Antibacterial peptides - key players in host defense at epithelial surfaces

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2010

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– key players in host defense at epithelial surfaces

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Akademisk avhandling

som, med vederbörligt tillstånd från Medicinska Fakulteten vid Lunds Universitet
för avläggande av doktorsexamen i Medicinsk Vetenskap, kommer att
offentligen försvaras i Lundmarksalen, Astronomihuset, Sölvegatan 27,
29e januari, 2010, kl 09.15

Fakultetsopponent

Docent Johan Bylund
Institutionen för Medicin
Avd för Reumatologi och Inflammationsforskning
Sahlgrenska Akademin, Göteborgs Universtitet

Organization	Document name
LUND UNIVERSITY Department of Clinical Sciences, Lund Division of Respiratory Medicine and Allergology Faculty of Medicine, Lund, Sweden	DOCTORAL DISSERTATION
	Date of issue January 29, 2010
	Sponsoring organization

Author(s)
Mette Eliasson

Title and subtitle

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Key words: Innate immunity, pharyngitis, mucosa, epithelium, Streptococcus pyogenes, antibacterial peptides, chemokines, CXCL9/MIG, IFN-γ

Classification system and/or index termes (if any):

Supplementary bibliographical information:	Language English
ISSN and key title: 1652-8220	ISBN 978-91-86443-21-4

Recipient's notes	Number of pages 124	Price
	Security classification	

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Lund 2010

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Cover image:
Control conditions (upper panel): Pharyngeal epithelial cells (left). Viable *S. pyogenes* bacteria in cell incubation medium (center), and on the cell surface (right). Stimulated conditions (lower panel): Antibacterial CXCL9/MIG (high-lighted in red) on the cell surface (left). Affected *S. pyogenes* bacteria in cell incubation medium (center), and on the cell surface (right).
Photos by Dr. Matthias Mörgelin and Pontus Nordenfelt.

Printed by Tryckeriet i E-huset.

ISSN 1652-8220
ISBN 978-91-86443-21-4

Lund University, Faculty of Medicine Doctoral Dissertation Series 2010:7

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Contents

List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (*I-IV*):

Paper I

Arne Egesten, **Mette Eliasson**, Helena M Johansson, Anders I Olin. Matthias Mörgelin, Andrea Mueller, James E Pease, Inga-Maria Frick, Lars Björck. The CXC chemokine MIG/CXCL9 is important in innate immunity against *Streptococcus pyogenes. The Journal of Infectious Diseases.2007 Mar 1;195(5):684-93*

Paper II

Mette Eliasson, Inga-Maria Frick, Mattias Collin, Ole E Sørensen, Lars Björck, Arne Egesten. M1 protein of *Streptococcus pyogenes* increases the production of the antibacterial CXC chemokine MIG/CXCL9 in pharyngeal epithelial cells. *Microbial Pathogenesis. 2007 Nov-Dec;43(5-6):224-33*

Paper III

Christofer Karlsson*, **Mette Eliasson***, Anders I Olin, Matthias Mörgelin, Anna Karlsson, Martin Malmström, Arne Egesten, Inga-Maria Frick. SufA of the opportunistic pathogen *Finegoldia magna* modulates the action of the antibacterial chemokine MIG/CXCL9, promoting bacterial survival during epithelial inflammation. *The Journal of Biological Chemistry. 2009 Oct 23;284(43):29499-508*

* both authors contributed equally to this work

Paper IV

Mette Eliasson, Anders I Olin, Matthias Mörgelin, Mattias Collin, Arne Egesten. Interferon-γ Induces a Peptide-Mediated Bactericidal Response in Human Pharyngeal Epithelial Cells. *Manuscript*

Abbreviations

AP	Antibacterial peptide
CTL	Cytotoxic T lymphocyte
FAF	*F. magna* adhesion factor
GAGs	Glucosaminoglycans
GAS	Group A streptococcus
GRO-(α/β/γ)	Growth-related oncogene (α/β/γ)
I-TAC	Interferon-inducible T-cell alpha chemoattractant
IFN	Interferon
IL	Interleukin
IP-10	Interferon-inducible protein 10
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MIG	Monokine induced by interferon gamma
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa B
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PG	Peptidoglycan
PRR	Pattern-recognition receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIC	Streptococcal inhibitor of complement

SLPI	Secretory leukocyte protease inhibitor
Stat	Signal transducer and activator of transcription
SufA	Subtilase of *Finegoldia magna*
Th	T helper
TNF	Tumor necrosis factor
TLR	Toll-like receptor

Introduction

The epithelial linings that cover the mucosal surfaces of the upper airways and the skin are under constant exposure to microbes. Bacteria of the skin- and mucosal microbiota are usually beneficial to the host and can provide protection by competing with pathogens for nutrients and space. Pathogenic bacteria express virulence factors such as adherence molecules, enzymes and toxins that enable them to colonize and invade epithelial surfaces. The host is therefore dependent on robust protection against pathogens.

The innate immune system provides a first line of defense against invading microbial pathogens. It is an evolutionary conserved system and, in contrast to the adaptive system which is restricted to vertebrates, genes involved are represented in vertebrates, invertebrates, and also in plants. It is older than the adaptive immune system, works unspecifically to invading pathogens, and does not generate immunologic memory (Lien & Ingalls, 2002; Medzhitov & Janeway, 2000). It takes three to five days for the adaptive system to reach sufficient number of produced and differentiated effector lymphocytes. In contrast, the innate immunity mechanisms are activated immediately upon microbial recognition (Bartlett *et al.*, 2008; Hippenstiel *et al.*, 2006; Kawai & Akira, 2009; Medzhitov & Janeway, 2000).

PAMPs and PRRs

The innate immune recognition of microorganisms is based on the detection of highly conserved structures called pathogen-associated molecular patterns (PAMPs). PAMPs are usually essential for the survival and pathogenicity of the microorganism and are shared by entire classes of pathogens (Medzhitov & Janeway, 2000). LPS (lipopolysaccharide), PG (peptidoglycan), LTA (lipoteichoic acid), and DNA are examples of common bacterial PAMPs. PAMPs are recognized by pattern-recognition receptors (PRRs) that have been selected over the course of evolution (Iwasaki & Medzhitov, 2004; Lien & Ingalls, 2002; Medzhitov, 2001; Pasare & Medzhitov, 2005). PRRs are present on cells involved in the initial microbial recognition, including epithelial cells, dendritic cells (DCs), monocytes/macrophages, neutrophils, and natural killer (NK) cells (Iwasaki & Medzhitov,

2004). In addition to PAMPs, endogenous molecules from stressed or damaged host cells may also be recognized by cells of the innate immunity (Matzinger, 2002). Through activation of NF-κB and MAPK signaling pathways, genes for a variety of effector molecules are transcribed, resulting in production of e.g. antibacterial peptides (APs), cytokines and chemokines (Akira *et al.*, 2001; Bartlett *et al.*, 2008; Hippenstiel *et al.*, 2006; Kawai & Akira, 2005; Medzhitov, 2001).

The Toll-like receptors (TLRs) are the best characterized PRRs in mammalian species, and they are the mammalian homologues of Toll receptors (Rock *et al.*, 1998), originally identified in the fruit fly, *Drosophila melanogaster* (Anderson *et al.*, 1985; Lemaitre *et al.*, 1996). The Toll receptors are evolutionary conserved and are found in plants, insects, worms and vertebrates (Iwasaki & Medzhitov, 2004; Lien & Ingalls, 2002; Medzhitov, 2001; Pasare & Medzhitov, 2005). Invasive bacteria that are not recognized by TLRs can be recognized via either NOD1 or NOD2, members of the cytosolic recognition family NOD (nucleotide-binding oligomerization domain) that detects bacterial cell wall constituents present in the cytosol of host cells (Kawai & Akira, 2009).

Innate immunity at epithelial surfaces

The innate immune responses that takes place in the mucosa and in the skin involve a variety of mechanisms and mediators, such as epithelial cells, DCs, monocytes/macrophages, neutrophils, complement, NK cells, cytokines, chemokines, APs, and the oxidative immune defense.

The epithelium – Inhaled microbes can be removed mechanically from the airways by ciliary movement and through coughing. Airway and oro-pharyngeal epithelium is also protected by mucus that constitutes a physical barrier protecting the epithelial surface by direct binding of microorganisms. IgA, present in mucus, saliva and sweat, inhibits bacterial binding to epithelial cells. The skin is further protected against microorganisms by its low water content, acidic pH, and antibacterial lipids. Antibacterial chemokines and peptides are constitutively produced in the skin- and airway-epithelium and can associate with mucins,

which place them in an ideal protective position against incoming microbes (Bartlett *et al.*, 2008; Felgentreff *et al.*, 2006). The epithelium not only forms a physical barrier, but is also important in the initial recognition of invading pathogens at epithelial surfaces. Upon recognition, the epithelium responds by producing inflammatory mediators such as cytokines, APs, and chemokines. The constitutive and induced production of antibacterial components emphasize the important role of epithelial cells in innate immunity, not only combating early and later phases of infections, but also preventing infection from developing in the first place (Boman, 2003; Hiemstra, 2001). The epithelium-derived molecules also have important roles in activating and directing cells of the adaptive immune system.

DCs – are phagocytosing and antigen-presenting cells (APCs). They are present in peripheral lymphoid tissue, such as the tonsils, and skin (Langerhans´ cells) where they constantly scan the surroundings, and present endogenous or exogenous antigens on their surfaces. Their major function is to activate T helper (Th) cells (Iwasaki & Medzhitov, 2004).

Monocytes/macrophages – are long-lived phagocytosing cells and APCs. They are present in blood (monocytes) and in tissues (macrophages). Upon microbial stimuli, they produce bactericidal reactive oxygen and nitrogen species (ROS/RNS). Macrophages are an important source of cytokines that trigger the recruitment and activation of inflammatory cells.

Neutrophils – are highly specialized but short-lived phagocytosing cells. They circulate in the blood and are recruited to the mucosa and skin upon a bacterial infection. They kill phagocytosed bacteria by releasing their granular content, consisting of a broad range of antimicrobial agents such as peptides and enzymes. An important feature of the neutrophils is their ability to mount a powerful oxidative burst in response to microbial and endogenous stimuli. Besides affecting the phagocytosed pathogens, ROS can also kill microbes in surrounding tissues (Rada & Leto, 2008).

NK cells – Under resting conditions, NK cells are found in blood and lymphatic vessels and they reside primarily in the spleen and in secondary lymphoid organs, such as the tonsils. NK cells contain granules with cytotoxic molecules, used for killing tumor cells or virus-infected host cells. NK cells interact with DCs in order to generate an appropriate immune response in both the lymph node and in the inflamed skin (Andoniou *et al.*, 2008). During the early innate immune response, activated NK cells are rapid producers of IFN-γ in response to various exogenous and endogenous stimuli (Billiau & Matthys, 2009; Schoenborn & Wilson, 2007).

Cytokines

During the initial response to infection, recognition of pathogens by PRRs on cells of the innate immune system results in a simultaneous production of cytokines and chemokines. Cytokines regulate the chemokine production either as antagonists or synergists, thus directing the type of immune response. Recruited innate immune cells amplify the release of cytokines and chemokines, thus sustaining the innate immune response that eventually results in recruitment of cells belonging to the adaptive immune response (Charo & Ransohoff, 2006; Gouwy *et al.*, 2005; Luster, 2002).

IL-1β and *TNF-α* – are the two most important cytokines in the early immune response**.** They are produced by a variety of cells, but Th cells and monocytes/macrophages are the main producers (Gouwy *et al.*, 2005; Strieter *et al.*, 2002; Veckman *et al.*, 2003). Other sources of IL-1β and TNF-α are keratinocytes and DCs (Giustizieri *et al.*, 2001; Kupper, 1990). IL-1β- and TNF-α-signaling result in the activation of NF-κB and an enhanced transcription of genes mediating innate immune responses (Lien & Ingalls, 2002; Strieter *et al.*, 2002).

Interferons (IFNs) – The interferons are major regulators of the innate immune system and are produced in response to a variety of stimuli. The type I IFNs (IFN-α, IFN-β) have antiviral activity and are induced by most human cells after exposure to viruses, double-stranded viral RNA, bacteria, and PAMPs (Kawai & Akira, 2005; Li *et al.*, 2009; Smith *et al.*, 2005). IFN-γ is the only type II interferons and is produced in response to cytokines,

primarily by NK cells (Li *et al.*, 2009) and to a lesser extent by other cells, such as Th1 cells, cytotoxic T lymphocytes (CTLs) (Billiau & Matthys, 2009) and mast cells (Marshall, 2004).

Important contributions of IFN-γ to the immune response include, priming of macrophages to produce proinflammatory cytokines such as TNF-α and IL-12, inducing the production of potent antimicrobials such as APs and reactive oxygen and nitrogen species in phagocytes. IFN-γ also induces the production of APs and chemokines in epithelial cells. Thus, IFN-γ not only optimizes cell-mediated effector functions of the innate immune system but also acts as a regulator of the adaptive immune response to bacterial agents (Billiau & Matthys, 2009; Schoenborn & Wilson, 2007).

The IFNs signal predominantly through the Jak (Janus kinase)-Stat pathway (Kawai & Akira, 2005). The IFN-γ receptor contains two chains, IFN-γR1and IFN-γR2. Functionally, IFN-γ acts as a homodimer and upon binding the receptor Jak1, Jak 2, and Stat1 become phosphorylated. Stat1 translocates to the nucleus where it binds to the IFN-γ-activation sequence in the promoter region of target genes (Kawai & Akira, 2005; Li *et al.*, 2009; Ohmori *et al.*, 1997; Pestka *et al.*, 2004). IFN-γ and TNF-α synergize to induce the expression of many gene products during inflammation. The synergy is dependent upon Stat1 and NF-κB. Motifs for these transcription factors are present in the promoter regions of many inflammatory genes, such as genes coding for chemokines (Mahalingam *et al.*, 2001; Ohmori *et al.*, 1997).

Cytokines direct the immune response

The innate and adaptive immune systems are two interdependent parts of a single integrated immune system and the response to pathogens requires the coordinated action of both systems (Luster, 2002). The move from innate to adaptive immune responses is, among other things, dependent on epithelial-derived products such as cytokines and chemokines. Naive CD4+ T cells are activated by DCs, and can differentiate into various effector lineages, including Th1, Th2, Th17, and regulatory T cells. The cell development diverges rapidly after antigen priming and is directed by specific cytokines (Figure 1). This results in

mature Th cells, efficient in producing cytokines that direct the immune responses mediated by effector cells. B cells and CTLs are key effector lymphocytes in the adaptive response and their migration to the site of inflammation is controlled by chemokines (Iwasaki & Medzhitov, 2004; Luster, 2002; Romagnani, 2004; Schoenborn & Wilson, 2007).

Th1 – The innate recognition of pathogens by DCs leads to production of the cytokines IL-12, IL-18, IL-2, IL-15, IFN-α/β, that activate NK cells. NK cells is the primary source of IFN-γ and they are able to secrete IFN-γ within hours due to their constitutive expression of IFN-γ mRNA (Schoenborn & Wilson, 2007). IL-12 and IFN-γ induce differentiation into Th1 cells, whereas IL-10 is a suppressor. Mature Th1 cells produce IL-2, TNF-β, and IFN-γ. IFN-γ is the predominant Th1 cytokine that stimulates and sustains an effective cell-mediated immune response against pathogens. IL-12 is also an important cytokine in the Th1 response demonstrated by its induction of IFN-γ-production in Th1 cells and CTLs (Luster, 2002; Schoenborn & Wilson, 2007).

Th2 – After the initial innate immune recognition, the production of IL-4 induces differentiation of naive CD4+ T cells into Th2 cells. In contrast, IL-4 is a negative regulator of Th17 differentiation, whereas IFN-γ suppresses the Th2 response (Harrington *et al.*, 2005; Romagnani, 2004). Mature Th2 cells are characterized by the production of IL-6, IL-10, IL-4, IL-5, and IL-13, where the latter three stimulate antibody-production by B cells. Th2 cells present in the tissue direct influx of effector cells participating in allergic immune responses, cells including eosinophils, basophils and mast cells (Gouwy *et al.*, 2005; Luster, 2002; Romagnani, 2004).

Th17 – Th17 cells are a recently discovered subset of CD4+ T cells, characterized by the production of IL-17A, IL-17F, IL-21, and IL-22. The differentiation of the Th17 lineage is a result of positive regulatory effects of TGF-β, IL-1β, IL-6, IL-21, IL-23 and absence of the negative regulatory effects of Th1 and Th2 cytokines. A number of extracellular pathogens induce mainly Th17 responses, and persons with a genetic inability to mount Th17 responses have recurrent infections in the skin and lung caused by *Candida albicans* and *Staphylococcus aureus*. The receptors for IL-22 and IL-17A are distributed on epithelial

cells of the skin and airways and activation induces the expression of several host defense genes, including human beta defensin (hBD)-2, Duox2, mucin, CXCL1/GRO-α, CXCL9/MIG, and CXCL10/IP-10. IL-22, IL-17A, IL-17F, and TNF-α can act both synergistically and additively to enhance the production of APs. Furthermore, IL-17A and IL-17F also synergize with TNF-α. IL-21 acts in an autocrine loop amplifying the Th17 cell response and its own synthesis. In addition, IL-21 stimulates IFN-γ production of both Th1 cells and CTLs. The effects of the Th17-produced cytokines and the distribution of their receptors, imply that Th17 cells may be important during bacterial infections at epithelial surfaces.

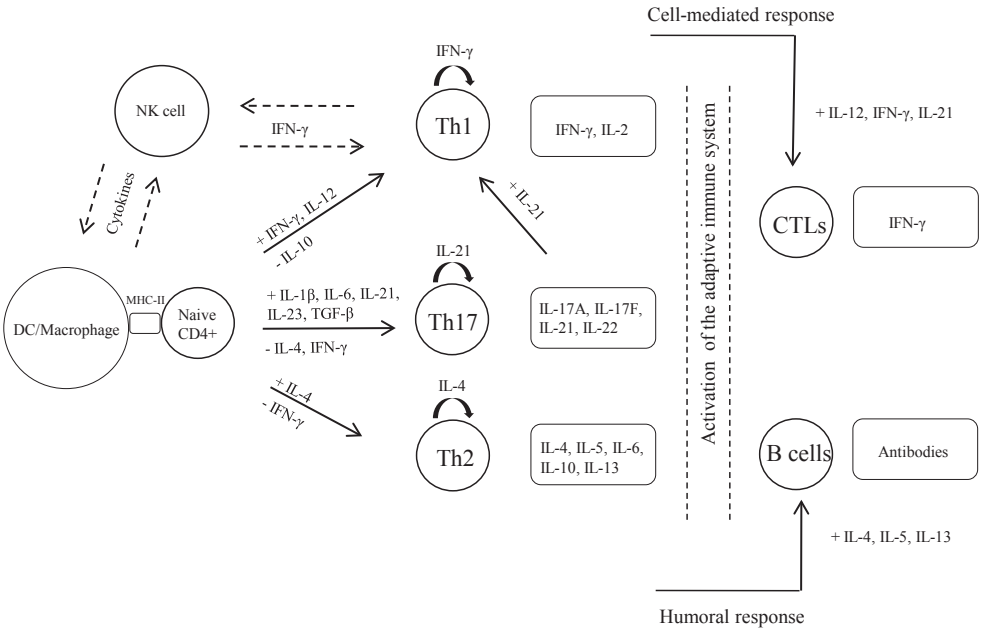

Figure 1. An overview of the differentiation of naive CD4+ cells into Th cells, and the cytokines involved.

Chemokines

Chemokines constitute a large family of cytokines with chemotactic properties. All chemokines regulate trafficking of leukocytes, during both health and disease (Baggiolini *et al.*, 1994; Billiau & Matthys, 2009; Luster, 1998).

Chemokines have a relatively low level of sequence identity, but their three-dimensional structures, functions, or receptor-binding profiles, are strikingly similar. In the N-terminal region, three antiparallel β-sheets are connected by disulphide bridges, a structure that is important for binding and activation of the corresponding chemokine receptor. The C-terminal region consists of an amphipathic α-helix, where one half of the circular structure is composed of nonpolar hydrophobic amino acids and the other side contains charged amino acids, thereby being hydrophilic (Baggiolini, 1998; Baggiolini, 2001; Luster, 2002; Turnbull *et al.*, 2001), (Figure 2).

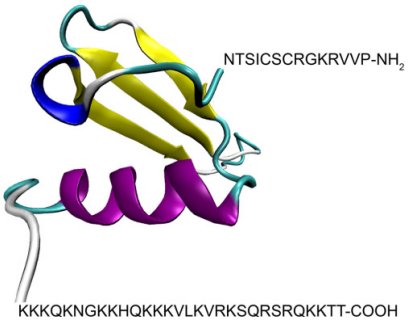

Figure 2. The predicted structure of the chemokine CXCL9/MIG. β-sheets in yellow, α-helix in purple.

Chemokines are small GAG-binding proteins consisting of roughly 70-130 amino acids. More than 50 chemokines have thus far been identified in humans (Baggiolini *et al.*, 1994; Baggiolini, 1998; Baggiolini, 2001; Luster, 1998). The majority are cationic proteins (pIs of ~9) at physiological pH due to a relatively high content of the amino acids lysine and arginine.

Chemokines have been divided into four families, according to the arrangement of conserved N-terminal cysteine motifs, C, CC, CXC, and CX_3C, where X is a non-conserved amino acid residue. The CXC and CC chemokines represent the largest families. The CXC

chemokines can be divided into ELR-positive and ELR-negative CXC chemokines, based on the presence or absence of a glutamic acid-leucine-arginin (ELR) motif, preceding the first two cysteines in the N-terminal region (Baggiolini, 1998; Cole *et al.*, 2001; Luster, 1998; Luster, 2002), (Figure 3).

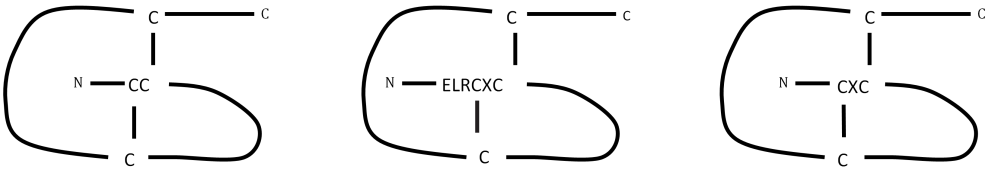

Figure 3. Schematic drawing of a CC chemokine (left), an ELR-positive CXC chemokine (center), and an ELR-negative CXC chemokine (right).

Receptor activation – Several chemokines can share the same receptor and several different receptors may bind the same ligand. The receptors can be restricted to a certain type of cell and to a cell state (activated and/or differentiated), and can be constitutively expressed, induced but also downregulated (Luster, 1998). Ten receptors for CC chemokines (CCR1-10), and six receptors for the CXC chemokines (CXCR1-6) have to date been characterized in humans (Luster, 1998; Murphy, 2002). Binding of a chemokine to its corresponding specific G-protein-coupled cell-surface receptor leads to cellular activation, an increase in intracellular calcium, and results in cellular responses (Luster, 1998; Murphy, 2002).

The presence of receptors on neurons, epithelial cells, and endothelial cells indicates additional roles for chemokines (Luster, 1998). In addition to leukocyte chemotaxis, some chemokines modulate angiogenesis, tumor growth, and inhibit stem-cell proliferation. Depending on the context, chemokines have different effects. For example, the ELR-negative CXC chemokine CXCL10/IP-10, inhibits neovascularization, tumor growth and metastasis, whereas the ELR-positive CXC chemokines, CXCL8/IL-8 and CXCL6/GCP-2, promote angiogenesis and tumor metastasis (Gijsbers *et al.*, 2005; Luster, 1998).

CC chemokines

Several members of the CC chemokine family, for example CCL20/MIP-3α and CCL28/MEC, are produced by epithelial cells (Sauty *et al.*, 1999). The CC chemokine receptors (CCRs) are preferentially expressed on activated lymphocytes of the Th2 phenotype, but also on eosinophils and basophils (Hoover *et al.*, 2002; Lazarus *et al.*, 2003; Luster, 1998; Luster, 2002).

ELR-positive CXC chemokines

The most studied member of this group is CXCL8/IL-8. It is produced by epithelial cells and neutrophils which thereby become activated in response to CXC8/IL-8 in an auto-/paracrine manner (Baggiolini, 2001; Sauty *et al.*, 1999). Other members of this chemokine family are CXCL1/2/3 (GRO-α/β/γ), which are produced by epithelial cells (Baggiolini, 1998; Luster, 1998). The ELR-positive CXC chemokines predominantly activate and recruit neutrophils through their receptors CXCR1 (CXCL8/IL-8) and CXCR2 (CXCL8/IL-8, CXCL1/2/3 (GRO-α/β/γ)). These receptors are also expressed on endothelial cells (Murphy, 2002).

IFN-γ-inducible ELR-negative CXC chemokines

Members of this family are the three closely related chemokines CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC (Cole *et al.*, 1998; Farber, 1997; Loetscher *et al.*, 1996). Upon IFN-γ-stimulation, these chemokines are produced by a variety of cells but predominantly by epithelial cells and monocytes (Cole *et al.*, 1998; Egesten *et al.*, 2007; Farber, 1993; Farber, 1997; Karlsson *et al.*, 2009; Loetscher *et al.*, 1996; Sauty *et al.*, 1999). Their receptor CXCR3 is expressed on Th1 cells, NK cells, DCs, mast cells and endothelial cells (Baggiolini, 1998; Loetscher *et al.*, 1996; Luster, 2002; Murphy, 2002).

IFN-γ is important for the optimal induction of CXCL9/MIG, but in the absence of IFN-γ, IFN-α/β can mediate production (Mahalingam *et al.*, 2001). IL-1β and TNF-α can synergize with IFN-γ and enhance the production of CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC (Egesten *et al.*, 2007; Lauw *et al.*, 2000; Sauty *et al.*, 1999).

Interactions with glycosaminoglycans (GAGs)

Heparansulfate, heparin, chondroitinsulfate, and dermatansulfate are GAGs found on cell surfaces and within extracellular matrix (Hoogewerf *et al.*, 1997). GAGs can be soluble or surface-bound and are composed of polysaccharide-chains attached to a protein core. Immobilized GAGs are essential for the biological activity of most chemokines (Johnson *et al.*, 2004; Proudfoot *et al.*, 2003; Shaw *et al.*, 2004).

Most chemokines are highly cationic peptides that interact with the negatively charged GAGs (Luster *et al.*, 1995; Prydz & Dalen, 2000). However, anionic chemokines also bind to GAGs, suggesting that the interaction is specific and not just electrostatic. GAG-binding sequences of chemokines and the corresponding motifs on GAGs have been identified, thereby regulating the interactions (Turnbull *et al.*, 2001). The preference for individual GAGs can vary between chemokines and certain chemokines can discriminate between GAGs (Handel *et al.*, 2005). GAG-binding may involve or induce oligomerization of the chemokines, suggesting that GAG-binding specificity may be a feature of chemokines that need to form oligomers in order to be active (Czaplewski *et al.*, 1999; Proudfoot *et al.*, 2003; Swaminathan *et al.*, 2003; Zhang *et al.*, 1994).

Oligomerization on GAGs establishes a local chemotactic gradient that efficiently attracts leukocytes (Hoogewerf *et al.*, 1997; Luster, 1998; Proudfoot *et al.*, 2003). The oligomerization on GAGs is also likely to be important on mucosal surfaces where antibacterial chemokines can be highly concentrated and form a bactericidal barrier against colonizing microbes (Egesten *et al.*, 2007; Luster, 1998). In addition, oligomerization of chemokines on GAGs on epithelial and endothelial cell surfaces may be a requirement in order to avoid being washed away from the local site under flow conditions (Proudfoot *et al.*, 2003). Some chemokines are able to interact with receptors as monomers (Rajarathnam *et al.*, 1994) and it is suggested that these are chemokines being produced within the extravascular space, which is not exposed to flow (Proudfoot *et al.*, 2003).

Antibacterial peptides (APs)

Under basal conditions, some APs are constitutively expressed by skin- and airway epithelia, and binding to GAGs can retain them on epithelial surfaces. The production of APs by epithelial cells and cells of the submucosal tissue, can also be induced in response to microbial pathogens, proinflammatory cytokines, growth factors, and injury (Bartlett *et al.*, 2008; Elias, 2007; Roupe *et al.*, 2009; Sorensen *et al.*, 2006).

APs have broad-spectrum effects on Gram-positive bacteria, Gram-negative bacteria, and also show activities against fungi and enveloped viruses. An important aspect is that they are active against bacteria resistant to conventional antibiotics (e.g. methicillin-resistant *S. aureus* (MRSA)) (Chambers, 2005). Despite the broad antimicrobial efficiency, bacteria of the human microbiota are relatively resistant to APs (Boman, 2000; Ge *et al.*, 1999; Karlsson *et al.*, 2009). Because of their multifunctional properties, these peptides are commonly termed antimicrobial peptides (AMPs). However, since fungi seldom cause survival problems for mammals, the driving force behind their evolution has probably been the antibacterial function. For this reason, Boman proposed that this family of peptides should be referred to as antibacterial peptides (APs) (Boman, 2003).

An important characteristic of APs is their combined cationic and hydrophobic properties, resulting in amphipathicity (Peschel & Sahl, 2006). The main target of APs is negatively charged bacterial membranes, to which APs are attracted electrostatically. The APs attain an amphipathic helical conformation, allowing them to insert into the hydrophobic face of the phospholipid bilayer (Tossi *et al.*, 2000), (Figure 4)(Brogden, 2005). It is unclear if the pore-forming activity is the primary killing mechanism, however, APs interacting with the membrane cause a reduced membrane potential, a disturbed osmotic balance, an increased pressure within the membrane, events resulting in lysis of the bacteria (Boman, 2003; Peschel & Sahl, 2006). In addition, many APs can neutralize the effect of LPS (Rosenfeld *et al.*, 2006) and some APs can act intracellularly, killing bacteria by interfering with DNA and protein synthesis (Boman, 2003).

Experimental data have shown that cholesterol, an essential component in the eukaryotic cell membrane, prevents damage to host cells (Boman, 2003).

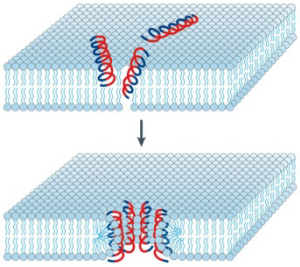

Figure 4. Illustration of APs forming a pore in the bacterial cell membrane. Modified from Brogden 2005.

Based on their 3-D structures, APs are divided into different classes where the main scientific focus has been on three classes. (1) Linear cationic peptides, free of cysteines, and often with an α-helical and amphipathic structure in solution (α-defensins, cathelicidins). (2) Peptides with three disulfide bonds giving the peptides a flat dimeric β-sheet structure (e.g. β-defensins), and (3) peptides with enrichment of certain amino acids (e.g. histidine-rich peptides) (Boman, 2003; Hancock & Diamond, 2000; Zasloff, 2002).

More than 800 different APs from animal and plants have thus far been characterized (Boman, 2003). Cecropins, isolated from moths and flies (Boman & Steiner, 1981; Hultmark *et al.*, 1980; Steiner *et al.*, 1981), and magainins, found in frog skin (Bevins & Zasloff, 1990; Zasloff, 1987) were among the first APs to be characterized in animals.

Defensins (α, β) and cathelicidins are the best characterized APs. Cathelicidins are found in all species investigated so far, but LL-37 is the only human cathelicidin, processed from the precursor protein hCAP-18. Epithelial cells synthesize and secrete LL-37 and the tissue specific β-defensins (Boman, 2003). The granules of neutrophils are loaded with several preformed APs, in particular α-defensins (Boman, 2003) and hCAP-18 (precursor of LL-37)(Borregaard *et al.*, 2007). Additional sources of APs include other blood cells, such as monocytes, platelets, and NK cells. APs can also be formed by proteolytis from larger proteins, such as lactoferricin from lactoferrin (Hancock & Diamond, 2000; Zasloff, 2002). The increased synthesis of APs after a breach in the epithelial barrier, indicates that APs

may have a role during epithelial repair, protecting the underlying tissue from pathogens until the epithelial barrier is restored (Hiemstra, 2001; Roupe *et al.*, 2009; Sorensen *et al.*, 2006).

The importance of APs has been questioned and there are some considerations to take into account when discussing their *in vivo* relevance. Most, but not all APs show decreased activity in the presence of salt at levels present in plasma. In cystic fibrosis, the salt concentration is increased, resulting in an environment that does not support optimal functioning of APs with recurrent bacterial infections as a consequence (mainly caused by *Pseudomonas aeruginosa*). In addition, most APs are inhibited by various substances such as plasma proteins, and soluble GAGs. However, there are many *in vivo* examples demonstrating important roles of APs in bacterial infections. Individuals suffering from Kostmann´s syndrome are born with only few neutrophils and acquire severe periodontitis and airway infections. Recent investigations have revealed that hCAP-18/LL-37 was missing in both neutrophils and saliva (Putsep *et al.*, 2002). Mice are normally highly sensitive to infection with *Salmonella typhimurium* but became resistant when transfected with the human enteric defensin hBD-5 (Boman, 2003).

Dual actions - antibacterial chemokines and chemotactic APs

APs present in humans at low (nanomolar) concentrations may exhibit other functions, including chemotaxis. During inflammation, certain chemokines reach antibacterial concentrations (micromolar), levels that are beyond what is required for chemotactic activity, and may act as APs. Amphipathic molecules with a high positive charge density are generally potent antimicrobials (Cole *et al.*, 2001). Most chemokines are positively charged at neutral pH, and Yang *et al* demonstrated that no chemokine with a pI lower than 9.0 was antibacterial against *E. coli*, indicating that cationicity is an important feature for antibacterial chemokines (Yang *et al.*, 2003). Additionally, the topological amphipathic

design, with clusters of hydrophobic and cationic amino acids organized in discrete surface areas, is an important structural characteristic for antibacterial chemokines (Yang *et al.*, 2003; Zasloff, 2002). These properties make it possible for the peptides to associate with, and disrupt bacterial membranes. However, there are examples of both linear and anionic peptides that possess antibacterial activity (Boman, 2003).

CC chemokines – Antibacterial activity has been ascribed to several CC chemokines. CCL20/MIP-3α is constitutively produced by many cells and is upregulated by proinflammatory stimuli such as LPS, IFN-γ, TNF-α, IL-1β (Eliasson *et al.*, 2007; Schutyser *et al.*, 2003). High production of this antibacterial chemokine by epithelial cells during mucosal inflammation points to direct antibacterial roles for CCL20/MIP-3α (Starner *et al.*, 2003; Yang *et al.*, 2003). Mucosa-associated epithelial chemokine (MEC), CCL28/MEC is produced constitutively in salivary glands and high concentrations are found in saliva. CCL28/MEC is effective against some strains of Gram-positive and Gram-negative bacteria, as well as *C. albicans*. This indicates that MEC/CCL28 has dual roles in mucosal immunity, both as a chemoattractant and also as a broad-spectrum AP when secreted into low-salt body fluids such as saliva (Hieshima *et al.*, 2003).

ELR-positive CXC chemokines – Antibacterial activity in the C-terminal region of epithelium and neutrophil-derived CXCL8/IL-8 has been reported (Bjorstad *et al.*, 2005; Yount *et al.*, 2007). CXCL1/2/3 (GRO-α/β/γ) are expressed by a variety of myeloid and epithelial cells after stimulation with proinflammatory cytokines (Becker *et al.*, 1994; Eliasson *et al.*, 2007) and have antibacterial activity (Yang *et al.*, 2003). CXCL6/GCP-2 is expressed by macrophages and epithelial cells and its antibacterial effect against Gram-positive and Gram-negative pathogens indicates that CXCL6/GCP-2 is important during mucosal infections (Linge *et al.*, 2008).

IFN-γ-inducible ELR-negative CXC chemokines – CXCL9/MIG, CXCL10/IP-10 and CXCL11/I-TAC are IFN-γ inducible chemokines. High production is seen in pharynx and airway epithelial cells and production is increased by IL-1β and TNF-α (Egesten *et al.*, 2007; Sauty *et al.*, 1999). All three have a broad antibacterial spectrum (Cole *et al.*, 2001;

Crawford *et al.*, 2009; Egesten *et al.*, 2007; Karlsson *et al.*, 2009). The antibacterial activity of CXCL9/MIG has been mapped to the C-terminal region (Egesten *et al.*, 2007) which is cationic, amphipathic and has a predicted α-helical structure, all features typical of APs. CXCL9/MIG has a higher antibacterial activity than CXCL10/IP-10 and CXCL11/I-TAC (Egesten *et al.*, 2007), and this might be explained by the more extended α-helical C-terminal of CXCL9/MIG. CXCL10/IP-10 and CXCL11/I-TAC have higher affinity for the CXCR3 receptor, thus these chemokines might be more important in regulating leukocyte trafficking while CXCL9/MIG has evolved towards an antibacterial function.

Several of the classical APs have chemotactic properties. For example, LL-37 can induce calcium mobilization in, and is chemotactic for monocytes, neutrophils and CTLs (De *et al.*, 2000). Humanα -defensin-1, and human β-defensin-2 are chemoattractive for monocytes/macrophages (Presicce *et al.*, 2009; Territo *et al.*, 1989) and CTLs (Chertov *et al.*, 1996).

Antibacterial redundancy among chemokines and peptides

It is important to note that, *in vivo*, APs do not exist alone but rather as a cocktail of multiple APs, each with overlapping spectra of activity. Their mode of action differs and by cooperatively attacking different targets in bacteria, one AP could unmask vulnerable structures for another AP.

In the airways, the presence of multiple factors increases the antibacterial potency of airway surface liquid (ASL). This was demonstrated by the synergistic actions of lactoferrin, lysozyme, and SLPI, some of the most abundant antibacterial components present in the airways (Singh *et al.*, 2000; Travis *et al.*, 1999). Synergistic effects of lysozyme and lactoferrin with other APs, e.g. LL-37, hBD-2, have also been confirmed (Bals *et al.*, 1998). Cole *et al* found that the microbicidal activity of nasal fluid is dependent on synergistic and additive interactions between lysozyme, lactoferrin, and APs (Cole *et al.*, 1999).

Oxidative innate immune defenses

In addition to the cell- and peptide-mediated responses of the innate immune system, phagocytes deliver microbicidal oxygen- and nitrogen-derivatives that target cellular components in bacteria, such as lipids, proteins and nucleotide bases (Fang, 2004; Hampton *et al.*, 1998).

In humans, the Nox (NADPH oxidase) family includes seven oxidase genes, Nox 1-5 and Duox 1-2 (Kuwano *et al.*, 2006). The Nox/Duox family delivers antimicrobial reactive oxygen species (ROS), including superoxide and hydrogen peroxide (Bartlett *et al.*, 2008; Rada & Leto, 2008). In phagocytes, hydrogen peroxide is converted to the microbicidal oxidant hypochlorous acid by myeloperoxidase (MPO). Hypobromous and hypothiocyanous acids are other potent MPO products (Winterbourn, 2008).

Inducible nitric oxide synthase (iNOS) generates reactive nitrogen species (RNS) derived from nitric oxide. Since nitric oxide readily reacts with superoxide to form the microbicidal peroxynitrite, there is a clear overlap between the two systems (Winterbourn, 2008). The Nox and the iNOS are the two most important pathways of the phagocyte-mediated microbial clearance (Fang, 2004; Winterbourn, 2008).

Recent studies suggest that iNOS, as well as several of the Nox/Duox family members, are expressed at high levels in epithelial cells and may be involved in innate immune functions at mucosal surfaces (Leto & Geiszt, 2006; Rada & Leto, 2008). Airway epithelial cells produce extracellular hydrogen peroxide, and the concurrent presence of lactoperoxidase (LPO) results in generation of bactericidal reactive oxidants, e.g. hypothiocyanite (Bartlett *et al.*, 2008; Leto & Geiszt, 2006; Moskwa *et al.*, 2007; Rada & Leto, 2008). Nox/Duox expression in epithelial cells is enhanced in response to microbes and proinflammatory cytokines (Rada & Leto, 2008; Sansonetti, 2004). For example, Duox2-expression, and Nox2-expression in airway epithelial cells (Harper *et al.*, 2005), and Nox1-expression in colon epithelial cells (Kuwano *et al.*, 2006), is highly induced by IFN-γ.

Streptococcus pyogenes, group A streptococcus (GAS)

Streptococcus pyogenes is a Gram-positive bacterium causing common infections such as impetigo and pharyngitis (Bisno & Stevens, 1996; Cunningham, 2000). Pharyngitis can be caused by a variety of bacteria and viruses, but GAS is the most common causative bacterium (Bisno, 1996; Fischetti, 1989). GAS can also cause severe clinical conditions such as invasive skin- and soft tissue infections, and septicemia (Bisno & Stevens, 1996; Bisno *et al.*, 2003; Cunningham, 2000; Nitsche-Schmitz *et al.*, 2007; Nowak, 1994; Stevens, 2000).

M protein and hyaluronic acid capsule

The cell membrane-anchored M protein appears as hair-like projections on the surface of the streptococcal cell wall and is composed of two polypeptide chains complexed in an α-helical coiled coil configuration that traverses the cell wall (Phillips *et al.*, 1981). M protein is released from the streptococcal cell wall spontaneously (Åkesson *et al.*, 1994) but also after proteolytic cleavage by the streptococcal cysteine protease SpeB (Berge & Björck, 1995). The initial step in colonization of the pharynx or the skin is adherence to epithelial cells (Cunningham, 2000). M protein facilitates bacterial adhesion to epithelial cells (Ellen & Gibbons, 1972) by binding to GAGs (Frick *et al.*, 2003b), mucins (Ryan *et al.*, 2001), and fibronectin (Cunningham, 2000; Nitsche-Schmitz *et al.*, 2007).

In order to invade deeper tissues, GAS has evolved numerous adhesins, including M proteins, and the hyaluronic acid capsule covering the bacterium. The adhesins bind extracellular matrix proteins and plasma proteins (Nitsche-Schmitz *et al.*, 2007; Okada *et al.*, 1994). In addition, GAS produces proteases and other enzymes that degrade tissue, thus providing access to nutrients and enhancing bacterial invasion (Nitsche-Schmitz *et al.*, 2007). M protein contains fibrinogen- , albumin- and IgG-binding regions that facilitate bacterial persistence in infected tissue (Herwald *et al.*, 2004; Åkesson *et al.*, 1994). A prerequisite for survival in tissues is the ability of pathogens to resist being killed by phagocytosis. The hyaluronic acid capsule contributes to antiphagocytotic properties of GAS (Comstock & Kasper, 2006; Fischetti, 1989; Foley & Wood, 1959; Moses *et al.*, 1997; Wessels & Bronze, 1994). In addition, different studies suggest that M protein-expressing

GAS can both resist being phagocytosed by neutrophils, and, in the case of phagocytosis occuring, survive in the phagosome by inhibiting its maturation (Fischetti, 1989; Foley & Wood, 1959; Moses *et al.*, 1997; Staali *et al.*, 2003; Staali *et al.*, 2006).

The N-terminal part of the M protein is highly variable, and as a consequence there are more than 100 different serotypes of GAS (Bisno *et al.*, 2003). Several different M protein serotypes have been isolated from severe, invasive disease. However, the dominating strain has been of the M1 serotype (Davies *et al.*, 1996; Holm, 1996). In addition, Davies *et al* demonstrated that an increased incidence of invasive disease parallels an increased proportion of infections caused by the M1 serotype (Davies *et al.*, 1996).

GAS not only adheres to epithelial cells but can also become internalized by epithelial and cells of lymphoid tissues (Dombek *et al.*, 1999; LaPenta *et al.*, 1994; Molinari *et al.*, 1997; Osterlund & Engstrand, 1995). Internalization may lead to persistence and a carrier state (Cunningham, 2000), as indicated by studies demonstrating viable intracellular GAS harbored in tonsils from patients with recurrent tonsillitis (Cunningham, 2000; Fischetti, 1989; Osterlund & Engstrand, 1997; Osterlund *et al.*, 1997). Internalized bacteria are able to survive antibiotics that act extracellularly, such as penicillins. Approximately 30 % of penicillin-treated children have surviving GAS isolated from the nasopharynx after treatment (Gerber, 1994; Osterlund & Engstrand, 1995). The role of M1 protein in internalization is not clear. Cue *et al* demonstrated that M1 acts as an invasin, when compared with M1-negative isogenic mutant (Cue *et al.*, 1998). In contrast, other groups did not observe a difference between $M1^+$ and $M1^-$ GAS in the uptake of bacteria by lymphoid tissue (Hyland *et al.*, 2009; LaPenta *et al.*, 1994). However, kinetic studies by LaPenta *et al* demonstrated that the internalization of $M1^+$ GAS by epithelial cells was more efficient than that of $M1^-$ (LaPenta *et al.*, 1994).

SIC and SpeB

Protein SIC (streptococcal inhibitor of complement) was originally isolated from the growth medium of GAS (M1 strain), and was found to inhibit the host-protective cytolytic function of the complement cascade *in vitro* (Akesson *et al.*, 1996). The protein is

associated with the cell wall but is released from the bacterial surface by proteolytic cleavage (Berge & Björck, 1995). SpeB is an extracellular cysteine protease and contributes to the virulence of GAS by its degrading of human immunoglobulins, fibronectin, and components of the extracellular matrix (Bisno *et al.*, 2003; Collin & Olsen, 2001; Schmidtchen *et al.*, 2001). Both SIC and SpeB have been shown to inhibit or inactivate APs. SIC block the antibacterial effect through binding inhibition (Egesten *et al.*, 2007; Fernie-King *et al.*, 2002; Fernie-King *et al.*, 2004; Frick *et al.*, 2003a), whereas SpeB actively degrades the APs (Egesten *et al.*, 2009; Karlsson *et al.*, 2009).

Finegoldia magna

The human microbiota consists of a diverse population of resident microorganisms (commensals) that may become pathogenic in response to an impaired epithelial barrier (opportunists). *Finegoldia magna*, formerly known as *Peptostreptococcus magnus*, is a Gram-positive anaerobic coccus. *F. magna* bacteria are commensals of the mouth, the upper respiratory and gastrointestinal tract, the skin and soft tissues, and the female genitourinary system, sites where they may also cause opportunistic infections (Murdoch, 1998). Acute and chronic skin wounds harbor a diverse microbiota and in 70-80% of both infected and noninfected wounds, anaerobic organisms are found. *F. magna* is a commonly isolated species, indicating that it is among the most virulent of the anaerobic bacteria (Bowler & Davies, 1999; Stephens *et al.*, 2003; Wall *et al.*, 2002).

It has been documented that Gram-positive anaerobic cocci and their metabolites interfere with wound healing by inhibiting fibroblast proliferation, inhibiting growth of keratinocytes and consequently reepithelialization (Stephens *et al.*, 2003; Wall *et al.*, 2002). *F. magna* bacteria produce enzymes interfering with components of the extracellular matrix (Karlsson *et al.*, 2007), thus disturbing matrix remodeling, and proteolytically active strains of *F. magna* are associated with infections causing tissue destruction (Krepel *et al.*, 1992).

SufA

The recently discovered SufA (Subtilase of *Finegoldia magna*) from *F. magna*, is a subtilisin-like serine protease, associated with the bacterial surface but also released during growth (Karlsson *et al.*, 2007). SufA interferes with innate immunity by cleaving antibacterial chemokines and peptides, a feature that may promote colonization and survival of *F. magna* (Karlsson *et al.*, 2007; Karlsson *et al.*, 2009). Cysteine-rich members of the defensins seem to be protected from degradation. On the other hand, the antibacterial α-helical structure of the CXC chemokine CXCL9/MIG (Egesten *et al.*, 2007) and the linear α-helical AP LL-37 (Bals & Wilson, 2003), which both lack cysteines, are degraded and inactivated (Karlsson *et al.*, 2007; Karlsson *et al.*, 2009).

FAF

F. magna adhesion factor (FAF) enables the binding of bacteria to the basement membrane between the epidermis and dermis, by interacting with the basement membrane protein BM-40 (Frick *et al.*, 2008). FAF is a surface protein but is also released by SufA (Karlsson *et al.*, 2009) and can bind to and inhibit the effect of antibacterial chemokines and peptides (Frick *et al.*, 2008; Karlsson *et al.*, 2009). FAF-expressing *F. magna* strains are more resistant to APs. Interestingly, FAF shows no affinity for the AP α-defensin and this peptide is not bactericidal against *F. magna* (Frick *et al.*, 2008).

Bacterial protection against antibacterial chemokines and peptides

The bacterial surface is negatively charged and the cationic feature of most antibacterial peptides and chemokines is believed to have evolved as a consequence of the long interplay during evolution. However, pathogenic bacteria have invented mechanisms to overcome the AP-mediated first line of defense. (Otto, 2009; Peschel & Sahl, 2006).

Strategies from bacteria to escape being killed by the APs have resulted in different mechanisms, including a change in the AP target to make it less susceptible. For example, many Gram-positive pathogens, such as GAS and *S. aureus* can modify teichoic acids with D-alanine, and decrease the net negative charge making the target less accessible for the cationic APs (Kristian *et al.*, 2005; Peschel *et al.*, 1999; Peschel & Sahl, 2006).

Another mechanism aims at destroying or inhibiting APs, preventing them from reaching the cytoplasmic membrane. Secreted bacterial proteases and proteins can efficiently digest and inactivate or inhibit APs. This is exemplified by SpeB and protein SIC from GAS, and SufA and protein FAF from *F. magna* (Egesten *et al.*, 2009; Fernie-King *et al.*, 2002; Fernie-King *et al.*, 2004; Frick *et al.*, 2003a; Karlsson *et al.*, 2007; Karlsson *et al.*, 2009; Schmidtchen *et al.*, 2002). However, APs that are less susceptible to digestion by bacterial proteases have evolved, and the presence of multiple disulfide bridges in the structure seems to render them more resistant (Egesten *et al.*, 2009; Peschel & Sahl, 2006). Another important observation is that bacterial proteases can degrade proteoglycans, thereby releasing GAGs that can bind and inactivate APs. These soluble GAGs can be found in clinical conditions such as chronic wounds (Baranska-Rybak *et al.*, 2006; Schmidtchen *et al.*, 2001).

Results and discussion

Paper I and II

The ELR negative CXC chemokines CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC are produced by a variety of cells both during health and disease, and were recently shown to be antibacterial (Cole *et al.*, 2001). In *Paper I*, the three chemokines where detected at high concentrations in tonsil fluid from patients suffering from streptococcal pharyngitis. CXCL9/MIG was found at the highest concentration, exceeding what is required for chemotactic activity (Xanthou *et al.*, 2003). Since the three chemokines can be produced by a variety of cells at mucosal surfaces, the source of the chemokines found in tonsil fluid could not be attributed to a single cell-type. Sauty *et* al have shown that airway epithelial cells produce the three chemokines *in vitro*. IFN-γ is necessary for the transcription, and TNF-α enhances their production (Sauty *et al.*, 1999). *In vitro,* we demonstrated that pharyngeal epithelial cells can produce the chemokines at high concentrations, either after stimulation with IFN-γ alone (*Paper II)*, or in combination with TNF-α (*Paper I)*. The levels of production *in vitro* were similar to what was seen in tonsil fluid, CXCL9/MIG being the predominant chemokine, followed by CXCL10/IP-10 and CXCL11/I-TAC.

– Epithelial recognition

The initial step in the innate immune response against bacteria is the recognition of PAMPs by PRRs on host cells. This recognition results in the production of cytokines, chemokines and APs.

LTA, PG (Bisno *et al.*, 2003; Schroder *et al.*, 2003), and protein M1 of GAS (Pahlman *et al.*, 2006), respectively, stimulates elaboration of cytokines via TLR2 on monocytes. In addition, GAS stimulates the production of the chemokines CXCL8/IL-8, CXCL9/MIG, and CXCL10/IP-10 in macrophages (Veckman *et al.*, 2003). LPS and LTA, respectively, increases the production of the chemokines hCAP-18/LL-37, and CXCL8/IL-8 in epithelial

cells from the upper respiratory tract (Nell *et al.*, 2004), and the production of the APs hBD-2 and hBD-3 in skin- and airway epithelia is induced in response to a variety of microbial pathogens as well as proinflammatory stimuli (Bartlett *et al.*, 2008; Sorensen *et al.*, 2005).

In *Paper I and II*, we stimulated pharyngeal epithelial cells with IFN-γ in combination with protein M1 or heat-killed GAS. The CXCL9/MIG production was dose-dependently increased in the presence of M1 and GAS, respectively. There was no difference in the response to M1-abscent mutant strains of GAS, demonstrating a broad epithelial recognition of GAS-derived PAMPs. The epithelial cells sense the bacteria and respond fast as indicated by an increase in CXCL9/MIG-mRNA already after three hours of stimulation with M1.

The upregulation of CXCL8/IL-8 and IL-6 expression in epithelial cells in response to GAS is mediated through NF-κB and MAPK signaling pathways (Tsai *et al.*, 2006). In *Paper II*, rapid phosphorylations of both p38 MAPK and NF-κB were seen after addition of M1 protein irrespectively of pre-incubation with IFN-γ. This demonstrates a constitutive, and not an IFN-γ-induced recognition of M1 protein. The enhanced CXCL9/MIG production after stimulation with IFN-γ was shown to involve NF-κB, as demonstrated by the decreased production after inhibition of NF-kB activation. Furthermore, microarrays revealed that, in response to IFN-γ and protein M1, genes coding for several antibacterial CC and CXC chemokines, e.g. CCL20/MIP-3α, CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, and CXCL1/GRO-α, CXCL2/GRO-β, and CXCL3/GRO-γ, were upregulated in pharyngeal epithelial cells.

– Antibacterial activity against GAS

CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC all showed a dose-dependent antibacterial activity against GAS, CXCL9/MIG being the most potent. Two regions with high isoelectric points (pIs) were identified in CXCL9/MIG, one in the N-terminal and one in the C-terminal part of the molecule. The latter region is cationic and amphipathic and has a predicted α-helical structure, all typical features of peptide-sequences exhibiting

antibacterial activity. A synthetic peptide from this region showed antibacterial activity comparable to that of intact CXCL9/MIG. Electron microscopy demonstrated gold-labelled CXCL9/MIG associated with the bacterial cell wall. Disruption of the bacterial cell wall and leakage of bacterial contents after incubation with CXCL9/MIG, demonstrates that CXCL9/MIG is bactericidal rather than bacteriostatic, and that the killing, at least in part, involves membrane-disintegration *(Paper I)*.

The physiological relevancy of APs is often discussed due to that the antibacterial effect of most APs *in vitro* is lost in 150 mmol l^{-1} salt, found in plasma. However, considering that the concentration of salt in oral fluid is 5 mmol l^{-1} (Aps & Martens, 2005), and that we could demonstrate antibacterial activity of the chemokines in 150 mmol l^{-1} salt, our results indicate that the antibacterial effect of the chemokines during streptococcal pharyngitis is highly relevant *(Paper I)*.

CXCL9/MIG was found both secreted into the incubation medium and also on the epithelial cell surface *(Paper I and II)*. In *Paper I,* I show that GAS was efficiently killed on the surface of pharyngeal epithelial cells stimulated with a combination of IFN-γ and TNF-α. CXCL9/MIG bound heparin with high affinity, a property shared by many APs. It has been proposed that antibacterial chemokines, forming oligomers when bound to GAGs, could serve as a concentrated antibacterial gradient at epithelial surfaces, thereby reducing the possibilities for bacteria to adhere (Luster, 1998). Knock-down of CXCL9/MIG by siRNA resulted in a reduced killing of GAS on the stimulated cell surface, implying an important antibacterial role of CXCL9/MIG at epithelial surfaces. We could also demonstrate an antibacterial activity in the incubation medium from pharyngeal epithelial cells stimulated with a combination of IFN-γ and TNF-α.

– Defense mechanisms of GAS

Bacteria and APs have evolved concomitantly for millions of years, hence bacteria have evolved mechanisms to defend themselves against APs. Protein SIC, released from GAS during growth, binds APs. In *Paper I,* SIC bound to CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC with high affinity, resulting in neutralization of the antibacterial activity. In

contrast, there was no interaction with the CXC chemokines CXCL1/GRO-α and CXCL8/IL-8. Interestingly, pre-incubation with SIC did not interfere with the chemotactic activity of CXCL9/MIG, CXCL10/IP-10, and CXCL11/I-TAC. Thus, if the antibacterial activity of APs becomes neutralized by factors released by the bacteria**,** there may be other ways for the host to overcome bacteria, for example by chemotactic recruitment of CTLs. In addition to neutralizing the effect of single APs, SIC dose-dependently reduced the antibacterial activity in incubation medium after stimulation of epithelial cells with a combination of IFN-γ and TNF-α. Protein SIC may explain the high frequency of GAS infections caused by strains of the M1 serotype. The unique variation in SIC between different M1 strains (Stockbauer *et al.*, 1998), and the ability to interfere with APs may facilitate the invasion at epithelial surfaces rich in APs. Previous studies show that a SIC-negative mutant M1 strain was less efficient in colonizing the mouse mucosal surface in the throat after intranasal infection (Lukomski *et al.*, 2000). We could demonstrate that SIC and heparin compete for the same binding site in CXCL9/MIG, and this attribute of SIC may reduce the ability of APs to bind to GAGs at epithelial surfaces.

Paper III

Bacteria of the human microbiota are generally regarded as beneficical to the host. *Finegoldia magna* is a commensal, but can cause opportunistic infections in the upper respiratory tract, in the skin and in soft tissues, locations where the virulent pathogen GAS often causes infection.

Keratinocytes produce various cytokines and APs in response to microbes, cytokines (Albanesi *et al.*, 2000; Schroder & Harder, 2006; Sorensen *et al.*, 2005) or injury (Nickoloff & Naidu, 1994; Roupe *et al.*, 2009; Sorensen *et al.*, 2006). In this study, IFN-γ-stimulated keratinocytes produced CXCL9/MIG and presence of GAS enhanced the transcription. However, different *F. magna* strains did not enhance the transcription, suggesting that this commensal is not inducing an immune response in epithelial cells. Similarly, Veckman *et al* demonstrated that CXCL9/MIG-production was observed in GAS-stimulated macrophages,

but not after stimulation with the nonpathogenic *Lactobacillus rhamnosus* (Veckman *et al.*, 2003).

The protease SufA, produced by *F. magna*, has previously been shown to cleave and inactivate APs, such as LL-37 and CXCL9/MIG (Karlsson *et al.*, 2007). In the present study, cleaved CXCL9/MIG was detected in supernatants from IFN-γ-stimulated keratinocytes infected with the SufA-expressing strains 505 and ALB8. No cleavage occurred when stimulating with the isogenic mutant CK05 that lacks SufA. Cleavage of CXCL9/MIG was a fast process and already after five minutes, multiple fragments had been generated. Although of various length, all fragments contained the previously identified bactericidal region of CXCL9/MIG, located in the putative amphipathic α-helix. SufA-generated CXCL9/MIG-fragments killed GAS efficiently, while *F. magna* 505 bacteria were unaffected. Both bacterial strains were highly sensitive to intact CXCL9/MIG. Interestingly, the ability of cleaved CXCL9/MIG to activate its receptor CXCR3 was retained.

The *F. magna* surface protein FAF was cleaved off and released from the bacterial cell surface by SufA. Previously, FAF has been shown to inhibit the antibacterial effect of the antibacterial peptide LL-37 (Frick *et al.*, 2008) and in the present study we could demonstrate that FAF bound CXCL9/MIG with high affinity, and dose-dependently blocked the antibacterial activity of CXCL9/MIG.

Paper IV

After recognition of bacteria, one of the earliest events in the innate immune response is the production of cytokines. IL-1β, TNF-α, and IFN-γ are three important proinflammatory cytokines that are induced in response to infections caused by bacteria, for example GAS (Goldmann *et al.*, 2007; Muller-Alouf *et al.*, 1997)

In *Paper I*, stimulation of pharyngeal epithelial cells, with a combination of IFN-γ and TNF-α, generated an antibacterial response against GAS. In this study, I compared the cytokine-

induced antibacterial response against GAS by stimulating pharyngeal epithelial cells with different combinations of IFN-γ, TNF-α, and IL-1β. Incubation media from cells stimulated with IFN-γ, alone or in combination with TNF-α showed high antibacterial activity. The combination of IFN-γ and IL-1β was less efficient, whereas no antibacterial effect was seen in the absence of IFN-γ. Previous studies have shown that the innate response in mice after intranasal infection with GAS, was dominated by interferon-responsive genes, and resulted in enhanced expression of CXCL10/IP-10 and production of CXCL9/MIG. GAS spread more readily from nasal lymphoid tissue to peripheral lymph nodes in IFN-γ knock-out mice (Hyland *et al.*, 2009). In another study, GAS-infected mice treated with anti-IFN-γ died from the infection, and mice unable to produce IFN-γ, were more susceptible to lethal infection (Raeder *et al.*, 2000).

In *Paper II,* microarrays revealed that many genes coding antibacterial chemokines and peptides were expressed and upregulated after exposing pharyngeal epithelial cells to IFN-γ. In this study, early findings indicated that the antibacterial effect was mediated by peptides and that antibacterial chemokines and/or APs could be important components. The threshold in the antibacterial effect seen after 24 hours of stimulation with IFN-γ, suggests that antibacterial chemokines and/or peptides accumulate in the cell incubation medium and reach a critical concentration over time. Electron microscopy images demonstrated a disrupted bacterial cell wall, and leakage of bacterial content, indicating that the bactericidal effect is mediated by membrane-disrupting actors. This also indicates that the effect of the cell incubation medium is bactericidal, not bacteriostatic. In *Paper I,* we demonstrated that protein SIC neutralized the antibacterial activity of incubation medium from pharyngeal epithelial cells stimulated with a combination of IFN-γ and TNF-α. In the present study, preincubation of medium from IFN-γ-stimulated cells with SIC led to a dose-dependent reduction of the antibacterial activity. This is yet another indication that the antibacterial activity present in the cell-incubation medium is peptide-mediated. Interestingly, in *Paper I,* we showed that SIC did not bind to the ELR positive CXC-chemokines CXCL1/GRO-α and CXCL8/IL-8, indicating a specificity where SIC may preferentially bind and inhibit chemokines that are efficient in killing GAS.

Characterization of antibacterial components in the cell incubation medium using LC-MS/MS and ELISAs displayed presence of several antibacterial chemokines and peptides/proteins, such as CXCL1/2/3 (GRO-α/β/γ), CXCL9/MIG, CXCL10/IP-10, CXCL11/I-TAC, CCL20/MIP-3α, NGAL, lysozyme, and SLPI. High antibacterial effect of several peptides against GAS, as exemplified by low MBC_{90}-values, was demonstrated. Of importance was the detection of lysozyme in the cell incubation medium, an antibacterial protein that can improve the bactericidal potency by synergistic interactions with other APs (Singh *et al.*, 2000). In *Paper I*, CXCL9/MIG was an important component of the bactericidal response on the surface of pharyngeal epithelial cells. In this study, CXCL9/MIG was important for the antibacterial activity in IFN-γ-stimulated cell incubation medium. This was demonstrated by a decreased killing of GAS after inhibiting the production of CXCL9/MIG in pharyngeal epithelial cells.

Recent research suggests that reactive oxygen and nitrogen species are important in the bactericidal defense at airway epithelial surfaces and that the response is enhanced by IFN-γ (Harper *et al.*, 2005; Moskwa *et al.*, 2007; Rada & Leto, 2008). However, I found no reduction in the bactericidal activity of IFN-γ-stimulated cell incubation medium after inhibition of either the oxidase-system or the NO-synthase. In addition, the *in vitro* model I used, consisting of mono-layered epithelial cells, lacks peroxidases, such as, lactoperoxidase (LPO) that could convert ROS and RNS to highly bactericidal products. This indicates that ROS and RNS have no or minor roles in mediating the bactericidal effect seen in this study.

Conclusions

- The epithelium not only serves as a physical barrier, but is important in innate recognition of pathogenic bacteria that may result in bactericidal responses.

- The epithelial response to pathogens affects the transcription, translation, and release of multifunctional peptides that are both antibacterial and chemotactic. Bactericidal activity can be found both on the surface of epithelial cells and also released to the environment, representing a powerful local host defense mechanism against colonizing and invading pathogenic bacteria.

- Inflammatory mediators direct the epithelial response. The cytokine IFN-γ induces an antibacterial response in epithelial cells, demonstrating its importance, orchestrating the first line of defense against pathogenic bacteria at epithelial surfaces.

- During evolution, pathogenic bacteria have evolved defense mechanisms in order to circumvent peptide-mediated host defenses, for example by release of AP-neutralizing proteins and AP-degrading enzymes.

- The ability of *F. magna* to avoid recognition by epithelial cells, and, as a consequence, the antibacterial response including APs, may serve to create an ecological niche for bacteria of the human microbiota during pathogen-induced immune responses.

Acknowledgements

Thank you

– Arne Egesten, my supervisor, for your time, trust and encouragement. Thank you for sharing your impressive knowledge, you are truly a human encyclopedia. I have really appreciated sharing room with you, your good sense of humor and your weird stories about migrated relatives.

– Mattias Collin for sharing your valuable time and for your enthusiasm in trouble-shooting. I have enjoyed our brain-storming meetings.

– Ole Sørensen, my co-supervisor, and **Inga-Maria Frick** for teaching me melt curves and growth curves, for great know-how in science and for good collaboration.

– Lars Björck for your generosity and comfortable leadership, contributing to an inspiring workplace with lots of laughs.

– Pia for being a good friend, for sharing the same high-quality-sense of humor, for assistance in the lab and for reminding me of "how a good mother ought to behave".
– **Maria B** and **Matthias M** for great EM-pictures. –**Anders O** and **Johan M** for proteomics. – **Anita** for "being there" with invaluable help, advice and After Eight.

– All present and former B14-persons, – **Andreas, Anneli, Artur, Axel, Daniel, Emma, Fredrik, Gopinath, Hans, Heiko, Ingbritt, Ingrid, Jakob, Jill, Jörgen, Karin, Kristofer, Lisa, Magnus O, Magnus R, Malgorzata, Malin, Marta, Martina, Mikael, Mina, Monica, Mukesh, Praween, Rolf, Sara S, Sara K.S, Silla, Sofie, Sonja, Tobba, Torsten, Ulla, Victoria** and **Wasen.** Thank you all for contributing to a great atmosphere with your positive, helpful, and interesting personalities!

– Oonagh and **Barbara** for friendship, for being endless sources of inspiration (and KI-gossip), and for your advice in science. **– Anna** and **Maria A** for dinners, talking, and encouragement.

– Jakob and **Bo** for unforgettable entertainment at the Christmas party 2007!

– Markus for being a great off-pist companion, and accompanied by **Adam, Pontus, Erik** and **Christofer,** thanks for many laughs and fruitful conversations about travels, skiing, Zlatan, movies, Innate immunity, music, Zlatan, skiing, movies, and….. Zlatan.… Drinking coffee with you has been well-invested time!

– **Former PhD students Sara, Helena** and **Björn** for great times at B14, and thanks Björn for all laughs while juggling and playing table-tennis.

– **Jessica, Ullis, Johan**, **Björn,** and **Martin** for many good years in Linköping and for making them so unpredictable!

– **Good old friends** for being good and old, and for coping with my absence these last months, I promise to return to Planet Social in February. Thank you Anna, Paula, Linda for your unexplainable interest in [strĕp'tə-kŏk'əs pī'ə-jĕn'es] during my friday-seminar-rehearsals.

– **My** f**amily** for being who you are: caring, easy-going and fun to spend time with!

– **Ella** for making me focus on Curious George instead of science, for letting me sleep until late in the mornings, and for being such a lovely little thing!

– **Simon** for your patience, your infinite source of energy, your love, and for lots of fun during these years. Thank you also for Wednesday-cakes, and for being the maid of my dreams!

Populärvetenskaplig sammanfattning på svenska

Människans övre luftvägar och hud är utsatta för sjukdomsframkallande bakterier. Epitelceller utgör en barriär, epitel, som gör det svårt för bakterierna att ta sig in i kroppen. Epitelet har även visat sig ha en viktig roll i det inledande, ospecifika immunsförsvaret. Vid närvaro av bakterier och/eller om det uppstår en skada på epitelet, inleds ett inflammatoriskt svar i epitelcellerna som resulterar i produktion av inflammationsdrivande ämnen, t.ex. cytokiner och kemokiner. Cytokiner är små ämnen som ansvarar för kommunikationen mellan immunförsvarets celler. Kemokiner är cytokiner som aktiverar och sätter vita blodkroppar i rörelse. Kemokiner produceras av en mängd olika celltyper, i både normala tillstånd och under sjukdom. Vissa kemokiner kan döda bakterier och epitelet tillverkar även andra små proteiner som kan döda bakterier som kallas antibakteriella peptider (AP). Dessa antibakteriella ämnen fungerar som kroppsegna antibiotika. Effekten är snabb och bakterier kan avdödas innan det specifika immunförsvaret med aktivering av vita blodkroppar och produktion av antikroppar hunnit komma igång. Den bakteriedödande egenskapen, i kombination med att de tillverkade ämnena även aktiverar och dirigerar vita blodkroppar, visar epitelets viktiga roll i det inledande immunförsvaret mot bakterier.

Halsfluss kan orsakas av bakterien *Streptococcus pyogenes*, grupp A streptokock (GAS). GAS kan även orsaka mer allvarliga sjukdomar som angriper hud och mjuka vävnader och om bakterierna kommer ut i blodomloppet kan det leda till livshotande tillstånd. Epitelets försvarsmekanismer är därför av kritisk betydelse för att begränsa och eliminera bakteriernas spridning. Ofarliga bakterier i vår normalflora kan bli sjukdomsframkallande som följd av en epitelskada, virusinfektion, eller hos personer med nedsatt immunförsvar. Normalflore-bakterien *Finegoldia magna* kan under sådana förhållanden bland annat orsaka infektioner i hud och mjuka vävnader .

Sammanfattning av delarbete I, II, IV

CXCL9/MIG, CXCL10/IP-10 och CXCL11/I-TAC är kemokiner vars produktion är beroende av IFN-γ och förstärks av TNF-α. IFN-γ och TNF-α är cytokiner som tillverkas i kroppen vid infektion..

I arbete I kunde vi mäta höga nivåer av de tre kemokinerna, framförallt CXCL9/MIG, i tonsillvätska från patienter med streptokock-halsfluss. Dessa nivåer var mycket högre än de som krävs för att rekrytera vita blodkroppar till infektionsplatsen. I en modell av streptokock-halsfluss såg vi att epitelceller, motsvarande de i halsen/tonsillerna, tillverkade de tre kemokinerna efter stimulering med IFN-γ och TNF-α. Epitelcellerna kunde även känna närvaro av GAS och dess ytproteiner, med ökad tillverkning av de tre kemokinerna som följd (Arbete I och II).

GAS som placerades på ytan av celler som stimulerats med IFN-γ och TNF-α eller i lösningen som cellerna stimulerats i avdödades effektivt (Arbete I och IV). Om jag stimularade celler med TNF-α utan IFN-γ närvarande överlevde GAS (Arbete IV). Detta tyder på att cytokinen IFN-γ har en viktig roll i det epiteliala immunsvaret mot bakterier. Eftersom CXCL9/MIG var den kemokin som tillverkades i störst mängd, användes syntetiskt framställt CXCL9/MIG i avdödningsförsök mot GAS. CXCL9/MIG var effektiv i avdödningen och när jag blockerade tillverkningen av CXCL9/MIG i epitelceller, fann jag att bakteriavdödningen minskade avsevärt (Arbete I och IV). Analys av den antibakteriella lösningen visade att många antibakteriella kemokiner och peptider fanns i höga koncentrationer. Dessa kemokiner och AP användes i avdödningsförsök där jag fann att de dödade GAS effektivt (Arbete IV). GAS kunde öka sin egen överlevnad genom att frisätta ett protein, SIC, som hämmade både CXCL9/MIG och den avdödande aktiviteten i lösningen som celler stimulerats i (Arbete I och IV).

Resultaten visar att epitel i övre luftvägarna kan känna igen grupp A streptokocker och dess ytproteiner med ökad antibakteriell aktivitet som följd. GAS kan i sin tur försvara sig mot det antibakteriella svaret genom att frisätta proteinet SIC. Cytokinen IFN-γ måste vara närvarande för att epitelcellernas svar ska bli antibakteriellt. Antibakteriella peptider och kemokiner är ansvariga för en stor del av bakterieavdödningen, där ffa den IFN-γ-beroende kemokinen CXCL9/MIG är viktig.

Sammanfattning av delarbete III

Epitelceller i huden reagerade inte på närvaro av normalflore-bakterien *Finegoldia magna*. I motsats reagerade de på den sjukdomsframkallande bakterien GAS med hög avläsning av CXCL9/MIG-genen som följd. Intakt CXCL9/MIG dödade *F. magna* och GAS, men *F. magna* frisatte ett protein (SufA) som kunde klyva CXCL9/MIG. De resulterande fragmenten kunde inte längre döda *F. magna* men var fortfarande effektiva mot GAS. FAF, ett *F. magna*-protein som är viktigt för bakteriens vidhäftande till vävnader, frisattes från bakterien med hjälp av SufA och hämmade den avdödande effekten av CXCL9/MIG.

Resultaten visar att *F. magna*, i motsats till GAS, inte startar ett immunsvar i epitelceller i huden. Försvarsmekanismerna hos *F. magna* kan förklara hur normalflore-bakterier kan överleva i hud och på slemhinnor trots närvaro av bakteriedödande ämnen som tillverkats i det immunsvar som väckts av en sjukdomsframkallande bakterie som t.ex. GAS.

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