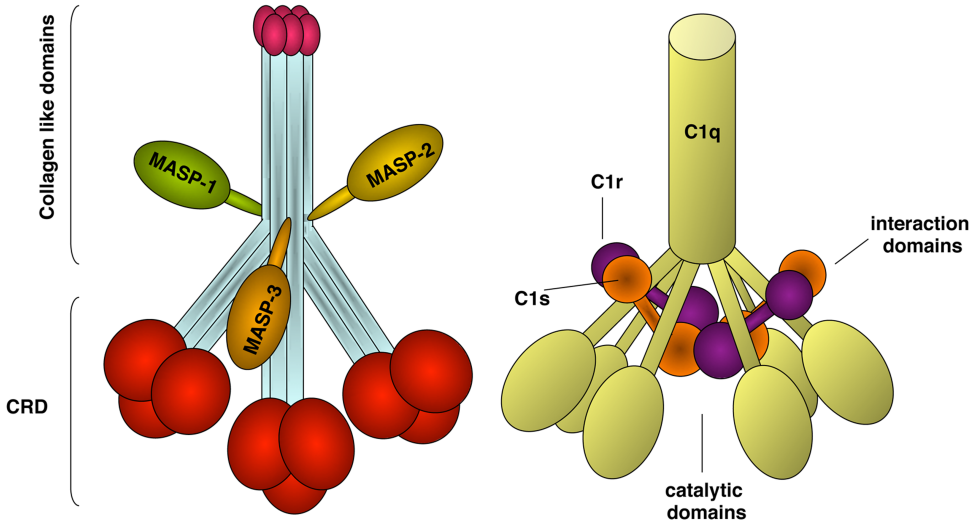

Fig.3. Structure of MBL and C1q. MBL and C1q are composed in a similar way. MBL is build of subunits containing a collagen-like domain and a carboxy-terminal carbohydrate-recognition domain (CRD). The trimeric form of MBL is shown but it may form more oligomeric forms. MBL subunits are held together by disulfide bonds and the collagen like domains form a triple helix. MBL associates with serine proteases MASPs. The classical pathway initiator complex is assembled from oligomeric recognition protein C1q and protease components, tetramer (C1s–C1r–C1r–C1s). C1q is a hexameric protein formed by six monomers - a triple-helical structures similar to collagen. These structures assemble with their N-terminal tails to form a molecule with one thick 'collagen-like stalk' and six globular heads protruding toward the C-termini.

Similar events as in the classical pathway take place upon lectin pathway activation, yet the nature of initial pathogen recognition and the actual PRRs are different. In the lectin pathway the recognition function is mediated by members of either of the two families, collectins (mannose-binding lectin, MBL or collectin K-1) or ficolins (ficolin-1, -2 or -3). Both of the families recognize various pathogens, but also apoptotic and necrotic cells, and other changed self-structures. MBL was the first protein described to activate this pathway. It is an oligomer composed like C1q – three polypeptide chains polymerize together to give rise to subunits, that further associate in 2-6 to form characteristic structures of collectins: N-terminal 'collagen-like stalk' region and C-terminal heads (for a review see [42]) (Fig.2). Through its multiple C-terminal C-type carbohydrate recognition domain (CRD) in the heads, collectins recognize specific carbohydrates groups, such as mannose and N-acetyl-glucosamine (GlcNAc) on microbial surfaces [43]. They have a weak affinity toward monosaccharides but strong one toward sugars organized in a pattern fitting with simultaneous binding of multiple CRDs, as it is the case for many bacterial surfaces [43]. Collectin K1 resembles MBL in structure and can bind to mannose and fucose in Ca^{2+}-dependent manner as well as many microorganisms [44].

Both, collectins and ficolins, individually associate with MBL-associated serine proteases, five of which have been described: MASP-1, -2 and -3 (MASPs), MAp44 and MAp19. The MBL/MASPs complex resembles C1 complex in both structural and functional ways (Fig.3). After collectin/ficolin binding to microbial surface moieties, the MASP proteases are activated and cleave C4 (MASP-2) and C2 (MASP-1 and -2) [45]. Hence, the lectin pathway activation results in the formation of classical pathway convertases C3 (C4b2a) and C5 (C4b2a3b). MASP-1 plays an essential role in the activation of a zymogen form of MASP-2 [46], and has also been postulated to be engaged in a direct C3 cleavage [47]. The role and activation of MASP-3 are still unconfirmed, yet it was proposed to act as a potential negative regulator of lectin pathway by competing with other MASPs [48]. Two truncated splice variants of MASP genes, MAp19 and MAp44, have also been suggested to attenuate lectin pathway activity. Ficolins also associate with MASPs and their overall structure is similar to C1q and MBL. They are composed of a short N-terminal region with one or two cysteine residues, followed by a collagen-like domain, a short link region, and a subsequent fibrinogen-like domain [49]. Again, the binding of collagen-like domains allows formation of trimeric subunits, which then assemble together into active oligomers through the binding

of subunits via disulfide bridges at the N-terminal regions [49, 50]. Ficolins recognize acetylated carbohydrates, through the C-terminal fibrinogen-like domain [49, 51, 52]. They bind mainly to the fitting patterns of the terminal GlcNAc residues, present in surface polysaccharides of a variety of pathogens, but they can also recognize acetylated albumin. Three members of the ficolin family have been described in humans, such as ficolin-1 (M-ficolin), -2 (or L-ficolin) and -3 (H-ficolin or Hakata antigen) [53-55]. Ficolin-2 and -3 are produced in the liver and found in serum, whereas ficolin-1 is mainly produced by peripheral blood leukocytes and bone marrow cells and found on their surface, but its presence in serum has also been reported. Collectins and ficolins have rather low serum concentrations (between 0.5 to 20 μg/mL, depending on the protein and the individual).

Complement effector mechanisms and its instructive functions in generation of adaptive immunity

Complement is an economical system not only due to its broadly specific PRRs recognizing molecular patterns shared by different microorganism, but also due to meticulous use of all activated components, generated by the cascades. All cleavage products of complement participate in different effector functions, aimed on incapacitating and destroying intruders and activating various immune responses – nothing is wasted in this system (Fig.4).

The MAC formation – direct lysis of microorganisms

All three distinct complement pathways converge to the formation of C5 convertase, an enzyme complex that activates the terminal pathway, leading to the end product of complement activation, MAC (Fig.2). The assembly of MAC is initiated when labile C5b molecule binds to C6 component to generate C5b-6 dimer. This dimer then interacts with C7 to produce C5b-7 trimer. The conformational changes within this complex result in an exposure of hydrophobic sites promoting high affinity for lipids [56]. The hydrophobic contact allows insertion into a membrane of a target microorganism, mediated by non-covalent and relatively nonspecific interactions. The membrane-bound C5b-7 complex serves as a receptor for C8, and the tetrameric C5b-C8 complex already allows some target membrane leakage. However only after C9 polymerization, the full

MAC lytic activity is achieved [57]. Initially, C9 interacts with C8 α-chain. Binding of the first C9 mediates incorporation of additional C9 molecules (n=1-18). The membrane attack complex restructures lipid organization in a target cell membrane thereby altering its permeability and mediating cell lysis. For a long time it was assumed that MAC is relevant for killing of Gram-negative bacteria, whereas Gram-positive species as well as fungi are resistant to lysis due to their thick cell wall. Unfortunately, even within Gram-negative species there is not that many that are successfully eliminated by complement-mediated lysis, owing to multiple mechanisms of complement evasion employed by different pathogens. Nevertheless, the role of MAC is perhaps the best recognized for *Neisseria*, since individuals with genetic deficiencies in terminal complement components are prone to invasive infections of *Neisseria meningitides* and *N. gonorrhoeae* [58]. Paradoxically, despite their thick protective cell walls Gram-positive bacteria invest extreme potential into controlling terminal pathway, and as long as the initial step of C5a generation constitutes obvious threat for all pathogens (see *Anaphylatoxins*), the relevance of MAC inhibition by these bacteria remains unclear.

Anaphylatoxins – mediators of inflammation

Even though complement cannot destroy most of the pathogens on its own, it facilitates their uptake and destruction by professional phagocytes, which next to complement constitute another major arm of innate immunity. Most important of them are macrophages and neutrophils. Macrophages are long-living cells present below the surface of all areas in the body, which are exposed to the environment, e.g under the skin, in the lungs or around the intestines. They watch for invading microbes and if they spot one, they provide initial inflammatory cytokines (interleukin-1, IL-1 and tumor necrosis factor-α, TNF-α), digest a pathogen and display its fragments to adaptive immune cells (hence macrophages are antigen presenting cells, APCs). Neutrophils circulate in the blood in large numbers (about 20 billions in total) and since all the parts of the body are laced with blood vessels, they can quickly bring reinforcement in the areas that are under attack. They just need to get informed that something is going on and within half an hour they will exit the blood and become activated. Neutrophils utilize multiple killing mechanisms, allowing killing of the pathogen both intracellularly (by engulfment, phagocytosis, respiratory burst, degranulation

of vesicles filled with multiple AMPs and hydrolytic enzymes) and extracellularly, via formation of NETs (neutrophil extracellular traps, web-like structures of genetic material with various antimicrobial compounds attached along, that can trap and kill the target). Neutrophils are short-lived blood leukocytes that in the absence of infection or inflammation usually go into apoptosis within 1 day from exiting bone marrow. Pro-inflammatory signals extend this halflife up to 5 days but they will anyway die while performing their antimicrobial functions, contributing to pus formation inside infected tissues. Simply, once all the detrimental processes in the neutrophils are unleashed, it is just safer for a body to get rid of highly activated, possibly raptured cells and produce the new ones. In addition to phagocytes, there is a number of auxiliary immune cells that help to mediate the inflammation. These cells include basophils and mast cells that have granules containing a variety of mediators, released when the cells are triggered. Mast cells populate submucosal tissues and lie close to blood vessels, and some of their mediators act on the cells in the vessel walls. Basophils are functionally similar to mast cells, but are mobile, circulating cells. Parasitic infections are counteracted by another group of blood cells, eosinophils. Innate immune system can also directly kill cells infected with certain pathogens (especially viruses), and this function is provided by natural killer (NK) cells, which are particularly important in early stages of viral infection. However, since one virus-infected cell can produce thousands of new viruses, more potent weapon is required to contain the infection, and this is one of the main reasons why vertebrates developed adaptive immunity, with antibodies that can lock onto the outer surfaces of viruses and prevent them from infecting new cells.

Complement provides major alarm signals and activators of both type of phagocytes, as well as all auxiliary immune cells. In the cleavage of C3 and C5, smaller fragments (C3a and C5a) are cut off from native molecules and released to the fluid phase. They constitute very potent anaphylatoxins – molecules with both chemoattractant and immune-modulatory functions that form a concentration gradient attracting different cells to the infection site. As they are extremely potent, carboxypeptidases present in serum and tissues quickly convert and incapacitate most of C3a and C5a by removing their C-terminal Arg residue into desarginated C3a (C3adesArg) and desarginated C5a (C5adesArg)[59]. Both anaphylatoxins act through G protein-coupled receptors, C5aR and C3aR, respectively, which contain seven transmembrane segments distributed throughout the whole protein. In the case of C3aR, only C3a (and not

C3adesArg) has been reported to bind and activate the receptor [60]. In contrast, both C5a and C5adesArg have been shown to trigger activation of C5aR; however, C5a has about 100-fold stronger affinity for C5aR than C5adesArg, and the latter retains only about 1-10% of C5a activity [61]. Both receptors are found on macrophages and neutrophils, eosinophils, basophils, mast cells and, at different levels, on non-myeloid cells, including bronchial and alveolar epithelial cells, vascular endothelial cells, Kupffer cells and stellate cells (specialized phagocytic cells involved in the removal of old erythrocytes and ICs), sinusoidal epithelial cells in the liver, as well as astrocytes and microglial cells in the brain [62].

A group of cells with extremely high C5aR levels are neutrophils, and that is why these cells respond to very low concentrations of C5a by chemotaxis – fast movement toward the site of infection along the gradient of C5a. Upon C5aR stimulation rapid changes take place in the activated neutrophils, including cytoskeletal remodeling, upregulation of adhesion molecules allowing for the attachment to the endothelium and exiting the capillary, upregulation of complement receptors (CR1, CR3 and CR4) recognizing complement fragments coating a pathogen, granules release, and synthesis of cytotoxic, reactive oxygen species [63]. In addition, C5a prolongs neutrophil survival [64], and C3a contributes to the induction of neutrophil respiratory burst [65]. To assist in the process of immune cells recruitment and inflammation, C3a and C5a regulate vasodilation, increase the permeability of small blood vessels and induce smooth muscle cells contraction [66]. Moreover, C3a and C5a can attract and activate the mast cells and make them release inflammatory molecules such as histamine and the cytokine TNF-α, which cause similar effects [67]. C5a is a very potent chemoattractant for macrophages [68] and stimulates them to release pro-inflammatory mediators (IL-1 and TNF-α) [69], whereas C3a triggers oxidative burst in these cells [70]. Eosinophils, which are involved in protection against parasites, also respond to anaphylatoxins (for a review see [71]). C5aR triggering in the liver initiates the production of acute phase proteins involved in inflammation, such as C-reactive protein, the first identified PRR [72]. This way complement initiates the inflammation both locally, but also systemically. A third receptor of anaphylatoxins, C5a-like receptor 2, C5L2, has been described that binds C5a and C5adesArg, but its role is so far unclear and controversial [73].

The larger fragments of complement cleavage products, C3b and C4b, not only constitute parts of convertases, but on their own act as opsonins – coat whole bacteria (viruses, fungi), which are then marked for recognition by phagocytes and other immune cells, carrying receptors for these fragments. Both C3b and C4b are actually further processed by serum factor I and its cofactors: C3b to iC3b, C3dg and C3d, and C4b to C4c and C4d - those fragments are no longer able to participate in complement amplification, yet function via complement receptors (CR) 1-4.

CR1 is a widely expressed receptor with an affinity toward C3b, C4b and to a lesser extent, iC3b. It is found on blood erythrocytes as well as monocytes, macrophages, neutrophils, NK cells, B cells and some T cells. It can also bind C1q, which in addition to its PRR function serves as an opsonin, of particular importance in the removal of apoptotic cells [74]. CR1 plays a crucial role in the removal of soluble antibody-antigen ICs coated with C3b from the circulation, which could otherwise deposit in small capillaries or kidney glomeruli and lead to their destruction by inflammatory responses. C3b-bearing ICs bind to erythrocytes via CR1 and are transported to the liver for removal by Kupffer cells [75]. Erythrocytes compete with phagocytes in binding ICs via CR1, hence they prevent their inappropriate activation. In addition CR1 also serves a cofactor for factor I – accelerates conversion of C3b to C3dg, thereby facilitating attachment of the opsonized target to another complement receptor CR2. This way pro-inflammatory status of ICs stimulating monocytes and neutrophils changes toward a potential B cell stimulus via CR2 [76]. CR1 on phagocytes allows them to recognize foreign target coated in C3b, iC3b and C4b molecules, engulf it and destroy it, however triggering phagocytic activities via CR1 usually requires additional signal provided by other opsonin (e.g. C1q, MBL) or fMLP (N-formyl-methionyl-leucyl-phenylalanine), strongly chemoattractive peptide derived from various bacteria [77].

More potent in stimulating phagocytes are CR3 and CR4 (Fig.4), both present on neutrophils, monocytes/macrophages, as well as eosinophils, basophils, NK cells, Kupffer cells, microglial cells and platelets. CR3 is also found on follicular dendritic cells (FDCs). CR3 and CR4 both bind iC3b in Mg^{2+}-dependent manner. Stimulation of neutrophils and monocytes via these receptors results in phagocytosis, production of ROS and, in the case of neutrophils, release of the

specific granules. CR3 on eosinophils takes part in the IgE antibody-dependent cytotoxicity towards parasites and their production of ROS. CR3/4 play roles not only in the phagocytosis but along with LFA-1, facilitate leukocyte adhesion to endothelial cells and their subsequent extravasation and migration to the sites of inflammation [78, 79]. A novel receptor of C3b and iC3b, CR of the Ig superfamily (CRIg), has been recently described to contribute to the clearance of pathogens and apoptotic cells. CRIg is expressed by a subset of tissue resident macrophages [80], but it is not only a phagocytic receptor. Binding of CRIg to C3b also inhibits both alternative pathway C3- and C5-convertase activity [81].

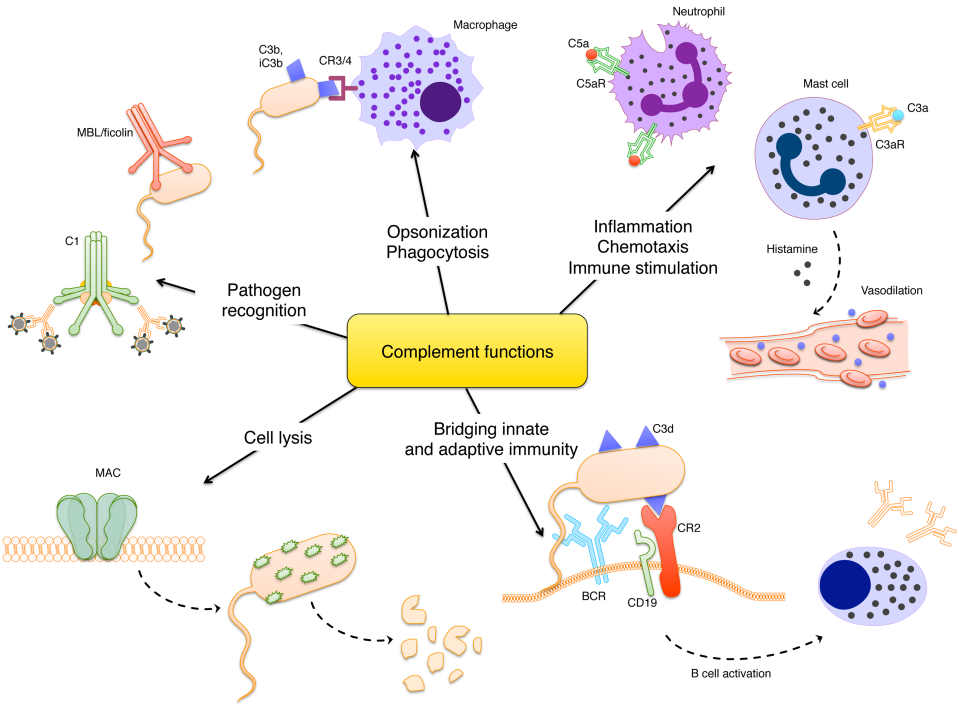

Fig.4. Complement functions. Complement fulfils various essential functions in the organism. It recognizes pathogens and leads to their destruction by direct complement-mediated lysis (MAC), or by facilitating their uptake by phagocytic cells. It activates various immune cells and stimulates their migration to the infection sites. It also regulates the permeability of blood vessels. Finally, it orchestrates the adaptive immunity. This schematic drawing was prepared using the Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

One more complement receptor, CR2, is the key molecule that bridges

complement with adaptive immune responses, and guarantees proper induction of humoral (antibody-mediated) immunity (Fig.4). Given its role, one can expect that it is present on major adaptive immune cells – mature B cells (approximately 8000 per cell), some T cells and FDCs in lymphoid organs. In addition it can be found on pharyngeal epithelial cells, astrocytes and platelets (in humans). To summarize the role of CR2, we have to briefly describe the basis of adaptive immune response. The key cells in this system, T and B lymphocytes (T cells and B cells) are derived from bone marrow stem cells, but T cells then develop in the thymus, while B cells develop in the bone marrow (in adult mammals). Both bone marrow and thymus constitute primary lymphoid organs. B cells express specific antigen receptors (B cell receptor, BCR, immunoglobulin molecules) on their cell surface during their development – each B cell possess specific surface receptor to a particular antigen. B cells released from bone marrow circulate in their naïve form and become activated after encountering their specific antigen that clusters BCRs on their surface and associated signaling molecules. Then they will multiply and differentiate into plasma cells, which produce large amounts of the immunoglobulin (same as the surface one, hence, of the specificity toward initial activating antigen), but in a secreted form. In order for that to happen, it is not enough to find an antigen that will cluster BCRs. A secondary 'co-stimulatory' signal is required, which is provided either by helper T cell (Th cells; T-cell dependent immunity) or a danger signal (e.g. INF-γ; T-cell independent immunity). Before Th cell can help, it also must be activated. Like B cells, T cells have surface exposed receptors, TCRs, which recognize their cognate antigens. Those antigens need to be presented to them by antigen presenting cells (APCs) – capable of displaying peptides of previously encountered pathogens on specialized surface molecules, MHC I or MHC II, and at the same time providing essential co-stimulation, required for adequate T cell activation. Three classes of cells can serve as APCs, activated macrophages, activated B cells and activated dendritic cells. The latter group of cells is the most important in antigen presentation, they are found all over the body beneath epithelial barriers and all the time they take up extracellular fluid. Normally DCs express low level of MHC but if there is an infection, activating signals will be provided from other cells (such as TNF from macrophages) or from PRRs on the surface of DCs (e.g. TLRs), which will lead to increased ingestion of foreign material and presentation of antigens in the context of MHC. These antigens within MHC are presented to T cells, which recognize them via TCRs, whereas co-stimulation is provided by crosslinking of other

molecules from both types of cells, most commonly B7/CD80 on APC and CD28 on T cell. Activated Th cell will then express CD40L, a molecule matching CD40 on the surface of B cell, and can send a co-stimulatory signal to a B cell recognizing its cognate antigen via BCR.

Since all pathogens are recognized by complement, they are subsequently opsonized with C3b and its derivatives, iC3b and C3dg. These fragments alert not only phagocytes but also adaptive immunity. B cells express large amount of CR2 on their surface so they can recognize an invader opsonised with C3d. If at the same time their BCRs become cross-linked, by binding of several microorganisms or by the repeated patterns of antigens on a single pathogen surface, BCRs and CR2 clamp together on the B cell and the activatory signal is greatly amplified (Fig. 4). CR2 therefore is called B cell co-receptor, as it sensitizes B cells to antigens tagged as dangerous by innate immunity, enhances signaling via the B cell antigen receptor, lowers the threshold of B cell activation and provides an important survival signal, overall facilitating activation of adaptive immunity [82, 83]. CR2-mediated activation of B cells is particularly important in inducing immunity toward pathogens not seen by the immune system before. CR2 also promotes uptake of C3d-coated antigens by B-cells, facilitates antigens processing and enhances antigen presentation by B cells to T cells [84, 85].

Other functions of complement in orchestrating adaptive immunity

In addition to DCs found around the body and presenting antigens to T cells, there is another group of so-called follicular DCs (FDCs). Those cells are found associated with B cells in lymphoid follicles in secondary lymphoid organs (lymph nodes, spleen and mucosal-associated lymphoid tissues – MALT). The FDCs function to display antigens to B cells. Antigens opsonized by complement as well as antibodies (generated later in the battle) are delivered to secondary lymphoid organs via blood and lymph. FDCs express three receptors for C3 fragments (CR1, CR2 and CR3) [86] as well as a receptor for the Fc portion of IgG, FcγRIIb [87]. These receptors allow FDCs to pick up and retain large amounts of opsonized ICs so that they can stay in the organism for a while and be displayed to activated B cells [88]. Cross-linking BCRs by cognate antigens presented by FDCs retains B cells in the lymphoid follicle, activates them and induces their proliferation – so called germinal centers of intensive B cell development and proliferation are formed. B cells proliferating in the germinal centers are very prone to apoptosis

and they die quickly, unless they will receive proper 'rescue' signal. Complement has been shown to play a crucial role in providing anti-apoptotic signal for proliferating B cells via CR1 and CR2 on their surfaces [89], in addition to cooperative signal from activated Th cells (via CD40L). After the period of proliferation some of these B cells become plasma B cells – they leave germinal centers and secrete antibodies to the circulation. Naïve B cells after initial activation produce mainly IgM, of greatest complement activating capacity - it is not a coincidence since complement is still crucial early in the infection. Later when B cells mature they have an opportunity to switch to other antibody classes of other functions: IgG – great opsonins stimulating phagocytes as well as mediating cytotoxicity of infected cells by NK cells, IgA – most abundant in the body, produced by B cells guarding mucosal surfaces, or IgE – bound to mast cells and responsible for reactions to allergens. The class switch is dictated by cytokine environment, e.g. upon INF-γ stimulation B cell will produce IgG3 – effective against bacteria and viruses. Complement receptors on FDCs were shown to provide important signal in this phenomenon [90, 91]. Some B cells do not become plasma cells but undergo somatic hypermutation – mutation in the part of antigen-binding region of the antibody, which increases, decreases or does not affect its affinity. Higher affinity allows proliferating B cells to be stimulated more easily by cross-linking antigens presented by FDCs, and those B cells are rescued from apoptosis. The key role of CR2 in retaining antigens for proper clonal selection and affinity maturation of activated B cells emphasizes the paradigm that innate immunity is essential for regulating adaptive immune responses (for detailed review see [92]).

Regulation of complement

Due to spontaneous activation of alternative pathway as well as everyday exposure to microorganisms from the environment, complement activation goes on in our body on daily basis. To prevent unintended injury of own tissues, our organism uses a complex set of plasma proteins (factor I, factor H, C4BP, C1 inhibitor) and cell-bound regulators (MCP, DAF, CR1, CD59 and CRIg) to restrict complement at all key steps in activation cascades. Most of complement inhibitors are composed of numerous complement control protein (CCP)-domains, of similar 3D fold in different proteins despite quite high differences in

their sequences. During the recognition phase, C1 inhibitor (belonging to the serpin family of protease inhibitors) controls the serine proteases of the classical pathway (C1r, C1s) and the lectin pathway (MASP-1 and -2) [93-96]. The main steps of complement activation, C3 and C5 convertases are at the same time the major check-points in the cascades, regulated by two mechanisms – accelerating decay of convertases, or cleaving soluble and surface-bound C3b and C4b into fragments no longer able to participate in cascades propagation. As mentioned earlier, factor I is responsible for the latter mechanism, but to function properly it requires cofactors enhancing its enzymatic activity. The classical and lectin pathway are regulated by C4b-binding protein (C4BP), the main soluble inhibitor participating in the cleavage of C4b by factor I and capable of accelerating decay of the C3- and C5-convertases [97, 98]. The alternative pathway is controlled by factor H, which accelerates the decay of C3bBb convertase, and serves as a cofactor in C3b proteolysis by factor I [99, 100]. Without factor H spontaneous activation of alternative pathway leads to the consumption of C3 and factor B [101]. The main cell-bound cofactors are MCP (membrane cofactor protein present on all nucleated cells, CD46) and CR1, participating in the proteolysis of both C3b and C4b. MCP synergizes with another membrane-bound protein, DAF (decay accelerating factor) – DAF binds to C3b and C4b and decays the convertases, while MCP facilitates cleavage of this fragments by factor I, resulting in efficient protection of host cells [102]. Host cell surfaces are further protected by the attachment of factor H, which appears to be particularly important for tissues expressing low number of complement regulators or completely lacking them, such as kidney glomeruli basement membrane [103]. In addition, five factor H-related proteins (CFHR 1-5) have been identified, capable of binding C3b and C3d, and supporting factor H in convertase decay and cofactor functions, as well as possessing other activities, e.g. inhibiting terminal pathway (for a review see [104]). Downstream of convertases, MAC formation on host cells is prevented by CD59, widely expressed molecule binding C8 and C9 and inhibiting pore formation. Furthermore soluble proteins clusterin and vitronectin bind to the C5b–7 complexes and prevent their insertion into cell membranes.

The combination of all various integral surface-attached and fluid phase inhibitors ensures that host cells and biomembranes are kept intact [105, 106]. If complement cascade becomes deregulated, this leads to immunopathology and autoimmune diseases. Such situation can occur when a gene encoding complement inhibitor is mutated, resulting in decreased or dysfunctional protein

expression. Even small changes in a single inhibitor can disturb the whole regulatory network and lead to complement attack at self-surfaces. This is particularly pronounced for factor H and factor I – if any of them is missing or entirely dysfunctional, the alternative pathway is vigorously activated and leads to secondary complement deficiency via overconsumption of C3 and other components. On the other hand, even discreet heterozygous mutations in factor H and factor I can lead to diseases such as atypical hemolytic uremic syndrome or age-related macular degeneration [107, 108]. Taken together, complement inhibitors are vital for keeping homeostasis of the organism dealing with bacteria every day.

The fast inhibition of complement is provided due to the fact that factor I circulates as an active protease in plasma and other body fluids – it can directly process C3b and C4b into inactive fragments, yet to ensure specificity they need to be bound to a cofactor such as factor H. Factor I, like other complement proteins, is synthesized mainly in the liver, as a multidomein protein composed of two chains (heavy and light) linked together via a disulfide bridge [109]. The serine protease domain is contained within the light chain [110]. Most of the plasma serine proteases, including complement proteins and coagulation factors, circulate as inactive proenzymes and become activated via a cleavage at the N-terminal part of serine protease domain, upon exposure to a certain trigger. Once activated, they are quite rapidly inhibited by endogenous protease inhibitors such as α-2-macroglobulin or the serpins. Factor I, together with another complement serine protease, factor D, are exceptions from this rule, as they are produced already in the cleaved and active forms. Yet, they do not exhibit any unspecific activity and do not react with any endogenous inhibitors. Their protease domains require further conformational changes in order to reach optimal activities [111]. This happens upon the binding of these serine proteases to their substrates contained within larger protein complex, such as C3b bound to factor H for factor I, or C3b bound to factor B for factor D [111].

Two major cofactors increasing the affinity of factor I to C3b and C4b and its enzymatic activity circulate in plasma at high concentrations. C4BP is the largest physiological complement inhibitor, consisting (in its dominant form) of seven identical α-chains and one β-chain linked together in one central core at their C-termini by disulfide bridges [112]. All C4BP molecules containing β-chain are associated with vitamin K-dependent, anticoagulant protein S [113]. In addition to the inhibition of classical and lectin pathway, C4BP has been shown to act as a cofactor in the degradation of C3b in fluid phase, thereby inhibiting the

alternative pathway [114]. Yet, the major inhibitor of the latter pathway is factor H, synthesized as a single polypeptide chain composed of 20 CCP-domains. While C4BP functions mainly as a soluble inhibitor, factor H inhibits complement both in fluid-phase and at cell membranes. The carbohydrate content of host membranes allows factor H to distinguish self-surfaces from a pathogen. Factor H has an affinity for glycosaminoglycans and terminal sialic acid of host cell membrane glycoproteins and in the vicinity of these molecules its binding to C3b deposited on host surfaces is increased [115, 116]. In contrast, if C3b is bound a bacterial cell wall, factor H has a low affinity, but instead binding of factor B is preferred, promoting alternative pathway activation. Unfortunately, human pathogens evolved to modify their surfaces in ways allowing them to acquire well-accessible host complement inhibitors. They can increase the content of sialic acid in their cell walls or express surface molecules capable of binding C4BP, factor H or factor I [117, 118]. Of note, paper III of this thesis shows that some pathogens can acquire factor I and its cofactors at the same time, for pronounced inhibition of all complement pathways.

Novel functions of complement regulators

Functions of complement inhibitors, especially those bound to the cell surfaces reach out beyond inhibiting complement cascades, and unraveling their novel roles has been an exciting subject of current studies. A particular focus is put on MCP/CD46, which is a transmembrane molecule possessing one of the two (Cyt-1 or Cyt-2) non-homologous cytoplasmic tails, containing signaling motifs. There is growing evidence implicating vital role of CD46 in an impressive number of different cellular activities and functions, comparable to none of other complement inhibitors (for a review see [119]). Importantly, CD46 participates in T-cell mediated immunity and tolerance and is a very potent co-receptor of T cells, providing another direct link of innate and adaptive immune responses. Cross-linking CD46 on naïve human peripheral blood $CD4^+$ T lymphocytes in conjunction with TCR (CD3) induces proliferation greater than stimulation by the classic co-stimulatory molecule CD28 [120]. In contrast to CD28/CD3 mediated stimulation resulting in generation of pro-inflammatory Th cells and enhanced DC activation, CD3/CD46-activated T cells produce high amounts of IL-10, a hallmark of T regulatory type 1 (Tr1) cells [120]. T regulatory cells suppress

bystander T-cell proliferation and are involved in shutting down immune responses after they have successfully eliminated invading organisms, and also in preventing autoimmunity.

Interestingly, expanding number of pathogens chooses CD46 as an adhesion receptor and/or cell entry molecule[121]. Its nearly ubiquitous and relatively high-level expression pattern can allow for infection of large panel of cells. But what pathogen more likely aim on is co-opting one or more of CD46 complement regulatory activities, signaling capabilities, or internalization mechanisms. Alternatively CD46 might play a protective role as a PRR, as demonstrated in the study showing that CD46 binding by pathogens (*Streptococcus pneumoniae* and measles virus) can induce their direction to autophagy pathway [122]. Autophagy is a self-degradative mechanism required at a basal level for intracellular clearance and recycling of cytoplasmic contents, but also constitutes an important innate immune mechanism to control infection. In case of *S. pneumoniae* CD46-mediated autophagy led to its more efficient elimination, whereas consequences for measles virus were not clearly determined [122]. In addition, CD46 has been implicated in the maintenance of intestinal epithelial cell junction and barrier integrity [122]. Further research is necessary to obtain more information regarding CD46 involvement in induction of autophagy in different cell types, as well as its role for infectivity/elimination of various pathogens. In this thesis you will find novel data regarding CD46 involvement in keeping integral gingival epithelial barrier in oral cavity (paper V).

In the light of all functions of complement system described above and many others activities not mentioned here, it is not really adequate to call such a powerful immune defense 'complement', since it does not complement but rather triggers, orchestrates and bridges all our immunity components. Yet, many bacteria find their ways to infect us, proving that none of our defenses are unbreachable. More strikingly, bacteria utilize very clever strategies of complement/immune evasion in order to survive and exploit various niches of our organism. And complement often turns out to have 'double-edged' sword nature in infections and diseases.

Periodontitis forms and prevalence

Periodontitis (peri = around, odont = tooth, -itis = inflammation) is a term used to describe a number of inflammatory diseases affecting the periodontium — that is, the tissues that surrounds and support the teeth. Periodontal diseases are characterized by a progressive loss of alveolar bone, periodontal ligament, and other tooth-supporting tissues due to chronic inflammation in gingival pockets (Fig. 5). In severe cases, the disease can lead to a complete loss of the dentition [123]. The stimulus for the initiation of the disease is the presence of numerous bacterial species that form biofilm (dental plaque) on the tooth surface and interact with host defenses (epithelial barriers, complement, phagocytes). As a result of the complex bacteria-host interactions there is a constant activation of inflammatory response, manifested by the infiltration of immune cells, release of host cytokines, chemokines, proteolytic enzymes and other mediators, which together with bacterial factors, induce the tissue damage.

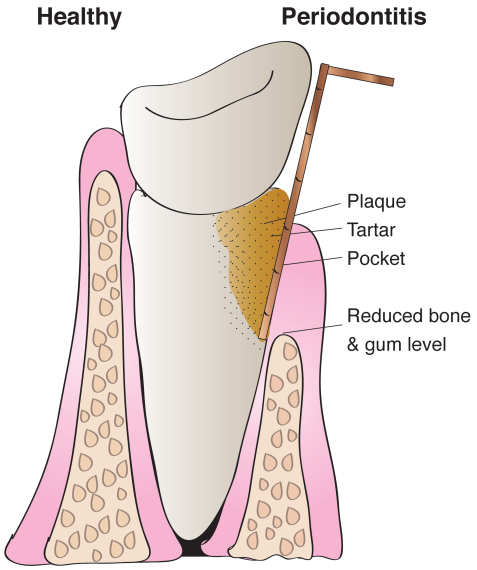

Fig.5. Periodontitis symptoms. Periodontitis is an infection-driven disease characterized by an accumulation of bacterial plaque on the margin between the teeth and the gingiva. The calcified dental plaque forms tartar. Due to the chronic inflammation induced by the bacteria, the teeth supporting structures (gums and periodontal bone) deteriorate, resulting in pocket formation and bleeding. In severe cases, the disease leads to a complete loss of affected teeth.

The mildest form of periodontal disease, gingivitis (gingival inflammation without any bone loss and no pockets deeper than 3 mm) affects more than 50% of adult population and periodontitis (3 or more teeth with pockets ≥ 4 mm) is present in 30% of adults, with approximately 8% of severe cases suffering complete loss of a dentition [124]. The host defenses are permanently engaged in capturing the infection and alarming the organism, so chronic inflammation takes place in the gingival pockets. Neglecting the basal oral hygiene for a couple of days results in gingivitis. That condition can be characterized by the occurrence of bacteria colonizing the teeth through specific adherence interactions, accumulating as a dental plaque due to effective attachment, and causing moderate chronic inflammation. However there is no destruction of teeth-supporting tissues and the condition is reversible by simple, appropriate oral hygiene. When the inflammation extends deep into soft and hard tissues, a periodontal pocket starts to be formed and filled with bacteria, the teeth loosen and there is alveolar bone resorption, the situation becomes largely irreversible and it is known as periodontitis [123]. The periodontitis basically occurs in either chronic or aggressive form. The chronic periodontitis is the most frequent and can be characterized by slow or moderate rate of development. It affects usually adult population and can either be local or generalized (affecting the whole dentition). No matter what the extent of the condition is, the etiology and the mechanisms of tissue destruction seem to be the same in both cases. In contrast, the aggressive form is less common and it concerns mainly young people. Similarly to the chronic form, the aggressive condition can also be local or generalized, but the etiology and the periodontium devastating mechanisms are divergent. The local aggressive periodontitis affects adolescent individuals and is associated with the high level of antibodies against periodontal pathogens. In generalized aggressive form, yet, the humoral response is not marked and the affected population is averagely close to 30 year old. The chronic periodontitis can be characterized by the correlation between the size of tissue destruction and the level of microbial plaque and tartar on the teeth whereas there is no such correspondence in the aggressive form in which usually the biofilm is in fact thinner than in chronic cases [125].

Human oral cavity as a microbial habitat

Human mouth is constantly exposed to the changing environment due to a variety food consumed everyday. The oral cavity possesses one of the largest microbiomes in our organisms, composed of more than 700 bacterial species [126] The mouth has one particularly unique feature in the organism due to the existence of non-shedding surfaces (teeth), providing excellent niche for microbial colonization. In contrast, desquamating mucosal surfaces including tongue, cheeks, palate and gingiva are relatively poorly colonized. As soon as the tooth erupts, or is cleaned, its enamel surface is covered with a matrix of both host (mainly salivary) and bacterial (e.g. secreted proteins) products [127]. The tooth surface itself offers several distinct locations, providing attractive habitat for microbes. Particularly, the areas between adjacent teeth and in the gingival crevice become more heavily colonized, as they are protected from normal removal processes, such as mastication, salivary flow or oral hygiene. In addition, the gingival crevice (the space between the teeth and the gingiva) is bathed with a serum-like exudate, gingival crevicular fluid (GCF), rich in proteins, glycoproteins, nutrients, as well as host defenses, such as antibodies, complement or phagocytic cells [128]. With increased growth of the bacterial plaque and their spreading down the crevice followed with inflammation, the flow of GCF enhances, favoring the growth of those species that are better adapted to these novel conditions. Many of these bacteria are asaccharolytic, obtaining energy from proteins. They often act in concert as consortia performing sequential degradation of protein molecules to methane, H_2S, H_2 and CO_2 [129]. The essential co-factors (like haemin for black-pigmented anaerobes) are obtained from human heam-containing molecules such as haemoglobin, haptoglobin or haemopexin. Freshly cleaned teeth are directly covered with a film of molecules from the saliva, the main fluid that dictates the conditions in the mouth – the temperature 35-36°C and moist at a pH o 6.75-7.25, which are optimal for growth of many microorganisms. The properties of saliva influence the whole ecology of the mouth, due to its ionic composition (buffering pH and participating in the remineralization of the enamel) and organic components providing attachment platform, selective nutritional conditions, or facilitating aggregation of bacterial species [130]. Tooth surfaces contain pits and fissures, allowing some protection of the bacteria. The initial microflora colonizing the teeth is mostly dominated by facultative anaerobic Gram-positive bacteria, especially streptococci. Yet,

advanced plaque has a lot more complex composition and architecture, and contains various species interacting together and acting as one organized community.

Historical perspective on the identification and classification of oral bacteria

The first examination of oral bacteria dates back to around 1680, when Antony van Leeuwenhoek (1632–1723) [131], a Dutch dry goods merchant, observed and described microorganisms isolated from his tooth plaque, with a primitive microscope. In his notebook, he recorded "I didn't clean my teeth for three days and then took the material that has lodged in small amounts on the gums above my front teeth…… I found a few living animalcules." He sketched the observed microbes and, when verified with current knowledge, these drawings represent the most abundant bacteria resided within oral cavity, including cocci, spirochetes, and fusiform bacteria. To date, extensive animal and clinical studies implicated these oral microorganisms with two common diseases, dental caries and periodontitis. Yet, even long before the visual observations of microorganisms, about 5000 BC the Sumerians blamed certain form of living described as 'tooth worm' as a causative agent of dental caries [132]. The actual identification of this 'tooth worm' absorbed a passionate dentist, W. D. Miller, who spent long hours in the laboratory of Robert Koch aiming to find 'the germs' responsible for tooth decay. He published his finding in 1980, in the book entitled '*Microogranisms of the Human Mouth*' and proposed a 'chemoparasitic' theory, according to which in a susceptible host frequently consuming fermentable carbohydrates oral microorganisms convert them into acid, which then demineralises tooth enamel [133]. Yet, limited microbiological cultivation and isolation techniques of the 19th century never allowed him to identify the exact causative agent of dental caries. This finding was partially accomplished in 1925, by J.K. Clarke, who isolated a bacterium from dental caries site, named *Streptoccocus mutants*, and demonstrated its ability to ferment several sugars and produce acidic pH of 4.3 in glucose broth [134]. However, he did not prove that *S. mutants* actually induced caries, what was experimentally showed later in 1960 [135].

Other than dental caries, another major human oral disease is periodontitis, widely

regarded as the second most common disease worldwide. The early studies implicating oral bacteria in the pathogenesis of this disease were conducted in a hamster animal mode. Administration of penicillin inhibited periodontitis in hamster implying an involvement of a bacterial agent [136], while the infectious nature of this disease was shown by demonstration of its transmissibility [137]. For a long time periodontal disease researchers attempted to isolate a specific microorganism from a complex microbial plaque that would be a sole causative agent of periodontitis, following the guideline of Koch's postulates. The major challenge they encountered was the isolation and cultivation of oral species in laboratory conditions. Soon they realized that the majority of the oral bacteria are anaerobic (= killed by air) and fastidious microbes. Great advancement in the anaerobic culture came in 1960s, with the invention of anaerobic glove boxes – a primitive version of now widely used anaerobic chambers, used first by Socransky [138] and Rosebury *et al* [139]. These improved anaerobic cultivation techniques combined with optimized complex culture media allowed to develop pure cultures of more than 300 different oral bacteria species within past 40 years, including clinical isolates from both supra-gingival and sub-gingival dental plaque taken from healthy and diseased sites [140]. The studies on healthy volunteers who agreed to withdraw tooth-brushing for a pro-longed period revealed direct link between accumulating dental plaque and the development of gingivitis, mild form of periodontal inflammation [141, 142]. After 28 days without essential oral hygiene in periodontally healthy volunteers there was a rapid accumulation of bacterial plaque on the teeth, and gingivitis developed in all subjects in 10–21 days. These changes were reversible when tooth-brushing was re-introduced. The authors analyzed smear preparations of plaque samples taken during the 28-day time course. The initially colonizing bacteria on the teeth belonged to the groups of gram-positive cocci and rods, followed by gram-negative cocci and rods, then fusobacteria and filaments, and finally spirilla and spirochetes. The appearance of clinical gingivitis correlated with the appearance of the gram-negative forms. Other studies on the microbial succession in oral plaque development confirmed these findings [143-145].

Some years have past and many other culture-based and molecular methods delivered massive information about the exact nature of species involved in periodontitis, revealing dramatic compositional changes in periodontal plaque during transition from health to the disease [146-149]. Scientists attempted to make a classification of periodontal bacteria. The most popular one has been a division into color-coded clusters proposed by Socransky *et al* in 1998 [150], and updated few

years later [151]. This division distinguishes several complexes of bacteria and reflects their succession in the oral plaque and their association with the symptoms of the disease. In this classification the bacteria composing the dental plaque were divided into six clusters ('yellow', 'purple', 'blue' or *Actino*-, 'green', 'orange' and 'red'), based on the structural characteristics of the biofilm extending away from the tooth surface. *Streptococci* species including *S. sanguis* and *S. oralis* composed the 'yellow' population whereas *Actinomyces odontolyticus* and *Veillonella parvulla* formed the 'purple' one. Together with the *Actinomyces*, those species were thought to be early colonizers of the teeth. The main postulated role of the pioneers was to express receptors for host ligands to enable rapid and firm attachment to the host surface. The next complex, designated with green, included *Capnocytophaga* spp, *Campylobacter consisus*, *Eikenella corrodens*, and *A. actinomycetemcomitans*, the bacteria contributing to the initial alterations in the habitat. The 'bridging species' formed the orange cluster: *Fusobacterium* spp., *Prevotella* spp., *Micromonas micros*, *Eubacterium* spp., and *Streptococcus constellatus*. That cluster included the species capable of using and releasing nutrients in the biofilm, as well as expressing cell surface molecules facilitating binding to early colonizers, and the members of the red complex. Finally, the red cluster was a consortium of three anaerobic Gram-negative species: *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, considered the most significant pathogens in periodontal disease progression, since there has been a marked correlation between the number and prevalence of these bacteria and periodontitis clinical parameters [150-152]. These three consensus periopathogens (in particular *P. gingivalis*), as well as members of the orange cluster also associated with periodontal lesions, have been heavily studies *in vitro*, aiming on the identification of their key virulence mechanisms [152].

The emerging periodontal pathogens

The implementation of culture-independent molecular methods for bacterial identification, such as 16S rDNA amplification and high-throughput sequencing, revealed the existence of novel species in the periodontal plaque and shed a new light on the compositional changes of oral biofilm in the disease [153-156]. In addition to the previously recognized periopathogens, novel poorly or non-cultivable bacteria were found, such as the Gram-positive *Filifactor alocis* and species in the

genera *Prevotella*, *Megasphaera*, *Desulfobulbus*, *Synergistes* and *Selenomonas* [154, 155, 157-159]. Many of them show equally good or even better correlation with periodontitis than the classical well-recognized periobacteria. In particular, *F. alocis* was present in diseased periodontal pockets in higher numbers, while it was hardly detectable in healthy or periodontitis-resistant patients, which was in contrast to the traditional periopathogens often found in healthy individuals [157, 160, 161]. These studies proposed that *F. alocis* can be a marker organism for periodontitis [157, 160], and hence it has become an exciting subject of current investigations.

Current understanding of periodontal disease

The qualitative and quantitative changes in the oral plaque during transition from health to the disease have been quite well documented by now. Yet, it is not entirely understood what triggers these changes in people maintaining standard oral hygiene throughout their lifetime. It is known from the studies of the edentulous oral cavity of infants prior to tooth eruption that anaerobic species are present already in the absence of teeth, with *Prevotella melaninogenica* being found most frequently, in 70% of infants [162]. As mentioned earlier, many major periopathogens are found in healthy individuals of different ages, indicating the co-existence of these bacteria in a commensal state with the host. At some point or period in host lifetime, these bacteria change in their numbers and relative proportions, and induce chronic periodontal inflammation, which may lead to teeth loss as a result of the destruction of the supporting alveolar bone [123, 163]. Periodontitis is a highly prevalent disease, presenting substantial economical burden [164]. Furthermore, given a strong association of periodontal bacteria with other diseases, such as cardiovascular disease, diabetes or rheumatoid arthritis, it becomes evident that efficient periodontal treatment would be of great medical benefit to general health [165]. Elucidating the molecular interactions between pathogens and the host is the only way increasing our understanding of the disease and taking us closer to the therapeutic solutions. What is currently believed is that healthy periodontal plaque exists in the state of homeostasis with the host, and different factors (e.g. environmental, deregulated host responses, bacterial-triggered processes) can contribute to its disruption leading to the pathogenic events and disease [163, 166]. In order to maintain this homeostatic status, periodontal tissues are kept in controlled low grade inflammatory state, which allows to

confine the bacterial plaque to the gingival margin and keep limited flow of GCF, substantial to supply the periodontium with essential nutrients [128]. However, manipulation of host responses by the bacteria can certainly tip the balance toward exacerbated inflammation, which in turn favors the growth of those species that are better adapted to inflammatory conditions, resulting in the dysbiosis (that is a change in the relative abundance of different microbes compared to their proportions in the healthy state) of oral plaque. Hence, the periodontitis etiology is currently explained with 'The polymicrobial synergy and dysbiosis model', highlighting joint effort of various bacteria composing oral plaque in the pathogenic mechanism [163]. Sadly enough, our main defense mechanism against the bacteria, the complement system, turns out to be a 'double-edged sword', and is proactively utilized by periodontal pathogens to manipulate the conditions in the gingival crevice, as evidenced by numerous studies, including papers composing this thesis (I and III-VI).

The central role of complement evasion by bacteria in periodontitis

If oral plaque is left undisturbed, the bacteria multiply and try to expand their niche. The natural expansion occurs down the tooth root to the gingival crevice, which offers both, better protection against removal forces and a constant flow of nutrients in GCF. GCF is a mixture of molecules originating from the blood, host tissues and subgingival plaque, such as electrolytes, small organic molecules, proteins, cytokines, specific antibodies, bacterial antigens, and enzymes of both host and bacterial origin [128]. Its cellular components include desquamated epithelial cells and transmigrating leukocytes, i.e. polymorhopnuclear cells, monocytes/macrophages and lymphocytes [128]. Along with the nutrients, various plasma components are extruded to the gingival crevice, including complement proteins, which increase in their concentrations once bacterial biofilm manages to proliferate (e.g. during poor oral hygiene). The bacteria stimulate constitutive signaling of innate immune receptors, and keep the periodontal mucosa in a state of 'physiological inflammation' leading to the continuous production of antimicrobial proteins and tissue repair factors. At the inflammatory conditions complement is found at 70-80% of its serum concentration, with elevation of certain components reflecting their local production [167, 168].

Periodontal bacteria have to be appreciated for their fine-tuned adaptations allowing them to co-exist with the host in the dynamic inflammatory environment. Essentially, these bacteria attempt to establish a balance between complement inhibition, leading to decrease in their immune clearance, and activation, providing flow of nutrients in the inflammatory exudates ([169, 170], paper I and IV). They have different capabilities to resist complement attack or thrive under inflammatory conditions, yet they can act synergistically to enhance their growth and survival. It appears that they act as a community, whereby each bacterium plays a specific role, and more resistant strains protect the susceptible ones against immune defenses. In this regard, red complex bacteria *P. gingivalis* and *T. forsythia* are both resistant to the bactericidal activity of human serum, due to the presence of a surface anionic polysaccharide (known as A-LPS) [171, 172] or surface glycoprotein layer (S-layer) [173], respectively. Yet, they appear to produce secreted proteases, which further protect them against complement, but more importantly can diffuse to distant locations and have a profound effect on the whole milieu and bystander species.

Complement inhibition by periodontal bacteria

The vital role of proteases for various organisms has been well-recognized within the last 30 years, during which the perception of these enzymes from proteins responsible for non-selective amino acids recycling evolved toward appreciating well-regulated protein degradation in response to various changing environmental conditions. It is clear that proteases are involved in countless cellular activities and extracellular processes in *Eukaryotes*, but at the same time their roles in microorganisms has become equally well-explored. In fact many bacteria express whole panels of proteolytic enzymes, and in particular proteases of human pathogens appear to be their key virulence factors. The functions of proteases in bacteria range from acquiring essential nutrients from host proteins and cleaving host extracellular matrix components or coagulation proteins allowing dissemination, through inactivating various cellular receptors and deregulating signaling networks, to a very specific degradation of key innate immunity components, such as complement [174].

The latter function appears particularly well-evidenced for major periodontal bacteria. The expression of proteases affecting complement is not unique to the red complex bacteria, as it is also found among orange cluster bacteria - equally

well associated with more advanced plaque and more severe disease [150]. These proteases appear to have a biphasic effect on complement system, act synergistically and share few unique activities. Regarding complement-inhibiting activities of these enzymes, the cysteine proteases of *P. gingivalis*, known as gingipains [169], streptopain-like protease of *Prevotella intermedia*, interpain A [175], as well as two metalloproteinases of *T. forsythia*, karilysin (paper I) and mirolysin (paper IV) diminish bactericidal activity of human serum. This can increase survival of sensitive bacteria in serum/GCF. *P. gingivalis* produces Arg- (RgpB, HRgpA) and Lys-specific (Kgp) gingipains, which cause a degradation of the central complement component C3, thereby inhibiting all the complement pathways [169]. Similar activity was observed for interpain A of *P. intermedia* [175]. Interestingly, we found that *Tannerella forsythia* produces two metalloproteinases, karilysin (paper I) and mirolysin (paper IV), which both appeared to degrade recognition molecules of the lectin pathway, MBL and ficolins, as well as C4, and to block terminal complement pathway by degradation of C5. As these proteases exert synergistic action on complement, both within the same species and between the species ([175], paper I and Paper IV), and these bacteria are found together at the infection sites, their resultant effect will most probably lead to the substantial inhibition of complement. In particular, we can consider that the recognition (MBL, ficolins) and opsonization (C3b, C4b) will be diminished, leading to decreased lysis of bacteria and abolished uptake by phagocytes. Furthermore, binding of soluble host complement inhibitors by *P. gingivalis* (C4BP) and *P. intermedia* (factor I, factor H and C4BP), contributes to complement inhibition on the surface of these periodontal bacteria ([176] and paper III). Another member of the red complex, *T. denticola* binds a full-length factor H to its surface, which is subsequently processed by its serine protease dentilisin to generate shorter factor H remaining attached to the bacterial surface [177, 178]. It is not known if the truncated version of factor H bound to *T. denticola* remains active and inhibits complement; alternatively inactivation of factor H by dentilisin could promote local complement activation. In fact, periodontal bacteria seem to have figured out that certain level of complement activation can be of benefit for them. Gingipains [169], interpain A [175] and mirolysin (paper IV) share a unique activity of activating the C1 complex in serum, resulting in the deposition of C1q on inert surfaces or on the bacteria themselves. These events might be particularly advantageous when oral bacteria try to establish infection and stimulate a flow of nutrients in GCF, given that they have multiple protective mechanisms rendering them resistant to complement-

mediated lysis. At this stage some sensitive bacteria might become eradicated, contributing to the dysbiosis observed in periodontitis. Once major periodontal bacteria reach higher concentrations, consistent with increased levels of their proteases, they can destroy C3, C4 and other complement molecules, contributing to the protection of by-stander species.

Complement activation by periodontal bacteria

The aforementioned bacterial proteases share one more feature – they all preferentially cleave α-chains of their substrates C3, C4 and C5. Specifically, gingipains (especially HrgpA and Rgp) act in a C5 convertase-like manner and generate biologically active C5a [169, 179]. Similarly, interpain A from *P. intermedia* acts on C3 and releases C3a, as confirmed by N-terminal sequencing of generated fragments [175]. Furthermore, we have demonstrated that both karilysin (paper I) and mirolysin (paper IV) are able to cleave C5 and release C5a, consistent with the generation of substantial chemotactic activity in heat-inactivated human plasma, attracting neutrophils. The bigger opsonic fragments C3b, C4b or C5b are then further degraded by these proteases, while anaphylatoxins, potent mediators of inflammatory responses [180] can exert reactions leading to the destructive breakdown of tissues and release of nutrients for the bacteria. C5a mediates vasodilation, increases vascular permeability and exudation of fluid rich in various nutritive components, along with extravasation of inflammatory cells, especially neutrophils [180]. However bacteria can also exploit C5a signaling for immune subversion, as discussed below.

Complement hijacking by periodontal bacteria

The release of anaphylatoxins by bacteria was surprising to us and other researchers, who observed the same phenomenon. Even though there are potential benefits of inflammation, the attraction of phagocytes to the site of infection seemed a suicidal strategy at first. Yet, once again periodontal bacteria appear to have that part under control. This is particularly well evidenced for *P. gingivalis*. This bacterium does not only generate C5a via direct action of gingipains [169], but also by gingipain-mediated activation of prothrombin to thrombin [181], which in turn activates C5 convertase [182]. Only wild-type *P. gingivalis* is able to release C5a *in vivo*, whereas its mutant deficient in all three gingipains is devoid of such

activity [183]. Contradictory to the expected eradication of the bacterium at such pro-inflammatory conditions, the survival of *P. gingivalis* in the presence of C5a is enhanced due to the following mechanisms. Firstly, activation of C5aR by gingipain-generated C5a stimulates G_i-dependent intracellular Ca^{2+} signaling, which synergizes with normally weak TLR2-mediated cAMP response activated by *P. gingivalis* [179]. The resultant C5aR-TLR crosstalk generates high levels of cAMP and leads to the activation of protein kinase A, and the latter inactivates the glycogen synthase kinase-3. This signaling pathway in mouse macrophages impairs the nitric oxide-dependent killing of *P. gingivalis* [179]. Secondly, the same subversive crosstalk also inhibits TLR2-induces IL-12p70 (IL-12), the key cytokine that induces INF-γ and stimulates phagocytes, while up-regulating bone-resorptive cytokines IL-1, IL-6, IL-17 and TNF [183]. As a result, in a mouse model of periodontitis, *P. gingivalis* can escape immune clearance and cause periodontal destruction [183]. The conditions generated by *P. gingivalis* are favorable not solely for this pathogen, but appear to benefit the whole microbial community and lead to quantitative and qualitative changes of oral plaque [184]. In fact, *P. gingivalis* administered to specific-pathogen free mice stably colonizes the murine oral cavity, albeit at very low levels (<0.01% of the total bacterial counts), and changes the whole oral commensal microbiome into a dysbiotic one [184]. This mechanism leading to the destructive inflammatory disease is dependent on complement C5aR, since it does not occur in C5aR-deficient mice [183-185]. Similarly mice deficient in TLR2 appear to be resistant to periodontal bone loss after oral administration of *P. gingivalis* [186]. Interestingly, *P. gingivalis* did not evoke a disease in germ-free mice, despite successful colonization, implicating a requirement for commensal microbiota in periodontal destruction [184]. Due to the fact that *P. gingivalis* present in very low numbers can exert such community-wide effects and induce a disease, it was proposed a 'keystone' species, in analogy to the role of crucial apex stone in the ancient arch [184]. Initiating crosstalk between complement and TLRs to counteract their normally antibacterial functions appears to be one of the key adaptation of *P. gingivalis*, presumably acquired during long co-existence with human host.

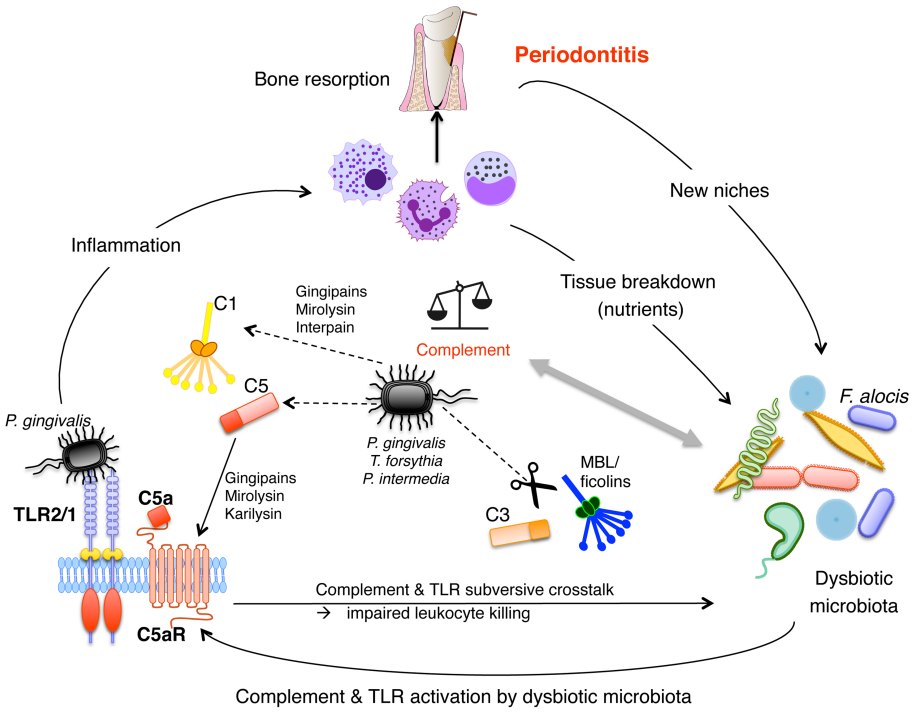

Fig.6. Complement manipulation by periodontal bacteria. Periodontal bacteria, such as *P. gingivalis*, *T. forsythia*, *P. intermedia* manipulate complement by acquiring host inhibitors and producing proteases of a dual complement-aimed activity. These proteases act synergistically and degrade key complement components, such as MBL, ficolins, C3 and C4. On the other hand, they can also stimulate complement by activating C1 and causing C1q deposition on different surfaces and bacteria, and by cleaving C5 and releasing biologically active anaphylatoxin, C5a, hence inducing local inflammation. The pro-inflammatory conditions are particularly beneficial at early stages of the infection, by inducing the flow of serum-derived exudate, rich in nutrients. The disruption of local homeostasis in the gingival crevice favours the growth of immune resistant bacteria, e.g. *F. alocis*, which takes advantage of the inflammatory milieu. Furthermore, some bacteria, such as the keystone pathogen of oral plaque, *P. gingivalis*, learnt to bridge host immune defences and induce the subversive crosstalk of Toll-like receptors with Complement C5a Receptor, resulting in the inhibition of leukocyte killing. This leads to the incapacitation of phagocytes and further growth of oral plaque, which in turn exacerbates inflammation. Adapted from Hajishengallis et al.[204] and modified by implementing some of the findings described in this thesis. In this schematic drawing some elements were prepared using Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

PPAD

Interestingly, *P. gingivalis* does not only produce proteases of C5-convertase like activity, but also expresses an enzyme that can modulate C5a activity. In this regard, this bacterium secretes peptidylarginine deiminase (PPAD), converting Arg residues in polypeptide chain into citrulline (Cit) [187]. In human, PADs are engaged in posttranslational modifications of proteins, e.g. by introducing citrullination to chemokines [188] or antibacterial peptide LL-37 [189]. However, in stark contrast to mammalian enzymes, PPAD of *P. gingivalis* preferentially modifies C-terminal Arg residues, presumably to neutralize the positive charge at the C-terminus of (poly)peptide fragments generated by cleavage of various proteins by its Arg-specific gingipains [190]. Given an essential role of terminal Arg for the activity of C5a [191], its modification by PPAD affecting the biological function of the anaphylatoxin offers attractive opportunities of modulating inflammatory milieu for *P. gingivalis*. In keeping with this, the citrullination of C-terminal Arg in C5a by PPAD was observed *in vitro*, consistent with the loss of its chemotactic activity toward neutrophils (unpublished data, Bielecka E. *et al*). This would indicate that *P. gingivalis* is capable of hijacking the whole potential of human anaphylatoxins C5a and controlling it on many levels (from a release through subverting its function via C5aR to inactivating its biological activity). Alternatively, citrullinated-C5a may have yet unexplored novel functions. Interestingly, PPAD has been proposed to provide a mechanistic link for the well known association between periodontitis and rheumatoid arthritis [192]. Infection of wild-type *P. gingivalis* exacerbated the collagen-induced arthritis in a mouse model, and the aggravated and early-onset disease symptoms were attributed to the induction of increased levels of autoantibodies to collagen type II and citrullinated epitopes. PPAD-null mutant did not elicit similar host response, hence indicating that the activity of bacterial PPAD in an organism can have systemic consequences.

P. gingivalis *versus other periodontal bacteria*

The studies in mice, supported by the correlation of *P. gingivalis* with diseased periodontal lesions in humans, indeed place this bacterium on a central position in the oral biofilm, orchestrating periodontium damage. Yet animal models of human diseases always have certain limitations and we can find gaps in this type of studies addressing periodontitis too. The aforementioned experiments [183, 184, 193], were

performed in the presence of commensal flora natural to mice, in the absence of pathogens other than *P. gingivalis* normally found in humans simultaneously, strictly associated with periodontitis. It is fair to assume that changes in the oral flora due to the presence of *P. gingivalis* cannot solely be attributed to the immune evasion (shutting down complement or incapacitating macrophages) by this pathogen. Studies addressing formation of oral plaque point out various synergistic interactions between different species that can enhance their colonization, persistence, or virulence [150], and these interactions certainly will look different in the human oral plaque when compared to mice. In this regard, the recent data show that the introduction of *P. gingivalis* into a healthy multispecies biofilm alters the pattern of community gene expression (for example, causing upregulation of chaperones, ABC-transport systems, putative transposases, and proteins related to growth and division, as well as numerous transcription factors) [194]. Furthermore, given strong association of all three members of red complex with periodontitis in humans, we cannot neglect the pathogenic mechanism of *T. forsythia* or *T. denticola*, which are still largely unexplained when compared to *P. gingivalis*. Considering the polymicrobial nature of periodontitis, identification of virulence mechanisms contributing to immune homeostasis disruption shared by different key periopathogens could give rationales for developing effective therapies. For instance, if proteases of C5a convertase-like activity are the key pre-requisites of keystone pathogens, their identification in other long-recognized pathogens of human periodontitis, such as *T. forsythia* (paper I and IV) gives further rationales for the central role of complement subversion by periodontal bacteria. Yet, it remains to be elucidated if C5aR-TLR subversive crosstalk can occur in the presence of *T. forsythia*. Of note, similarly to the infection of TLR2-deficient mice by *P. gingivalis*, oral administration of *T. forsythia* in such mice showed diminished capacity of this bacterium to induce alveoral bone loss, while there was a significant destruction in wild-type mice [195].

Neutrophils in periodontitis

Another arising question is if bacteria induced C5aR-TLR crosstalk can affect other cell types, and more importantly if it can take place in human cells. Interestingly, neutrophils are dominant cellular population in the gingival sulcus, where they tend to form a specific structure called a 'leukocyte wall' along the margins of the periodontal plaque [128]. Their accumulation in periodontal pockets and infiltrating the junctional epithelium is one of the hallmark features of periodontitis and they appear to be involved in the immunopathogenesis of the

disease [196, 197]. Similarly to deregulated complement activation, deviations in neutrophil activity (both impaired or exacerbated activity and/or recruitment) result in the disrupted homeostasis in the periodontium and manifest in humans by various forms of periodontitis [197, 198]. Local production of C5a by bacterial proteases could certainly affect both the migration and the activity of neutrophils in the gingival crevice. The impact of C5a on neutrophils is not straightforward, as it strictly depends on the exact levels of the anaphylatoxins. While low levels of C5a induce neutrophils chemotaxis and increase their antibacterial activity, the high ones evoke their 'immune paralysis' demonstrated by inhibited migration and diminished performance [199, 200]. Hence, taking into account synergistic action of several periodontal bacterial proteases, there is a good probability for local generation of high C5a levels, which could corrupt neutrophils populating the periodontium. If these cells are further affected by bacteria bridging C5aR with TLR responses, it remains to be elucidated. Nevertheless, as C5a appears to act in favor of key periopathogens and the bystander species, there are ideas of trying to counteract its signaling by implementing C5aR antagonists in periodontitis therapy [201]. In a murine model, local intragingival administration of C5aR antagonist leads to the reversal of *P. gingivalis*-induced dysbiosis, inhibits inflammation and prevents bone loss [185]

Keystones and pathobionts

In the light of the data described above, there emerges a new trend of looking at the oral pathogens from the perspective of their functions in the community [202]. Accordingly, the species capable of breaking down the host-microbe homeostasis at low colonization levels would have a role of keystone pathogens, as proposed for *P. gingivalis* [179, 202, 203]. Subsequently, massive periodontal destruction would then be mediated by pathobionts, commensals that begin to thrive under inflammatory conditions and evoke disease-associated symptoms [184, 202]. For example, *F. alocis*, highly prevalent and abundant in diseased periodontal sites with little detection in healthy or periodontitis-resistant individuals would appear to play a role of the pathobiont, following those definitions [161, 204, 205]. The numbers of this bacterium increases in the disease and it exhibits very strong correlation with clinical parameters (paper VI), consistent with the induction of periodontal damage upon its proliferation. However, we cannot exclude that it does not play any role in the early course of a disease, by deregulating host defenses. The battery of virulence factors of this bacterium have just begun to be studied, and interestingly 15 different proteases have been identified in its genome, as well as an arginine

deiminase [206], an enzyme present also in *P. gingivalis*, as mentioned earlier, but otherwise not described in *Prokaryotes*. Regarding the virulence mechanisms, in paper VI we demonstrate that *F. alocis* have a great potential of inhibiting complement, as well as may offer protection to bystander species, which at least in principle could result in disruption of local homeostasis and a dysbiosis of oral plaque. Therefore, it can be quite hard to attribute strict roles to particular bacteria, as they may appear to have mixed functions.

Inflamm-aging and periodontitis

Another intriguing issue is why *P. gingivalis* is frequently found at low numbers in healthy individuals, without inducing the disease-associated dysbiosis? And a question related to the latter – since periodontitis in majority of the cases affects elderly population, what changes in the host and/or oral bacterial community with age, that the homeostatic balance becomes disrupted? There have been studies trying to explain the increased susceptibility of elderly to infections or other diseases associated with aging, such as cardiovascular diseases, autoimmune disorders, rheumatoid arthritis or cancer. Many studies propose a link between these syndromes, but the mechanistic explanations are in most cases not very well supported [190, 207-209]. One such link between them might be immunosenescence, i. e. age-related alterations in the immune system leading to the impairment of its functions [210]. Those changes are associated with continuous exposure to a variety of stressors and a concomitant progressive increase in pro-inflammatory status, which are major characteristics of the aging process, consequently called inflamm-aging [210]. A number of in vitro studies point out various alterations in immune cells functions or signalling related to aging, that could play a role in age-associated diseases, such as periodontitis, as reviewed in [211]. It is not clear however to what extent these changes may be relevant *in vivo*, where all the cells are involved in various interaction with each other and can respond very differently, depending on the local environment. Furthermore, age-related changes are heterogeneous between individuals and sometimes the genetically programmed alterations are hard to dissect from the changes induced by varying environmental and lifestyle factors, such as use of medications, antibiotics or diet. It appears that aging is a progressive degenerative process tightly intertwined with inflammatory responses, yet *per se* it is not a disease [212]. Therefore the cause and effect questions are still not answered for periodontitis or other age-related syndromes.

Another aspect addressed by researchers is how periodontal bacteria interact with gingival epithelial barrier and if they can spread to deeper tissues. The gingival sulcus is layered with junctional epithelium, which lacks differentiation and keratinization, and is known for its high cellular turnover. Desquamation processes occur apically toward the bottom of the sulcus, while mitotic activities are enhanced in DAT cells constituting the suprabasal layers of the junctional epithelium, directly attached to the tooth surface [213]. Intriguingly, studies regarding the colonization strategies of *P. gingivalis* in primary epithelial cell cultures of gingiva showed that physiological structuring of junctional epithelium constitutes a logical route for the bacterium [214]. Hence the bacterium invading DAT cells disseminated in the junctional epithelium intercellularly to the bottom of the sulcus during the regular process of proliferation. Further, *P. gingivalis* has been shown to prolong host cell survival and enhance the proliferation of the infected host cells, which would allow the organism to establish itself in the gingival epithelium, and to contribute to disease [215]. These studies further support the hypothesis that *P. gingivalis* has co-existed with the host long enough to acquire evolutionary adaptations facilitating both extracellular and intracellular growth. Not only epithelial but also endothelial cells were found to be susceptible to *P. gingivalis* invasion [216, 217]. It has been speculated that the invasion of coronary artery endothelial cells by oral bacteria can be a contributing factor to the link between periodontitis and cardiovascular disease [218]. The mechanism of *P. gingivalis* adhesion to, and invasion of, host cells is of great interest and several effector molecules have been pointed out. The initial binding is mediated by the major fimbriae of the bacterium interacting with integrins on epithelial cells [219, 220]. Bacterial internalization involves microtubule and microfilament structures of the cells [220, 221]. Several virulence factors contribute to the bacterial intracellular persistence, as reviewed in [222]. Yet, considering quite rare occurrence of periodontal bacteria in locations distant from oral cavity, their spreading at normal conditions is quite efficiently prevented by the main barrier, gingival epithelial cells. It would be interesting to know how exactly the integrity of this barrier is maintained. In paper VI we propose one potential mechanism of antibacterial defense in gingival epithelial cells. We show that major periopathogens, *P. gingivalis*, *T. forsythia* and *P. intermedia* bind CD46 molecule and we address in detail the role of activation of CD46 on gingival epithelial cells by periopathogens. While we found that the initial attachment and cell invasion of primary gingival cells by *P. gingivalis* was

decreased, when we silenced CD46 expression in these cells, the presence of CD46 during the interaction of bacteria with gingival cell was found to determine their final fate inside the cell. Hence, normal primary gingival cells or the control siRNA transfected ones limited the invasion of intracellular bacteria efficiently, whereas the CD46-deficient cells allowed them to persist at significantly higher levels. These results led us the conclusion that certain bacterial clearance mechanisms are activated in oral epithelial cells upon CD46 activation. There is substantial evidence in the literature indicating that the antibacterial role of CD46 can be based on induction of autophagy by this receptor, as shown for measles virus or GAS [122]. When induced upon intracellular pathogen invasion, autophagy can play an important role in controlling infection [223]. Regarding periopathogens, there is evidence about the involvement of autophagy in infection of cells with *P. gingivalis* [224], but the exact mechanisms has not been explained so far. Furthermore, given various immunomodulatory functions of CD46 on different cell types, our findings certainly open up a whole new field of studies elucidating the interplay between oral bacteria and cellular subpopulations in the gingival crevice.

Taken together, the more we know about the fascinating interplay between periodontal bacteria and host defenses, the more questions emerge. It is a great challenge to identify the key immune defense mechanisms that could be addressed in therapy (either counteracted or enhanced) to restore the local homeostasis once it is disrupted.

Opportunistic pathogens

The transient and resident colonizers of the skin and mucosa

In contrast to the bacteria colonizing our mucosa persistently throughout our lifetime, such as periodontal species, there are numerous bacteria that are continually being inoculated transiently on our skin/mucosa. The skin is being colonized by various microorganisms on daily basis, but virtually most of them are unable to multiply and usually die. Only few resident organisms are able to proliferate, not just merely survive, on the skin. These resident species constitute normal flora of the skin and are usually associated directly or indirectly with the sweat and sebaceous glands, providing warm and humid niches. The secretions of the skin glands contain excellent nutrients for the bacteria, such as urea, amino acids, salts, lactic acids and lipids. The normal flora of the skin is dominated by the Gram-positive bacteria restricted to a few groups. These include several species of *Staphylococcus* and a variety of both aerobic and anaerobic corynebacteria. The resident microorganisms are more or less constant, however various factors can affect the nature, extent and composition of normal flora. Under certain circumstances, these bacteria can cause opportunistic and even life-threatening diseases.

The features of *S. aureus* infection

One example of an opportunistic colonizer, extremely well adapted to the challenges posed by our natural barriers and immune defenses is *Staphylococcus aureus*. The factors governing the transition of *S. aureus* from a mere colonizer to a severe pathogen are carefully studied, especially due to the increasing incidence of fatal bloodstream infections. It happens rarely that *S. aureus* is acquired from an

external source and causes a severe infection; usually such cases concern open wound infections. More commonly, an individual is infected by the bacteria colonizing his or her skin, or mucosal surface [225, 226], easily transferred to other tissues injured either mechanically or damaged due to previous exposure to viral infection (e.g. in upper airways). *S. aureus* can be found on the mucosa lining the nose, throat, vaginal wall, and gastrointestinal tract. About 20% of the human population are persistent carriers of *S. aureus*, and another 60% are colonized transiently [227]. The definition of transient carrier is generally described as a single positive culture on a nasal swab versus at least two consecutive positive cultures within one week apart (persistent). Colonization is more frequent among younger children, and patients with HIV and diabetes [228]. Usually *S. aureus* remains within the host in a commensal state, but in the invasive form can cause a wide spectrum of clinical manifestations, ranging from skin-limited abscesses and wound infections, to life-threatening diseases, including pneumonia, bacteremia, sepsis, endocarditis, or toxic shock syndrome [229]. It is associated with both community-acquired and hospital-acquired infections, and has become a major public health threat due to the increasing prevalence of multiple antibiotic resistant strains, such as methicilin-resistant *S. aureus*. The emergence of vancomycin-resistant strains brings back the terrifying spectre of fatal bloodstream infections from the pre-antibiotic era, and emphasizes a need for the development and implementation of new treatment strategies.

In terms of human virulence, *S. aureus* is perhaps the most successful bacterium, as it produces a large arsenal of tightly regulated virulence factors that can be exploited in different host environments [230]. It appears that various proteins produced by this pathogen have multiple roles - they bind several host ligands and plasma proteins, modulate complement and coagulation, as well as promote adhesion, colonization or dissemination. Surprisingly, the pathogen maintains fine control of virulence expression, and for the most part rarely causes severe infection in previously healthy individuals. According to the generally accepted hypothesis, dissemination of *S. aureus* takes place via transition from adhesive to migratory/invasive phenotypes producing various extracellular proteins. This process has been shown to be dependent on, amongst other things, proteolytic enzymes, which cleave tissue adhesion molecules [231, 232]. Two global gene regulators control this transition - the accessory gene regulator (*agr*) quorum sensing system supports more invasive phenotype of *S. aureus* [233, 234], whereas the pleiotropic virulence determinant regulator, SarA, is responsible for proteases and other

virulence factors repression [235]. Owing to its central role in innate immunity and its bridging function to adaptive immune responses, the complement system is a key target for evasion strategies of *S. aureus*. Already as a colonizer, in the adhesive form, *S. aureus* must deal with complement factors that can be produced locally in the skin/epithelium [236, 237]. However, during dissemination into the bloodstream the bacterium is exposed to far more challenging conditions, especially in terms of complement activation.

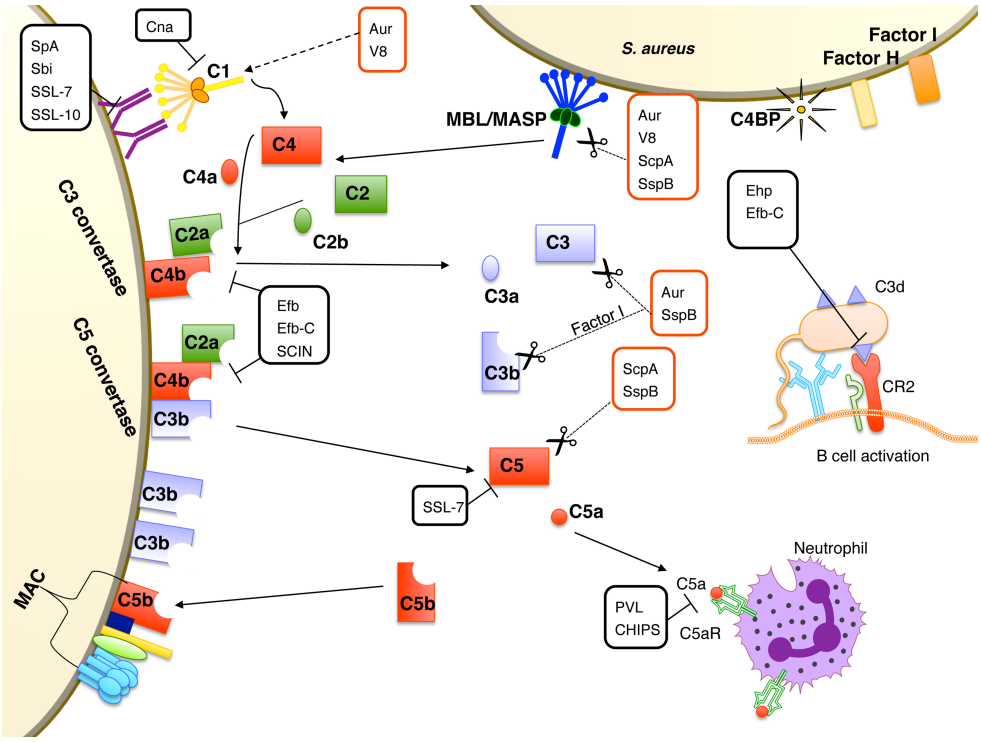

Fig.7. Factors of *S. aureus* modulating complement activity. In black frames: various complement inhibitors. In red frames – staphylococcal proteases. Various staphylococcal molecules inhibit complement at the level of recognition, C3 activation, C5 activation, or act on complement receptors. Staphylococcal proteases have a dual role, as they both inhibit and activate complement. See details in the text. In this schematic drawing some elements were prepared using Biomedical-PPT-Toolkit-Suite of Motifolio Inc., USA.

Although, as a Gram-positive bacterium with thick layer of peptidoglycan this pathogen is insensitive to complement-mediated lysis, the three activation

pathways of complement should, in principle, ensure its quick recognition and opsonization for efficient phagocytosis, while anaphylatoxins activate various immune responses. Yet, *S. aureus* developed many mechanisms attenuating complement activation at every possible step. The arsenal of staphylococcal virulence factors acting of complement revealed in numerous studies highlights their role mainly as inhibitors of this powerful system (Fig. 7). In contrast, in our study (paper II) we demonstrate that *S. aureus*, via action of its proteases, not only inhibits complement-mediated processes, but also selectively activates some of them. When compared to proteases produced by periodontal bacteria, we observed several analogous modes of action of staphylococcal enzymes.

S. aureus – the master of complement evasion – the known facts and gaps in the understanding of staphylococcal infections

Staphylococcal proteins affecting complement at the activation level

One of the hallmarks of *S. aureus* biology is its ability to infect the human host repeatedly throughout life. A severe bacterial infection normally leads to mounted adaptive immune response within seven to ten days to limit the ongoing infection and prevent future reinfections. However, *S. aureus* avoids recognition by immunoglobulins, subsequent opsonophagocytosis and classical pathway complement activation, due to the presence of antibody binding proteins. To date, four such proteins have been identified, namely Spa (Staphylococcal protein A) [238], Sbi (second binder of immunoglobulin) [239], SSL7 (superantigen-like protein 7) [240] and SSL10 [241]. Further, *S. aureus* blocks classical complement pathway by binding C1q to Cna, a microbial surface component [242]. The data included in this thesis (paper II) demonstrate that proteases of *S. aureus* decrease the deposition of MBL and ficolins, and consistently block downstream complement activation via lectin pathway. Interestingly, in this study we also found that the deposition of classical pathway collectin, C1q, was not decreased (apart from a relatively small effect exerted by a cysteine protease ScpA), but rather increased by the action of bacterial proteases. Additionally, aureolysin (Aur) and serine protease V8 were found to cause the deposition of C1 from serum onto inert surfaces without the need of a specific C1 activator. The increased deposition of C1q in the presence of Aur occurred not only on blocked microtiter plates but also on the surface of *S.*

epidermidis. The latter organism is a commensal bacterium found on the skin and in the nasal cavity of humans, and has been specifically shown to block biofilm formation and nasal colonization by *S. aureus* [243]. Hence, we speculated that such *S. aureus* protease-induced deposition of C1q could render *S. epidermidis* more vulnerable to opsonophagocytosis, and would then result in its eradication, leaving the niche free for pathogen colonization. The role of C1q in the phagocytosis of bacteria, independently of C3b, has been demonstrated for several species [244, 245]. Taking into account the vital role of C1q in the nonphlogistic clearance of apoptotic cells, the attractive hypothesis emerges, whereby *S. aureus* promotes the uptake of commensal species (or perhaps its own) without boosting the inflammatory response. Similar events could be promoted by periodontal proteases, also shown to increase C1q deposition on bacteria (see above).

Targeting the central complement activation step, C3 conversion, by staphylococcal factors

Several proteins of *S. aureus* have been found to inhibit the central activation step of complement component C3, which simultaneously block the initiation of the alternative pathway, the amplification loop of complement, and the induction of downstream effector functions. The extracellular fibrinogen-binding protein (Efb) C-terminal domain (Efb-C) [246, 247] and the Efb homologous protein (Ehp) [248] bind to native C3 and some of its activation fragments (i.e. C3b, C3d), form stable complexes and trigger conformational changes in C3 and C3b. These events result in blocking of C3b deposition and the generation of anaphylatoxins via the C3 and C5 convertases [249]. Furthermore, the tight binding of both Efb-C and Ehp at a C3d region has been shown to affect the interaction of C3d with its major receptor CR2, thereby interfering with complement-mediated adaptive immunity functions [250]. Further inhibition on C3 level is provided by Aur, which blocks phagocytosis by converting C3 to active C3b, which then becomes more vulnerable to cleavage by host complement inhibitors factor H and factor I [251]. Of note, host complement inhibitor (factor I) and its cofactors (factor H and C4BP) are bound by *S. aureus* and contribute to complement inhibition on its surface [252-255]. C3b release due to cleavage by Aur is accompanied by C3a production, which is then further cleaved to smaller fragments in the presence of Aur and serum, and therefore does not induce neutrophil activation [251]. This seems to be a protective strategy of the bacterium since C3a, in contrast to C5a, has direct antibacterial activity [256]. We were able to confirm the action of Aur on complement in paper II.

Interestingly, we found that another protease of this bacterium, SspB does not cleave intact C3, but it does degrade C3b, both deposited on a plate and in fluid phase. We speculated that there could be a cooperative action between Aur and SspB, whereby Aur converts C3 to C3b, which is then degraded further by SspB. Alternatively, if joint action of Aur with factor I leaves the bacterium coated with iC3b, perhaps *S. aureus* benefits from consequent iC3b-mediated phagocytosis. Intriguingly, phagocytosis via iC3b-CR3 is often associated with attenuated killing mechanisms or even immunosuppressive signaling [257-260] and may be exploited by certain pathogens. For example, obligate intracellular pathogen *Mycobacterium* utilizes CR3 to promote its intracellular cell entry to macrophages [261]. Of note, *S. aurues* has been shown to survive in phagocytes [262-264] and the metalloprotease Aur was proven to be important in this process [265].

In addition to direct action of *S. aureus* on C3, the bacterium exploits the link of complement with coagualtion. Active plasmin has been shown to cleave and inactivate C3 and its fragments [266]. *S. aureus* can bind plasminogen to its surface proteins Efb, Sbi [267] and TPI (Triosephosphate isomerase; [268]), which is then further converted to active plasmin by Sak staphylokinase or by human uPA (urokinase plasminogen activator) [269]. Activated plasmin bound to the bacterial surface cleaves C3, C3b and iC3b as well as immunoglobulins and thus limits phagocytosis of *S. aureus*. The proteins of staphylococcal protein inhibitor family (SCIN-A, SCIN-B and SCIN-C) have also been shown to affect an activity of C3 convertase by 'freezing' its conformation and blocking its enzymatic action [249, 270]

Targeting the C5 convertase level and the terminal complement pathway by S. aureus

SSL-7 protein of *S. aureus* binds complement C5 with high affinity and blocks the activity of C5 convertase [249, 271]. Furthermore *S. aureus* directly blocks the effector functions of C5a anaphylatoxins by secreting chemotaxis inhibitory protein (CHIPS), which binds to C5aR [272]. In addition, recently, staphylococcal Panton-Valentine Leukocidin (PVL) has been demonstrated to bind human complement receptors C5aR and C5L2 and inhibit important immune cell functions [273]. Interestingly, we showed that cysteine proteases of *S. aureus*, ScpA and SspB, and the metalloprotease Aur are able to release biologically active C5a from C5 present in heat-inactivated human plasma (paper II). These data indicate that while *S. aureus* inhibits the physiological ways of C5a generation and its effector functions, it is also capable of releasing the anaphylatoxin under certain conditions. It is quite

hard to determine which effects are more important under physiological settings, and most likely this will depend on many factors, including local environment and infection phase (e.g. colonization vs dissemination). Given the central role of C5a in the immunopathogenesis of sepsis contributing to multiorgan failure and mortality [274], the release of C5a by *S. aureus* proteases and its outcome in infection is certainly worth further investigations. Particularly, it is known that neutrophils can undergo 'immune paralysis' during sepsis, the effect mediated mainly by excessive C5a levels [200, 275]. Increased local production of C5a at infection sites could reduce the number of functional neutrophils and facilitate the dissemination of *S. aureus*. Notably, SspB has been shown to affect the phagocytes, i. e. to induce apoptosis-like death in human neutrophils and monocytes by selective cleavage of CD11b [276]. In addition, SspB induces the engulfment of neutrophils and monocytes by macrophages, by both degradation of repulsion signals and induction of 'eat-me' signals on their surfaces [277]. Another interesting question would be if *S. aureus* could exploit a similar subversive crosstalk between C5a receptors (C5aR) and TLR receptor as *Porphyromonas gingivalis*, to impair intracellular killing in macrophages [179]. There is growing evidence demonstrating prolonged survival of *S. aureus* in the phagocytes [265, 278, 279], but the exact mechanisms have not been clearly elucidated. Staphopains seem to be potential candidates to study in this context, as they might contribute to the imbalances in C5a-C5aRA axis. Interestingly, combined inhibition of complement and TLR2 (CD14) resulted in the attenuation of the inflammatory response to *S. aureus* in the human whole blood model [280]. The effects of complement inhibition were attributed mainly to the disruption of the interaction between C5a and C5aRA. In another study, a novel C5a-neutralizing aptamer molecule attenuated inflammation, prevented organ failure and improved survival in a mouse model of sepsis [281]. Staphylococcal molecules targeting C5 convertase subsequently inhibit the terminal pathway/MAC formation. The relevance of this inhibition for *S. aureus* are unclear however, due to the fact that thick peptidoglycan layer normally shields this bacterium from the lytic action of complement. Recently it has been shown that sublytic MAC can trigger intracellular signaling in the cells leading to the activation of NLRP3 inflammasome, a large multiprotein complex, which plays a key role in the secretion of pro-inflammatory cytokines including IL-1-β [282]. Perhaps Gram-positive bacteria aim on controlling that step as well? Various pathogens have been shown to affect inflammasome activation by direct action on

its components [283], but bacterial terminal complement pathways inhibitors are interesting candidates to study in this context as well.

Major findings of this thesis

Papers I and IV

In these two papers we studied novel metalloproteinases of *T. forsythia*, karilysin and mirolysin, and we showed that they contribute to bacterial complement resistance by cleaving several key components of this system. Interestingly, both of the proteases were able to cleave similar substrates. They both degraded MBL, ficolin-2, ficolin-3 and C4, resulting in the inhibition of classical and lectin pathways of complement activation. At the same time they selectively activated certain complement-mediated processes. Namely, they targeted α-chain of complement component C5 to release biologically active anaphylatoxins C5a, activity of which has been largely implicated in pathogenesis of periodontitis. In addition, mirolysin, but not karilysin, was able to cause increased C1q deposition on inert surfaces or bacteria, in the absence of physiological activators. Interestingly, these two proteases acted synergistically on complement, providing enhanced effect, and in addition they exerted synergistic action on complement when combined with proteases of other major periodontal bacteria, found together with *T. forsythia* at the infection sites. All in all, these two papers expanded knowledge about the virulence factors of one of the key bacteria involved in periodontitis, and confirmed a current paradigm about fine-tuned manipulation of complement by well-adapted oral species.

Paper II

In paper II we studied *Staphylococcus aureus*, known for its impressive repertoire of complement inhibitors. At the moment when we started our study, nothing was known about the effects of staphylococcal proteases on complement system. We tested a whole panel of major proteases of *S. aureus*: cysteine proteases staphopain A and staphopain B, the serine protease V8, the metalloprotease Aur and two staphylococcal serine proteases D and E, and found that all but the last two are

potent inhibitors of human complement. We further identified their substrates within complement cascades. We observed some analogies in the action of staphylococcal proteases, when compared to the enzymes of periodontal species.
Most of the staphylococcal proteases targeted MBL, ficolins and C4, and inhibited the classical and lectin pathway. They also inhibited the alternative pathway, either by direct cleavage of C3, or in case of aureolysin, by generating C3b-like fragment vulnerable to the processing by human inhibitors factor H and factor I. We also showed that three of the proteases were able to release C5a, similarly to proteases of periodontal bacteria. Furthermore, *S. aureus* mutants lacking proteolytic enzymes were found to be more efficiently killed in human blood. These results gave an overview about the contribution of staphyloccal proteases to its complement evasion strategies, and revealed that *S. aureus* does not only aim on inhibition of this system, but may as well benefit from selective activation of certain complement-mediated processes.

Paper III

Further, we showed that another important periopathogen, *Prevotella intermedia*, acquires resistance towards complement by binding complement inhibitor found in human serum, factor I. This study was the first one to demonstrate such interaction within Gram-negative pathogens. We also demonstrated that this bacterium hijacks the whole fluid-phase inhibitory mechanism of human complement, as in addition to factor I it binds its two major cofactors C4b-binding protein (C4BP) and Factor H. These results demonstrate that *P. intermedia* is well-adapted to a human host.

Paper V

In this study we found that major periopathogens have the ability to bind to the membrane-bound complement inhibitor CD46, the molecule found on almost all human cells. Strikingly, even though this molecule improved initial attachment of bacteria to the epithelial cells, the final outcome was not beneficial for the bacteria – in cells without CD46 they persisted much longer without getting cleared, indicating activation of certain killing mechanisms upon CD46

stimulation. The phenomenon may be related to autophagy, which may be affected by CD46 as shown previously. In the continuation of this study, we are planning to elucidate what exact pathway is responsible for bacterial removal upon CD46 activation. In addition, due to involvement of CD46 in the astonishing number of cellular activities, the interactions of periodontal bacteria with this molecule in various cell populations can be interesting to study.

Paper VI

Recent findings implicated an involvement of a novel gram-positive *Filifactor alocis* in the pathogenesis of the periodontal disease. In our study, we have confirmed that *F. alocis* is very prevalent species in both chronic and aggressive periodontitis. It could only partially be removed with conventional periodontal therapy (scaling and root planing) and had a tendency for recurrence. Taking into account the key role of complement deregulation by periodontal bacteria, we focused on *F. alocis* capacity to manipulate this system. We pinpointed different strategies, such as production of proteases or non-productive binding of C3, employed by this bacterium. We further characterized the interaction between C3 and cell surface of *F. alocis* and we identified bacterial surface protein responsible for capturing C3 from serum. We are going to express the protein of *F. alocis* responsible for C3 binding in a recombinant form, and possibly some of the proteases, and further characterize their effect on complement.

Conclusions and future perspectives

Human pathogens have co-existed with the host for thousands of years and they have been fighting in an evolutionary battle against complement with great success. The mechanisms of pathogen complement evasion are astonishing and we can do nothing but learn from our enemies. *In vitro* studies allow for elucidating a molecular basis of host-bacteria interactions and they illustrate how complex and intertwined all the immune responses are. The pathogens have figured it out and they seem to control each and every step of complement activation, as well as keep all the links between complement and other defenses in check. What becomes evident is that in disease we often do not fight with bacteria but we try to alleviate our own immune system, which is derailed by pathogens.

The major challenge is to find a good balance between inflammation activation leading to the removal of pathogenic organisms, and inhibition, in order to limit the potential tissue destruction resulting from exacerbated inflammatory responses. Bacteria learned to strike a "golden mean" – if in doubt, look at periodontal pathogens and how they manipulate complement to get the essential nutrients but avoid immune clearance. We need to look very carefully which pathways and processes are deregulated by pathogens and try to find ways to breach their tactics. Bacterial complement inhibitors, for example, can serve as excellent structural and functional templates for new drugs, since they were carefully selected by evolution to provide hundred percent precision in action. There is also a reason why each and every successful human pathogens produces several complement inhibitors, aimed on controlling various steps of complement cascade, and more strikingly, often few inhibitors act on the same step. We may not see a point in producing several proteins of seemingly same functions, but a miniscule organism with a single chromosome and limited energy resources does not invest effort into producing any redundant molecules. Hence, there are always functions for all bacterial proteins; it is just a question of time to find them. The redundancy also indicates a vital importance of counteracting certain steps of host immune responses for the pathogen.

Importantly, we should look very carefully on the parallels in the pathogenic mechanisms of various human pathogens. Surprisingly, unrelated bacteria associated with distinct diseases, such as periodontal bacteria in comparison to *S. aureus*, appear to share certain mechanisms of immune evasion. When looking into this thesis work, this notion is exemplified by the activities of bacterial proteases, which in case of both *T. forsythia* and *S. aureus*, target similar steps of complement activation in analogous ways. Most of the studied proteases target collectins of the lectin pathway and degrade them – hence, is lectin pathway the most crucial in the recognition of bacteria and activating complement on their surface? Many of them activate C1q and increase its deposition on inert surfaces and bacteria – what mechanism stays behind this? Most of these proteases destroy the opsonins C3b – logically this brings a lot of benefit in limiting central complement step and inhibiting phagocytosis. Finally, many of these proteases are able to release biologically active C5a – certainly limited inflammation acts in favor of our enemies.

In order to find therapeutic solutions, we need to take more holistic view on the infectious and inflammatory diseases. We have to look on the whole groups of pathogens building biofilms and find their key components (e.g. keystone pathogens). We have to look on the genomes of bacteria and identify proteins of similar functions. We have to create networks, look on matrixes of genes, develop good predictive software etc. Research is already going in that direction, by combining various techniques and more systemic approaches when unraveling the complex nature of host–pathogen interactions. Hopefully continued studies will not only increase our understanding of the host-pathogen interplay, but also bring effective therapeutic solutions. Which points of the complement cascades should be intervened with, needs to be adjusted to a specific microorganisms, yet due to the parallels in the pathogenic mechanism, we may also find more general strategies. It appears, that in a number of infectious diseases, interfering with C5 or C5a-C5aR axis, gives promising results, like in periodontitis or sepsis, as mentioned above. In a recent outbreak of enterohemorrhagic *E. coli* (EHEC) in Europe, it turned out that off-label use of a drug, eculizumab, inhibiting the C5 activation into C5a and C5b-9, successfully treated the patients, giving rationale for further clinical trials [284]. Although eculizumab was developed to inhibit the abnormal activation of C5, in patients missing complement regulators with paroxysmal nocturnal hemoglobinuria (PNH) suffering from the destruction of erythrocytes, by analogy it worked also in a disease, in which the reason of

excessive complement activation was of bacterial origin. Identifying other commonalities within many complement-related pathologies or infectious agents can result in the development of other novel complement-targeted drugs, or finding new applications for the existing ones. We just have to keep an eye on what pathogens do in our organisms, as they do not sleep but move forward in this evolutionary arms race and we also have to constantly make us stronger.

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