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Vorstand: Professor Dr. med. Matthias Frosch

Ehem. Vorstand: Professor Dr. Dr. h. c. mult. Jörg Hacker

CEACAM3-mediated phagocytosis of human-specific bacterial pathogens involves the adaptor molecule Nck

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Lisa Peterson

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Referent: Professor Dr. Dr. h. c. mult. Jörg Hacker

Koreferent: Professor Dr. med. Dr. rer. nat. Bhanu Sinha

Dekan: Professor Dr. med. Matthias Frosch

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Die Promovendin ist Ärztin.

Meinen Eltern.

Contents

1 Introduction

`Immunity refers to the global ability of the host to resist the predation of microbes that would otherwise destroy it.´ Hoebe, Janssen et al. 2004

The 20th century with its discovery of antibiotics and the development of vaccination strategies has seen breakthrough medical advances in the battle against infectious diseases. However, despite extensive use of these drugs, microbial diseases continue to be a leading cause of morbidity and mortality worldwide (Finlay and Cossart 1997). With the emergence of antibiotic resistances, increasing global mobility and the lack of innovative antimicrobial strategies, pathogens thought to be defeated are resurfacing while novel pathogens are recognized in formerly unknown contexts. The astonishing ability of the microbes to resist immunological and pharmacological strategies and their continuous adaptation to changing environmental conditions has fuelled scientific interest in bacterial pathogenesis. The quest for understanding the workings of pathogens on a molecular level has revealed just how intricately intertwined microbial pathogens have become with their host´s immune system. In search for innovative, specifically targeted and thus possibly more efficient antimicrobial agents, infection biologists and immunologists are now deciphering the molecular crosstalk in the host-pathogen relationship as the basis of microbial disease.

Although ongoing research on various pathogens and their virulence mechanisms has revealed the high degree of strategic diversification, there are some common rules about the success of bacterial infection, which include

- Colonisation of the host by adherence to epithelial cells
- Variation of surface molecules to avoid humoral defence mechanisms
- Secretion of immune-escape factors such as IgA proteases to inactivate humoral factors

- Invasion of host cells
- Resistance to or survival in phagocytic cells and in the blood serum

On a molecular level however, different species display a unique repertoire of virulence factors and strategies to secure their specific niche (Finlay and Cossart 1997; Finlay and Falkow 1997). Many pathogens are also capable of `hijacking´ host signaling pathways and organelles to establish and maintain infection and warrant dissemination (Finlay and Cossart 1997; Gruenheid and Finlay 2003). While some species bank on unspecific and highly detrimental strategies, other pathogens specialize on long term colonization. The degree of host specificity thereby seems to correlate with the ability of the pathogen to preserve its habitat, a delicate balance of virulence and containment reflecting continual host-pathogen coevolution.

1.1 Neisseria gonorrhoeae

A model organism to study the balance of pathogen clearance and bacterial pathogenicity can be found in *N.gonorrhoeae*. Highly adapted to survive in its sole natural host the human being, it displays a wide array of pathogenicity factors and evasion strategies enabling it to breach the integrity of host tissues, escape immune surveillance by the human immune system and persist to multiply and disseminate to other host organisms. *Neisseria gonorrhoeae* and *N. meningitidis* are two exclusively human-pathogenic members of the family *of Neisseria,* gram-negative diplococci, specialised in the colonisation of mucous membranes. Non-pathogenic members of this genus include *N. cinerea*, *N. mucosa* and *N. subflava,* which form part of the physiological microflora. *N.gonorrhoeae* is the bacterial agent causing gonorrhea, a veneric disease in man mainly involving the mucous membranes of the urogenital tract. Although potentially curable, the disease is still one of the most commonly reported sexually transmitted diseases in the western hemisphere, with 25-40.000 suspected annual cases of gonorrhea in Germany and 339.593 cases documented in the United States in 2005. Moreover, prevalence of antibiotic resistance of *Neisseria gonorrhoeae* to common treatment regimens is

increasing, thus contributing to a recent rise in the incidence of gonorrhea in the USA, Canada and western european countries (Robert-Koch-Institut 2001; Robert-Koch-Institut 2004; CDC 2007; Newman, Moran et al. 2007). The sheer numbers of gonococcal infections world-wide, an estimated 60 million cases annually, as well as its association with other veneric diseases such as HIV-1 and chlamydial infections render it an important public health issue (Hillis, Nakashima et al. 1994; Cohen 1998; Tapsall 2005).

1.1.1 Clinical picture

Gonococci employ a panoply of specifically adapted strategies to evade, interact with and exploit the human immune system during the different phases of infection, mostly causing only moderate disruption of host tissue. Consequently, 10-50% (gender differences) of infections remain asymptomatic and thus undetected, enabling bacteria to persist within and be transmitted to other hosts over considerable periods of time. Only 15-30% cause complications, like acute pelvic inflammatory disease and sterility, and just 1-5% of all infections result in systemic dissemination leading to arthritis, dermatitis, endocarditis or even meningitis (Handsfield 1990; Tapsall 2002; Tapsall 2005). Acute disease is associated with a strong inflammatory response, whose hallmark is a purulent exsudate from cervix and urethra. This `gonorrhea´ consists mainly of gonococci attached to or ingested by neutrophils and monocytes, indicating that the innate immune system plays a major role in this immunologic response (Rest and Shafer 1989; Shafer and Rest 1989). Whether gonococcal association with human leukocytes results from a host defense strategy or represents a bacterial immune evasion strategy, has long been a matter of scientific debate (Veale, Finch et al. 1976; Drutz 1978; Mezzatesta and Rest 1983; Fischer and Rest 1988; Farrell and Rest 1990; Belland, Chen et al. 1992).

1.1.2 Adhesion and invasion - molecular processes of infection

Successful colonization of mucosal surfaces requires firm attachment to host surface structures (Finlay and Falkow 1997; Abraham, Jonsson et al. 1998) and

the evasion or inactivation of targeted antimicrobial agents. The mucosa, as a first barrier impeding invasion, features several protective mechanisms including an electronegative surface charge, cleansing by mucous flow and ciliae, exfoliation of surface cells as well as the secretion of H+, microbicidal peptides and IgA antibodies. Experimental evidence suggests that *Neisseria*, rather than breaking up tissue integrity, can access the intact epithelial layer by interacting with epithelial structures in a receptor-like fashion to induce host signaling pathways. On a molecular level, colonization of mucous membranes is modelled in distinct and highly conserved steps (Nassif 1999). These processes involve tailor-made virulence factors, expressed at different stages of the infective cycle (Tapsall 2002) which orchestrate the sequential interaction with host structures.

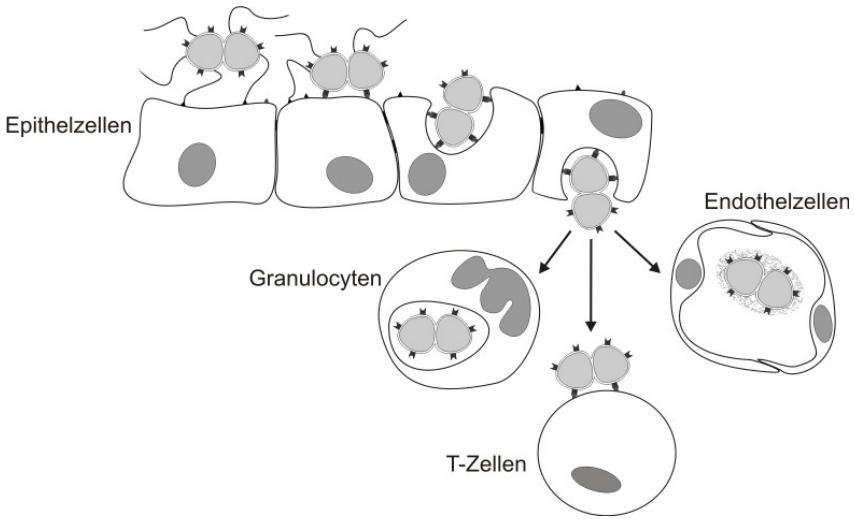


Fig.1.1 **Sequential interaction of pathogens with host structures.**
1. Primary anchorage by retractable type IV pili via CD46 2. Tight secondary adhesion via Opa-proteins binding to epithelial CEACAM1, CEA and CEACAM6. 3. Internalization and subsequent transcytosis to subepithelial spaces. 4. Systemic dissemination and exposure to cells involved in immune surveillance. (taken from: `CEACAM1: Contact-dependent control of immunity´, (Gray-Owen and Blumberg 2006))

Initial attachment to host cells is facilitated by a surface organelle of *Neisseriae*, the type IV pilus. Following this pilus-mediated adhesion, a more intimate contact develops, allowing for further bacteria-host cell interactions mainly

involving bacterial outer membrane proteins, the opacity (Opa) proteins. Opa proteins interact with host cell receptors of the CEACAM-family (Chen and Gotschlich 1996; Virji, Makepeace et al. 1996) or with heparan sulphate proteoglycans (Chen, Belland et al. 1995; van Putten and Paul 1995), and both interactions are associated with the invasion of the epithelial cell layer (Nassif 1999) (Fig. 1.1).

1.1.3 The pilus

The significance of the pilus for successful infection is reflected by the finding that non-piliated variants are not able to cause disease (Swanson, Robbins et al. 1987). Being a filiform polymeric structure equipped with specific adhesion molecules, the pilus allows for tight contact with the target cell from a distance (Swanson 1973; Heckels 1989; Virji, Kayhty et al. 1991). Intriguingly, pili are capable of retraction, thus establishing spatial proximity between bacterium and host cell, as well as being responsible for a form of bacterial motility, so-called twitching motility (Merz, So et al. 2000). The mechanic trunc of the pilus is made up of several subunits: a major PilE or Pilin protein subunit as well as various pilus-associated proteins (Meyer, Billyard et al. 1984). PilE is arranged repetitively to form a helical structure holding only a few copies of a 110 kDa adhesion protein named PilC at its tip and shaft (Rudel, Scheuerpflug et al. 1995). The epithelial receptor of PilC has been identified as the human CD46 protein or ´membrane cofactor protein` (Kallstrom, Liszewski et al. 1997). Physiologically a complement regulatory membrane glycoprotein (Lee, Bonnah et al. 2002), CD46 is ubiquitously expressed on nucleated cells and involved in the prevention of cell damage by autologous complement factors. However, it also serves as a pathogen receptor for *Neisseria*, *Streptococcus pyogenes* as well as measles virus and human herpes virus (Dorig, Marcil et al. 1993; Okada, Liszewski et al. 1995; Nassif, Pujol et al. 1999; Santoro, Kennedy et al. 1999). Attachment of piliated gonococci to target cells however, is not simply a mechanic process since engagement of CD46 results in tyrosine phosporylation-dependent signal transduction via its cytoplasmic tail, which is mandatory for adhesion (Kallstrom, Islam et al. 1998; Kallstrom, Blackmer Gill

et al. 2001; Tobiason and Seifert 2001; Lee, Bonnah et al. 2002). Once attached, other surface structures including lipooligosaccharides (LOS) and Opa proteins provide stabilizing interaction and induce the internalization process while pili are subsequently dismantled.

1.1.4 Opacity proteins

While pili are vital for initial attachment, bacterial *invasion*, the prerequisite of invasive disease, has been assigned to another family of neisserial integral membrane proteins: the **op**acity **a**ssociated outer membrane proteins, short Opa-proteins. Next to porins and lipooligosaccharides, Opa proteins are the main constituents of the outer membrane of gonococci. Named for their ability to induce intergonococcal adhesion causing an opaque phenotype of agar-grown colonies, these proteins are of paramount importance in the gonococcal – host cell interaction process (James and Swanson 1978; King and Swanson 1978; Swanson 1978). Although dispensable for primary attachment, they are essential for tight adhesion to and invasion of epithelial cells, and bacterial isolates from natural infections of the urogenital tract or rectum express at minimum one Opa protein. Moreover, *Neisseria* reisolated from human volunteers inoculated with non-opaque gonococci almost invariably have taken on an opaque phenotype upon recovery (Swanson, Barrera et al. 1988; Jerse, Cohen et al. 1994).

Opa-proteins constitute a highly diverse family of closely related, size-variable outer membrane proteins. Made up of eight transmembrane domains arranged as anti-parallel β-strands and four extracellular loops with both the N- and C-terminus situated in the periplasm, sequence diversity is predominantly observed within the first three loops, named semivariable loop (SV), `hypervariable domain1´ and `hypervariable domain2´ (HV1/2) (Bhat, Gibbs et al. 1991; Malorny, Morelli et al. 1998). By point mutations in these domains and modular exchange of domains between different Opa-proteins, new variants constantly emerge. The number of Opa gene loci varies between neisserial species and ranges from 3-4 in meningococci to 12 in gonococci. Moreover,

Opa protein expression undergoes phase variation, a genetically-based mechanism to modify the expression of virulence determinants. As a result, heterogeneous populations of Opa-expressing gonococci arise, with individual bacteria expressing none to multiple Opa membrane proteins. Apart from enabling bacteria to evade adaptive immune responses, differential expression of Opa proteins seems to account for their preferential interaction with the different tissues of the body, a phenomenon called cell tropism (Kupsch, Knepper et al. 1993).

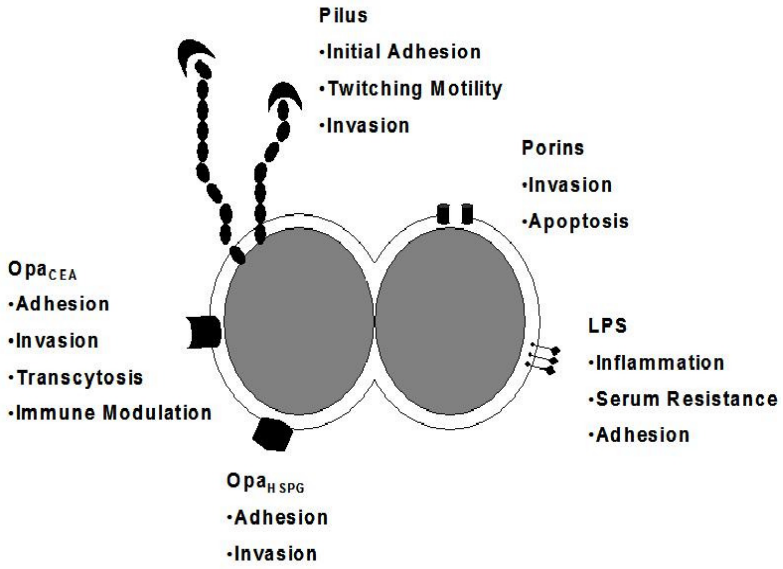


Fig.1.2 **Important factors of virulence of *N.gonorrhoeae***
a) The Pilus, mediates intitial attachment and establishes spatial proximity to enable close physical contact between bacteria and host cells.
b) The interaction of Opa-proteins with Cell Adhesion Molecules (CEACAMs) and Heparansulphateproteoglycanes (HSPGs) mediates invasion of epithelial cells.
c) LOS/LPS is capable of stimulating the generation of various cytokines in endothelial and immune cells, including TNF-α, IL 8 or IL-α. Moreover, sialylation of LOS increases serumresistance and may protect from phagocytosis by PMNs and recognition by the complement system.
d) Porins act as active gated ion channels to allow a calcium influx and may thus induce apoptosis in epithelial and phagocytic cells. The expression of certain serotypes seems to correlate closely with invasive, disseminated disease.
(modified after: `Host cell invasion by pathogenic *Neisseria´* (Dehio, Gray-Owen et al. 2000))

1.2 Interaction of gonococci with epithelia and endothelia

The invasion of epithelial cells is a common theme among bacterial pathogens and seems to serve several functions. Foremost, it represents an immune evasion mechanism, as gonococci are temporarily shielded from both humoral and cellular agents of the immune system. Secondly, endocytotic entry into epithelial cells has been shown to be part of vesicular transcytosis of bacteria to subepithelial spaces, used as a platform for replication and further spreading. There has also been speculation that epithelial shedding of bacteria-containing cells may promote spreading to potential hosts (Apicella, Ketterer et al. 1996), however recent evidence points to the specific inhibition of this innate immune mechanism of exfoliation by gonococci (Muenzner, Bachmann et al. 2008). To induce these infection-promoting events, bacteria communicate with host cells via cellular receptor proteins that physiologically serve intercellular adhesion or connect to the extracellular matrix. Well-known examples of such diversion of host cell mechanisms are the integrin-mediated internalization of *Yersinia spp.* or the cadherin-mediated uptake of *Listeria spp.* (Isberg, Voorhis et al. 1987; Gaillard, Berche et al. 1991; Mengaud, Ohayon et al. 1996). *N.gonorrhoeae*, as already mentioned, has been identified to interact specifically with HSPGs (Chen, Belland et al. 1995; van Putten and Paul 1995) and CEACAM molecules on eukaryotic cells to induce internalization, transcytosis and the modulation of gene expression (Chen, Belland et al. 1995; van Putten and Paul 1995; Chen and Gotschlich 1996; Virji, Watt et al. 1996; Chen, Grunert et al. 1997; Gray-Owen, Dehio et al. 1997; Dehio, Gray-Owen et al. 1998; Dehio, Gomez-Duarte et al. 1998; Muenzner, Bachmann et al. 2008).

1.2.1 Interaction of Opa-proteins with heparansulphate-proteoglycanes

Heparane-sulphate-proteoglycanes (HSPGs) are widely expressed cell surface proteins on eukaryotic tissues. Typical members of the HSPG family of proteoglycans are glypican, cerebroglycan, betaglycan and the members of the syndecan family. They are either GPI-anchored (glypicans or cerebroglycan) or contain a tyrosine-rich cytosolic domain (syndecans and betaglycan) and exhibit long covalently attached heparan- and chondroitin-sulphate-containing

glucosaminoglycane side chains, which are strongly negatively charged. They bind to a variety of growth factors and extracellular matrix proteins and thereby take part in the regulation of various cellular functions, including proliferation, migration, adhesion and cytoskeletal organization. Despite their wide distribution and extensive involvement in cellular processes, only few gonococcal Opa_{HSPG} variants like Opa_{50} of the MS11 strain and $Opa_{27.5}$ of the VP1 strain have been shown in vitro to be to capable of mediating efficient binding to and invasion of epithelial cells (Kupsch, Knepper et al. 1993; Chen, Belland et al. 1995; Dehio, Gray-Owen et al. 1998). Likewise, only two members of HSPGs, syndecan 1 and 4 (Dehio, Gomez-Duarte et al. 1998; Freissler, Meyer auf der Heyde et al. 2000) mediate gonococcal invasion of epithelial cells. Both HSPGs are syndecans carrying an intracellular tyrosine-containing domain and experimental evidence points to extensive signaling events triggered by Opa_{HSPG} carrying NGO. In Chang conjunctiva cells, this signaling cascade involves phosphatidylcholin-dependent phospholipase C (PC-PLC) and acid sphingomyelinase (ASM), producing the second messengers diacylglycerol (DAC) and ceramide (Grassme, Ireland et al. 1996; Grassme, Gulbins et al. 1997). In other epithelial cell lines e.g. CHO- or HeLa-cells an alternative pathway is triggered, involving the serum component vitronectin and activation of protein kinase C. Vitronectin binding to Opa_{HSPG} leads to the co-ligation of HSPGs and the vitronectin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin, or analogously $\alpha_5\beta_1$ via fibronectin in HEp2 cells (Duensing and van Putten 1997; Gomez-Duarte, Dehio et al. 1997; Dehio, Freissler et al. 1998; van Putten, Duensing et al. 1998), thus stimulating bacterial uptake in an α_v-integrin dependent manner. However, vitronectin and fibronectin are serum factors which usually have no access to mucous membranes and HSPGs seem to be restricted to the basolateral membrane in columnar and cuboidal epithelia, the target tissues of *Neisseria gonorrhoeae* (Carey 1997). It therefore seems unlikely that Opa_{HSPG} mediate apical acces to epithelia in vivo. Models that have addressed this problem mimicking in vivo conditions with polarized T84 monolayers propose a role in the colonization of the basolateral membrane as a platform to penetrate into deeper tissue layers and for reseeding of mucous

membranes (Mosleh, Boxberger et al. 1997; Dehio, Freissler et al. 1998; Wang, Gray-Owen et al. 1998). It has also been suggested, that the thinning and disruption of membrane integrity in the infection process may lead to an increased accessibility of HSPGs to gonococci (Apicella, Ketterer et al. 1996; Mosleh, Boxberger et al. 1997).

1.2.2 Interaction of Opa-proteins with CEACAM molecules

The vast majority of Opa-proteins mediating attachment and invasion of host cells interact with receptors of the family of **c**arcino**e**mbryonic-**a**ntigen-related **c**ell **a**dhesion **m**olecules (Bradley, Griffiths et al. 2005; Muenzner, Rohde et al. 2005; Pils, Gerrard et al. 2008). CEACAM-molecules, formerly known as CD66 proteins, are immunoglobulin-related glycoproteins (Thompson, Grunert et al. 1991; Skubitz 1995; Beauchemin, Draber et al. 1999; Kuespert, Pils et al. 2006). The human CEACAM family comprises 12 closely related proteins and several isoforms which mediate homo- and heterotypic interactions, thus contributing to cell-cell-adhesion as well as modulation of signal transduction. Interestingly, CEACAMs have been found to serve as cellular receptors for a variety of gram-negative bacterial pathogens including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae* and *Moraxella catarrhalis, Salmonella spp.* and *E.coli* (Leusch, Drzeniek et al. 1991; Chen and Gotschlich 1996; Virji, Makepeace et al. 1996; Hill, Toleman et al. 2001; Hill and Virji 2003).

All CEACAMs possess at least one amino-terminal immunoglobulin variable domain-like extracellular region (IgV-like domain) and it is this region which determines homo- and heterotypic interaction (Obrink 1997; Kuespert, Pils et al. 2006). Following this IgV-like domain, some members also have up to six Ig-constant-like domains (IgC) and either a transmembrane plus a cytoplasmic domain (CEACAM1, 3 and 4) or a GPI membrane anchor like CEA and CEACAM6, 7 and 8 (Beauchemin, Draber et al. 1999; McCaw, Schneider et al. 2003). Additionally, CEACAM1, 3 and 7 exist in different splice variants, which vary in the length of their cytoplasmic domain as well as in the number of their

extracellular constant-like domains (Nagel, Grunert et al. 1993; Hammarstrom 1999) (Fig. 1.3).

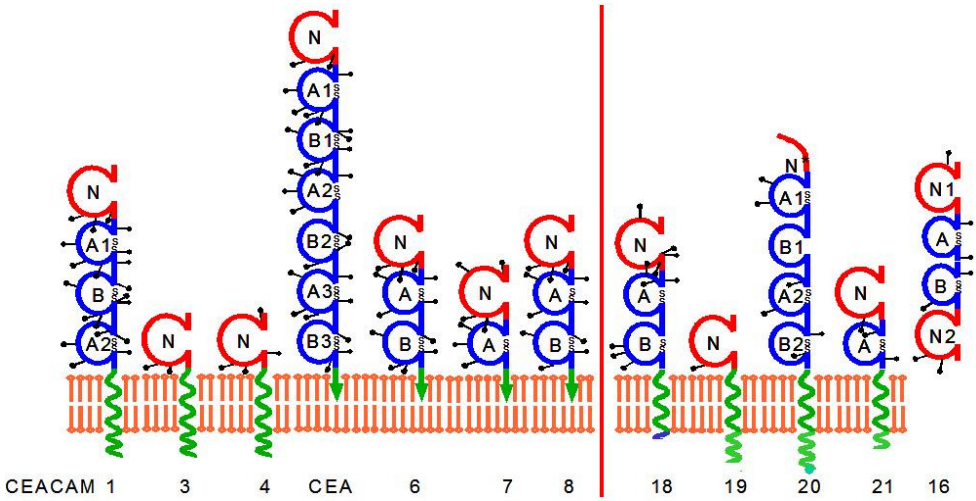


Fig.1.3 **Members and structure of the human CEA-family of cell surface molecules**
C - amino-terminal Ig-variable-like extracellular domain (IgV)
C - Ig-constant-like domains (IgC)
—● - glycosylation sites
CEACAMs are either GPI-anchored or possess a transmembrane and a cytoplasmic domain. Only seven of the twelve CEACAM-genes are actually expressed in humans, separated by the red bar from the pseudogenes of CEACAMs 16, 18-21
(adopted from the CEA homepage)

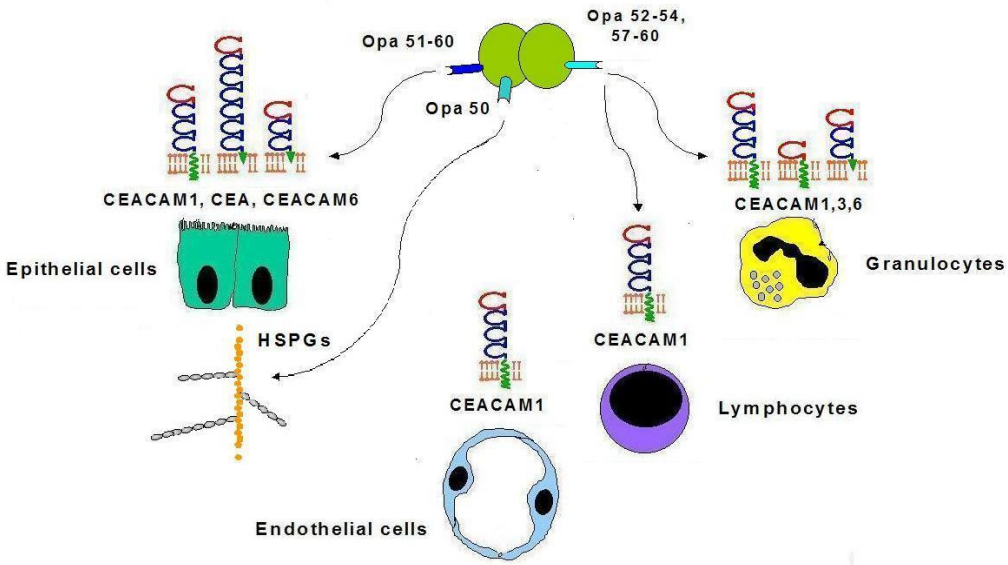
CEACAM 1, 3, 6 and CEA but no other member of this family are capable of interacting with one or more Opa proteins and are thus referred to as `CEACAM receptors´ (McCaw, Schneider et al. 2003; Hauck, Agerer et al. 2006). By contrast with the carbohydrate-mediated binding of Opa_{HSPG} to HSPG, and despite rich glycosylation of CEACAM molecules, this interaction is a pure protein-protein interaction (Bos, Grunert et al. 1997; Chen, Grunert et al. 1997; Bos, Kuroki et al. 1998; Billker, Popp et al. 2000; Muenzner, Dehio et al. 2000). Binding of all Opa_{CEA} proteins has been located to the non-glycosylated C´CFG face of the N-terminal Ig-like domain of CEACAMs (Bates, Luo et al. 1992; Virji, Watt et al. 1996; Billker, Popp et al. 2000; Kuespert, Weibel et al. 2007).

CEACAM family members are of wide tissue distribution, yet predominantly found on epithelial and hematopoetic cells (Hammarstrom 1999; Kuespert, Pils et al. 2006). Their extensive expression implies participation in numerous cellular functions, few of which however are understood to date. What we know is that these molecules physiologically are capable not only of cell-cell-adhesion via both homo- and heterotypic interactions (Benchimol, Fuks et al. 1989; Oikawa, Inuzuka et al. 1991; Kuespert, Pils et al. 2006) but have also been implicated in the regulation of processes such as the hepatic uptake of insulin, migration of PMNs, the regulation of endothelial cell proliferation as well as T-cell responses and tumor suppression (Stocks and Kerr 1993; Obrink 1997; Poy, Yang et al. 2002; Iijima, Neurath et al. 2004; Kuespert, Pils et al. 2006; Leung, Turbide et al. 2006; Leung, Turbide et al. 2008).

Interaction of CEACAMs with pathogenic ligands in a receptor-like fashion has been demonstrated for various bacterial pathogens (Chen, Grunert et al. 1997; Berger, Billker et al. 2004; Schmitter, Agerer et al. 2004; Hauck, Agerer et al. 2006). Interestingly, CEACAM-binding has also been shown for a viral pathogen in mice, mouse hepatitis virus (MHV), which seems to gain access to its host via the N-terminal domain of CEACAM1 (Dveksler, Dieffenbach et al. 1993; Godfraind, Langreth et al. 1995). Recently, a CEACAM1 deficient mouse was engineered, lacking this receptor (CEACAM1a$^{-/-}$). These `CEACAM1-knockout´ mice are completely resistant to MHV infection and no virus can be recovered from target tissues of MHV after inoculation with the virus (Blau, Turbide et al. 2001; Hemmila, Turbide et al. 2004).

Like HSPGs, CEACAMs are differentially expressed on a wide range of epithelial, endothelial and hematopoietic cells and Opa-dependent interactions have been observed with all of these cell types (Chen and Gotschlich 1996; Virji, Makepeace et al. 1996; Chen, Grunert et al. 1997; Gray-Owen, Dehio et al. 1997; Wang, Gray-Owen et al. 1998; Muenzner, Naumann et al. 2001; McCaw, Schneider et al. 2003). Expression patterns differ in type and quantity of expressed CEACAM members: while CEACAM1 and 6 display a wide tissue

distribution, CEA and CEACAM7 expression is limited to epithelial cells and CEACAM3, 4 and 8 are restricted to granulocytes. Most tissues thus coexpress CEACAM1 plus one or several other family members (Thompson, Grunert et al. 1991; Nagel, Grunert et al. 1993; Gray-Owen, Dehio et al. 1997) (Fig. 1.4).



Tissue	CEACAM1	CEACAM3	CEA	CEACAM6	HSPG
Epithelia apically	+		+	+	
Epithelia basolaterally					+
Endothelial Cells	+				
Lymphocytes	+				
Granulocytes	+	+		+	

Fig.1.4 **Opa protein binding affinity to CEACAM molecules and distribution of Opa-binding CEACAM molecules determines tissue tropism of gonococci**
Opa-proteins expressed by bacteria are antigenically distinct and have specific binding properties. Depending on the expression pattern of Opa-proteins, individual bacterial clones display distinct affinities for the tissues of the body. Likewise, the expression pattern of cellular receptors determines tissue tropism. (modified after Gray-Owen, S.D.)

The ability of CEACAMs to induce bacterial internalization cannot be attributed to a specific CEACAM-member, but has been demonstrated for CEACAM1, CEA and CEACAM 6 in the epithelial cell line HeLa (Chen and Gotschlich 1996; Virji, Makepeace et al. 1996; Bos, Grunert et al. 1997; Gray-Owen, Lorenzen et

al. 1997). Importantly, these CEACAMs are expressed apically on target tissues such as the endometrium and cervical as well as urethral epithelial cells, and are as such accessible to Opa_{CEA}-expressing gonococci, even at the early stages of infection. Gonococci not only access intact epithelia but traverse them to reach the subcellular stroma. This observation had been made early on using organ cultures derived from fallopian tubes and the nasopharynx (McGee, Johnson et al. 1981; McGee, Stephens et al. 1983; Stephens, Hoffman et al. 1983; Stephens and Farley 1991). Furthermore, mimicking in vivo conditions by using polarized epithelial monolayers, Wang and coworkers could show that apically-expressed CEACAMs mediate bacterial traversal across of both Opa_{CEA}-expressing *N.gonorrhoeae* or *E.coli*. Importantly, the integrity of epithelial tight junctions as well as the transepithelial electric resistance were maintained throughout, while intracellular enveloping of bacteria in phagosome-like structures was observed by electron microscopy. These data indicate, that bacteria pass transcellularly rather than paracellularly (Wang, Gray-Owen et al. 1998). Therefore, tissue damage observed in the course of infection seems to be secondary to the disease process, probably caused by the general inflammatory reaction as part of an innate immune response, since bacterial entry into host tissues neither requires disruption of epithelial layers nor causes it.

Recent studies have shown that recruitment of epithelial CEACAMs by pathogenic bacteria significantly modulates epithelial gene expression events. Importantly, these processes lead to an enhanced host cell adhesion to the extracellular matrix by de novo expression of CD105, a TGFβ1-receptor, thus decreasing cellular detachment (Bradley, Griffiths et al. 2005; Muenzner, Rohde et al. 2005). Infection-induced exfoliation is part of an important innate defense mechanism, effectively reducing the infectious load (Apicella, Ketterer et al. 1996; Mulvey, Lopez-Boado et al. 1998; Mulvey, Schilling et al. 2000; Mulrooney, Hong et al. 2001). Thus CEACAM engagement by *Neisseria* may represent an adaptation to counteract an early innate immune event.

It is interesting to note that natural infection of epithelia by gonococci induces the synthesis and release of proinflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, GM-CSF and membrane-cofactor protein-1 (Naumann, Wessler et al. 1997). The evolutionary and biological role of this mechanism has not entirely been elucidated, yet may be an `early warning system´ to alert the host of intruders even before bacterial products are available for direct stimulation of the immune system (Henderson, Poole et al. 1996). However, these inflammatory molecules have also been found in vitro to stimulate the upregulation of some CEACAM molecules, as shown for TNF-α and CEACAM1. Whether this represents an adaptation strategy of *N.gonorrhoeae* to enhance its adhesion and invasion into host tissues as seen with *Salmonella*, *Shigella* and *Listeria spp.* remains to be seen (Hess, Niesel et al. 1987; Galan, Pace et al. 1992; Eckmann, Kagnoff et al. 1993).

1.3 Interaction of gonococci with the human immune system

As seen, *Neisseria gonorrhoeae* has evolved several features which allow it to efficiently gain access to its host organism. However, while temporarily protected inside epithelia, gonococci face agents of the human immune system again upon entering subepithelial compartments or the blood stream. Several battlefields of infection and immunity in gonococcal infection have been identified, revealing that the exquisite adaptation of this pathogen to its human host is not least the result of a continual host-pathogen interaction, in which the ´selective force of the human immune system has shaped the pheno- and genotype of the bacterial pathogen` (Seger and Hamilton 1988; Brunham, Plummer et al. 1993).

1.3.1 Interaction of N.gonorrhoeae with elements of adaptive immunity

Adaptive immunity is a special feature of higher vertebrates and characterized by a tailor-made, yet slightly delayed immune response as well as the capacity to memorize previously encountered antigen. Cells conferring adaptive immunity are derived from the hematopoietic lineage and broadly subdivided into two classes: the **B**one-marrow-derived lymphocytes and **T**hymus-derived

lymphocytes. The main effector cells of cell-mediated immunity are T-cells while the humoral or antibody-mediated immune response is conferred by plasma-cell-turned B-lymphocytes that secrete specific immunoglobulins against foreign antigen. A range of immune cells termed antigen-presenting cells (APCs) including macrophages, dendritic cells and B-cells, facilitate the development of a specific immune response by presentation of foreign antigen to lymphocytes with costimulatory molecules and cytokines. While cell-mediated immunity is required for the recognition and destruction of host cells harbouring viral, bacterial, fungal or protozoan pathogens as well as malignantly transformed cells, humoral immunity is based on the secretion of specific antibody into extracellular fluids where they may bind to surface structures of pathogenic microorganisms and their products. This coating by antibody or complement, serves several functions: abrogation of adhesion to host structures, tagging for phagocytosis, a process also referred to as `opsonization´, and the activation of the complement system via the Fc-part of the immunoglobulin (Mandel 1976; Loos, Martin et al. 1989; Ravetch and Kinet 1991; Boackle 1993).

Important characteristics of the adaptive response are its antigen-specificity and its immunological memory provided by antigen-specific lymphocytes. A subsequent encounter with an already recognized antigen can reactivate `memory lymphocytes´, a subset of already determined yet dormant B- and T-cells, and induce their expansion, leading to a faster and stronger immunological answer (Sprent 1997). The basis for diversity and thus specificity of B- and T-lymphocytes for antigen lies in gene rearrangement of variable regions. Moreover, specificity of antibody is based on the diversification of immune receptors by somatic gene rearrangement after stimulation by antigen, so-called somatic hypermutation. By a process called clonal selection, the progeny with the highest affinity for presented antigen is subsequently selected to survive.

Despite these powerful immune mechanisms it is important to note, that gonococcal infection, in contrast to other venereal infectious diseases, has been

found to induce only a comparatively weak adaptive immune response (Blake and Wetzler 1995; Hedges, Mayo et al. 1999). This is certainly not due to a lack of immunogenic structures in *N.gonorrhoeae*, as severel surface elements of the pathogen, such as LOS, pilus and Opa-proteins have the potential to stimulate both the adaptive and innate immune response (Jones, Newland et al. 1980; Nicolson, Perry et al. 1987). Consistently, early on in the infection process, antibodies directed against the pilus can be found in the host serum. Yet, these antibodies only disable part of the bacterial population and do not protect against reinfection (Blake and Wetzler 1995). Moreover, reexposition of patients to gonococci does not produce a stronger immune response, suggesting, that an efficient immunological memory is not induced by previous gonococcal infection. Consequently, no efficient anti-gonococcal vaccine has been generated to date despite serum antibody responses against prototype vaccines (McChesney, Tramont et al. 1982; Boslego, Tramont et al. 1991; Johnson, Chung et al. 1991).

1.3.2 Antigenic and phase variation

This lack of an adaptive immune response is partly due to efficient immune *evasion* strategies, several of which have been elucidated so far. Most importantly however, gonococci avoid *inducing* a specific immune response and thus an immunologic memory by variation of their immunogenic surface structures mainly by two mechanisms: The switching between distinct phenotypes, referred to as `phase variation´, and the change in primary sequence of the variant structure, termed `antigenic variation´ (Meyer, Billyard et al. 1984; Seifert and So 1988; Johnson, Chung et al. 1991; Seifert 1996). Accordingly, even members of a gonococcal population derived from a clonal isolate have been found to vary greatly in their antigenic structures (Merz and So 2000).

Phase variation has been described for PilC, LOS and Opa-proteins. Both RecA-dependent and independent mechanisms are observed. In the case of Opa-proteins, it is a RecA-*independent* mechanism called **s**lipped-**s**trand

mispairing (SSM), which allows switching between distinct phenotypes (Fig. 1.5). SSM is a process that produces mispairing between mother and daughter strand during DNA replication. Genomic regions susceptible to SSM are those that contain short, repetitive DNA sequences of 6bp or less, designated **s**hort **s**equence **r**epeats (SSR), microsatellites, or `variable number of tandem repeats´. SSM at these regions will result in a change in the number of unit repeats, which can lead to altered gene expression at either the transcriptional or translational level, depending on the position of the repeats relative to the promoter and coding sequence (Meyer 1991; Davidsen and Tonjum 2006).

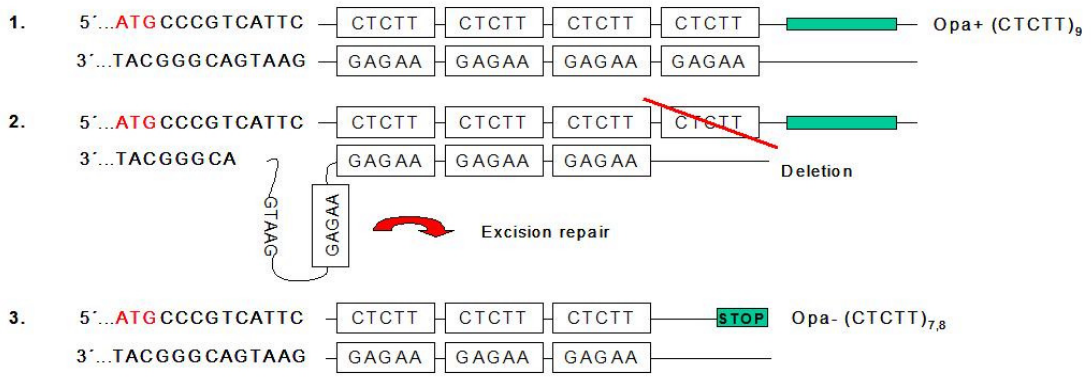


Fig. 1.5 **Phase variation of gonococcal Opa-proteins by slipped-strand mispairing**
1. The coding sequence of Opa proteins contains a variable copy number of a pentameric sequence (CR-unit: CTCTT). 2. During the process of replication, mispairing of these pentameric sequences with the antisense strand can occur, the resulting DNA-loop is subsequently excised and the unpaired pentamer removed by repair mechanisms. 3. The replicated DNA-sequence is one or several pentamers shorter and, by frame shift, may give rise to a stop-codon, the Opa protein is not expressed
(modified after Salyers and Whitt, 1994 and Davidsen and Tønjum, 2006)

Antigenic variation is found for the gonococcal pilus as well as the outer membrane LOS (Robertson and Meyer 1992; Meyer, Pohlner et al. 1994; van Putten and Robertson 1995), and is achieved by a RecA-*dependent* process in which genetic modules, so-called minicassettes, are recombined between transcriptionally active and inactive genloci. The inactive genloci represent a pool of variable domains and the active genloci the genetic scaffolding onto which the modules are placed, including the promotor as well as the coding sequence for the constant regions of surface structures. For the pilus by itself

an astonishing 10^6 possibilities for recombination has been estimated (Meyer, Mlawer et al. 1982; Gibbs, Reimann et al. 1989).

1.3.3 IgA protease

In addition to the great variability of immunogenic structures which circumvent in large part the adaptive immune response, gonococci possess another virulence factor directed specifically against the humoral response: the neisserial IgA protease. This endopeptidase has the capacity to break down human immunoglobulines, with a specificity for human type IgA1 (Plaut, Gilbert et al. 1975). By its action, IgA, the predominant antibody on human mucous membranes, is selectively cleaved into its specific subunits, each of which retains its binding capacity. While the antigen-binding segment may ´shield` bacteria from further immune attacks, the Fc-part respectively may engage Fc-receptors on phagocytic cells and block further interaction with opsonised antigen (Kilian, Mestecky et al. 1988; Kilian, Reinholdt et al. 1996). Yet, while IgA1 is the predominant isotype in the nasopharyngeal tract, IgA1 and 2 isotypes coexist in a 50:50 ratio in the urogenital tract, implying that not all of the present antibody can be inactivated. Furthermore, comparative studies of homologous proteins in other bacterial species showed either no IgA-specific protease function or no function specific for the IgA of the respective host organism (Kilian and Holmgren 1981; Kilian, Mestecky et al. 1988; Benjelloun-Touimi, Sansonetti et al. 1995). Moreover, clinical studies on the role of IgA1 protease suggested, that cleavage of IgA1 on mucous membranes is unlikely to be a significant factor of pathogenicity in female lower genital tract infections (Hedges, Mayo et al. 1998). Thus an alternative, yet pathogenicity-promoting function has been proposed for the IgA1-protease such as alteration or cleavage of lysosomal proteins, promoting the intracellular survival of gonococci (Kilian and Holmgren 1981; Hauck and Meyer 1997; Lin, Ayala et al. 1997; Ayala, Lin et al. 1998).

1.3.4 Interaction with T-lymphocytes and immunosuppression via ITIM

Recent data support the idea that gonococci actively interfere with lymphocytes to modulate the host immune response. It has long been observed that infection with *N.gonorrhoeae* increases the susceptibility to subsequent infection with HIV-1 or *Chlamydia trachomatis* (Hillis, Nakashima et al. 1994; Cohen 1998), and concomitant gonococcal infection leads to increased viral shedding of HIV-1 infected individuals (Fleming and Wasserheit 1999). Interestingly, adhesion of gonococci to CD4+-T-cells has been shown to arrest their activation and proliferation, rather than stimulating it (Boulton and Gray-Owen 2002). This effect can be ascribed to CEACAM1, the only representative of its family expressend on lymphocytes. CEACAM1 harbours a tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain (Chen, Zimmermann et al. 2001). Engagement of the CEACAM-molecule leads to tyrosine phosphorylation by Src-kinases and recruitment of tyrosine phosphatases such as SHP-1 and –2, or SHIP (Beauchemin, Kunath et al. 1997; Huber, Izzi et al. 1999; Boulton and Gray-Owen 2002) which, by dephosphorylating their cellular substrates, curb or suppress cellular signaling processes (Ravetch and Lanier 2000). Since CD4+-T-cells, so called `helper-T-cells´, have a vital function in directing acquired immunity, their inactivation seems a powerful means to curtail the adaptive immune response to gonococcal infection (Boulton and Gray-Owen 2002; Lee, Boulton et al. 2007). This immunosuppressive mechanism might also explain the transient decline in CD4+-T-cell counts, observed frequently in individuals with gonococcal infections (Anzala, Simonsen et al. 2000; Boulton and Gray-Owen 2002). Interesting data reveal, that even membrane blebs containing neisserial outer membrane proteins shed by *Neisseria spp.* in vitro and in vivo, are sufficient to induce such an immunosuppressive effect (Pettit and Judd 1992; Brandtzaeg and van Deuren 2002; Namork and Brandtzaeg 2002). Therein might lie a mechanism by which gonococci create `a zone of immunosuppression´, even at sites distal to infection (Lee, Boulton et al. 2007).

1.3.5 Interaction of N.gonorrhoeae with elements of innate immunity

In striking contrast to the comparatively inefficient adaptive immune response to gonococcal infection, a strong recruitment of phagocytic cells, polymorphonuclear neutrophils (PMNs) and monocytes, to the site of infection can be observed in gonorrhea (Ward, Glynn et al. 1972; Ward and Watt 1972; Handsfield 1990; Ryan 1990). The resulting purulent, polymorphonuclear neutrophil (PMN)-dominated discharge from the infected organ system consists mainly of granulocytes closely associated with or even containing internalized bacteria. It has long been a matter of debate, whether these bacteria are still viable, since morphologically they appear intact. Gonococci are known to persist in the host for months and this lead to speculations that gonococci, in analogy to CEACAM-mediated adhesion and invasion into epithelial cells, use granulocytes as vessels for persistance and transmission. Various intracellular pathogens including *Salmonella spp.*, *Mycobacteria spp.*, *Legionella* and *Shigella spp.* are capable of modifying the bactericidal apparatus of epithelial cells, for example by influencing phagosome maturation, intracellular transport and lysosomal membranes such that prolonged intracellular survival is possible (Garcia-del Portillo and Finlay 1995; Garcia-del Portillo and Finlay 1995). Some data indeed indicate, that gonococcal interaction with lipid raft-mediated vesicle trafficking may influence its intracellular localization, and cleavage of lysosomal proteins by the neisserial IgA protease might enable bacterial escape from phagosomes (Hauck and Meyer 1997; Schmitter, Pils et al. 2007). However, these conclusions have been drawn by studies on *epithelial* cell lines, yet the target proteins of the IgA protease in phagocytes seem to be more resistant to its enzymatic action. Accordingly, no intracellular survival of gonococci in *professional phagocytes* has been witnessed so far (Swanson et al. 1975; Veale et al. 1976; Witt et al. 1976; Drutz 1978; Casey et al. 1979; Cooper and Floyd 1982; Mezzatesta and Rest 1983; Parsons et al. 1986; Rest and Shafer 1989; Swanson and Isberg 1996).

PMNs are highly specialized cells, dedicated to the killing of invading microorganisms. Following the lead of chemoattractants, they leave the blood

stream to infiltrate the site of infection. A local ´instructive milieu`, created by secretion of cytokines, chemokines and interleukins (Akira, Takeda et al. 2001) orchestrates the ensuing inflammatory response, which ideally leads to the recognition and destruction of pathogenic microorganisms (Muzio, Polentarutti et al. 2000; Kapetanovic and Cavaillon 2007). Antigen recognition by PMNs is generally achieved by opsonization of antigen by specific antibody or complement and binding to specific granulocytic immune receptors, triggering a stereotypical sequence of signaling events involving a tyrosine-containing activation motif and the tyrosine kinase Syk. However, no efficient antibody response against gonococci has been described, and yet an efficient innate immune response may be generated to clear gonococcal infection, pointing to non-opsonin-dependent antigen recognition. This very ancient system has an important function in the early stages of infection and employs so-called PRRs (**P**attern **R**ecognition **R**eceptors), a set of conserved germline-encoded receptors that recognize largely invariant structures of microorganisms, so called PAMPs (**P**athogen **A**ssociated **M**olecular **P**atterns), including bacterial LPS, flagellin, unmethylated CpG DNA of bacteria and viruses and dsRNA (Iwasaki, Medzhitov, 2004). PRRs are expressed by a range of cells likely to encounter pathogenic structures, including macrophages, epithelial and endothelial cells (Girardin, Sansonetti et al. 2002). Three main families of PRRs have been recognized to date, including the Toll-like-receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) and retinoic acid-inducible gene 1-like receptors.

1.3.6 The opsonin-independent uptake of gonococci

It has been recognized for some time that it is an opsonin-*independent* phagocytic process which is responsible for gonococcal internalization by human granulocytes (Kupsch, Knepper et al. 1993). However, this process does not involve PRRs but is an Opa_{CEA}–receptor mediated process (Virji, Makepeace et al. 1996; Gray-Owen, Dehio et al. 1997; Hauck, Meyer et al. 1998). Human PMNs coexpress CEACAMs 1, 3, 4, 6 and 8, of which only 1, 3 and 6 serve as receptors for Opa-proteins. Granulocytic CEACAMs are stored

in primary and secondary granules and become exposed upon specific stimuli such as bacterial contact, thereby increasing gonococcal adhesion and internalization (Densen and Mandell 1978; Farrell and Rest 1990; Chen and Gotschlich 1996; Muzio, Polentarutti et al. 2000). Previous works had been conducted using the myelomonocytic cell line JOSK-M cells, which demonstrated that the infection of JOSK-M with OPA_{CEA} –expressing bacteria activates a distinct signaling cascade, involving extensive protein phosphorylation and rapid activation of acid sphinomeylinase (ASM) (Hauck, Grassme et al. 2000). Tyrosine phosphorylation has been attributed to the Src-PTKs Hck and Fgr followed by GTP-loading of the small GTPase Rac1, which leads to the necessary membrane remodeling. Activation of PAK and JNK mediate nuclear signaling and induction of stress-regulated genes (Gray-Owen, Dehio et al. 1997; Hauck, Meyer et al. 1998). However, as JOSK-M-cells like PMNs coexpress several CEACAM-members, the exact contribution of individual CEACAMs to these signaling processes was not clear (Virji, Makepeace et al. 1996; Hauck, Lorenzen et al. 1997; Hauck, Meyer et al. 1998; Schmitter, Agerer et al. 2004; Pils, Gerrard et al. 2008).

Further experiments with human 293T and HeLa cells expressing only a single type of receptor demonstrated, that it is CEACAM3, but not CEACAM1 or CEACAM6 which is responsible for the nonopsonic uptake of bacteria and their elimination (McCaw, Schneider et al. 2003; McCaw, Liao et al. 2004; Schmitter, Agerer et al. 2004; Pils, Gerrard et al. 2008). Interestingly, this member is restricted to human granulocytes and no endogenous ligand nor any mammalian homologue has been identified to date. Moreover, *Neisseria* internalized via CEACAM3, in contrast to CEACAM 1 or 6, are efficiently eliminated indicated by an oxidative burst in primary human granulocytes challenged with opaque gonococci (Schmitter, Agerer et al. 2004). Thus, the granulocyte-specific member CEACAM3 plays a dominant role in this context and may be regarded as a specific host-defense targeted towards CEACAM-binding bacteria.

1.3.7 The oxidative burst induced by Opa_{CEA}-carrying gonococci

The oxidative burst is one of two strategies employed by professional phagocytes to kill invading microorganisms. As opposed to the oxygen-independent mechanisms (Klebanoff 1992; Lehrer 1992), which mainly comprise soluble proteins contained in specialised organelles such as lysozyme, elastase, or the pore-forming defensins, it designates the generation of reactive oxygen (ROI) and nitrogen (RNI) intermediates which display strong microbicidal activity. These oxygen derivatives are produced by the activity of a membrane-bound, multi-component enzymatic system, the NADPH-oxidase. Its main constituents are a membrane-bound catalytic core cytochrome (cytochrome 558) with its $gp91^{phox}$ an $p21^{phox}$ subunits, as well as the cytoplasmic p^{67}phox and p^{47}phox components (Cross, Parkinson et al. 1985; Bokoch 1993; Diekmann, Abo et al. 1994; Edwards and Watson 1995). Upon the stimulus of bacterial products such as LPS or bacterial particles, the cytoplasmic components of the oxidase are recruited to the membrane-bound cytochrome: the NADPH-oxidase becomes activated and generates superoxide radicals (O_2^-). These radicals however only have a limited reach and short half-life and dismutate spontaneously or by the action of superoxide-dismutase to hydrogen peroxide (H_2O_2). By the action of the enzyme myeloperoxidase, more stable reactive metabolites, such as hypochlorite, are generated (Andrews and Krinsky 1986). The oxidative burst may be measured by various assays and is a reliable indicator of phagocytic activity. Several groups were able to show that gonococcal internalisation into human granulocytes is followed by a significant production of oxygen derivatives, indicative of phagocytosis (Virji, Makepeace et al. 1996; Schmitter, Agerer et al. 2004).

1.4 CEACAM3

Thus, CEACAM3 seems to represent the immunological answer to bacterial exploitation of epithelial CEACAM molecules (Chen, Bolland et al. 2001; McCaw, Schneider et al. 2003; Schmitter, Agerer et al. 2004; Kuespert, Pils et al. 2006; Schmitter, Pils et al. 2007; Pils, Gerrard et al. 2008). In line with this model, results of sequence comparison between CEACAM3 and other

CEACAM family members point to a chimeric origin of CEACAM3. While the extracellular domain displays significant homology with CEACAM1, the cytoplasmic domain resembles that of a phagocytic immunoreceptor, implying the bacteria-binding extracellular domain to be derived from different genes than the function-promoting intracellular domain. That no homologue has been identified to date in murine, canine or even other primate species, suggests that CEACAM3 is rather a recent evolutionary invention (Pils, Gerrard et al. 2008).

1.4.1 The role of the ITAM-motif in CEACAM3

CEACAM3, a 35kD transmembrane receptor, is a rather small member of the family, encompassing an N-terminal 34-residue signal peptide, an extracellular IgV-like domain, a hydrophobic transmembrane domain and a cytoplasmic portion. It is heavily glycosylated, like all CEACAMs, and exists in two splice variants, CEACAM3L and S, with a long and short cytoplasmic sequence respectively. The functional clue to CEACAM3-signaling is contained within this cytoplasmic portion (Irving and Weiss 1991; Irving, Chan et al. 1993; Gray-Owen, Dehio et al. 1997) which contains a sequence motif consisting of two tyrosine residues in a conserved spacing, reminiscent of an **I**mmunoreceptor-**T**yrosine-based **A**ctivation **M**otif (Fig. 1.6):

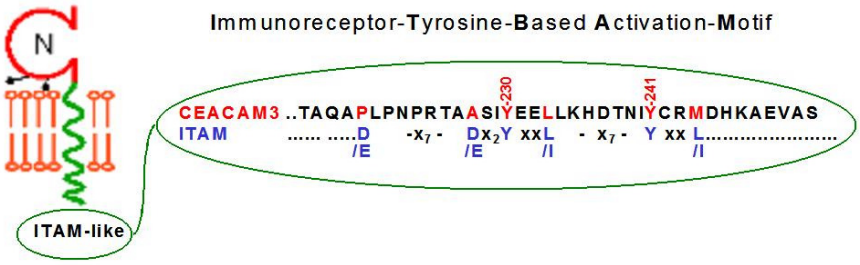


Fig. 1.6 **The ITAM-like amino acid sequence contained within the cytoplasmic portion of CEACAM3**
Minimal consensus ITAM sequence (in blue) and ITAM-like of CEACAM3 (in red) have been aligned for comparative purposes:
CEACAM3 displays two tyrosines in a conserved spacing 6-8 amino acids apart, at positions 230 and 241 respectively. While Y230 is paired with a leucine, much like in the consenus ITAM, Y241 pairs with a methionine. There are further differences in the surrounding amino acid sequence.

Moreover, CEACAM3 contains a short proline-rich motif which may serve as docking site for SH3 domains of molecules of signal transduction, and several predicted serine phosphorylation sites (Blom, Gammeltoft et al. 1999; Pils, Gerrard et al. 2008).

ITAMs as well as their inhibitory counterparts, ITIMs, are a common sequence motif in immunoreceptors such as the B-cell receptor (Igα and β-chains), T-cell receptor (ζ-chain) and immunoglobulin Fc-receptor (FcεR1γ-chain) (Cambier 1995). Many observed immunological functions, including the activation or inhibition of lymphocytes and NK-cells, cytokine release and foremost, immunoreceptor-mediated phagocytosis are transmitted and regulated by ITIM/ITAM-containing immunoreceptors. The minimal consensus sequence of ITAMs has been revealed as being two pairs of tyrosine - leucine/isoleucine repeats, six to eight amino acids apart (D/E^{-}x^{7}D/E^{-}x^{2}**Y**xx**L**x$^{6-8}$**Y**xx**L/I)** (Reth 1989; Letourneur and Klausner 1992; Romeo, Amiot et al. 1992; Koyasu, Tse et al. 1994; Cambier 1995). By chimeric coupling of ITAM-containing immunoreceptor cytoplasmic tails to random cell surface proteins, it could be demonstrated, that the cytoplasmic domain by itself is sufficient to induce receptor-associated signal transduction pathways and cellular activation (Irving and Weiss 1991; Irving, Chan et al. 1993). Sequential truncation of the cytoplasmic domain lead to the identification of the above cited consensus as the `minimal activation unit´ (Letourneur and Klausner 1992).

Since its identification, the canonical motif YxxL/Ix6-8YxxL/I has been found as a component of a number of different receptors and adapter molecules in hematopoietic cell lineages (Reth 1989; Flaswinkel, Barner et al. 1995; Humphrey, Lanier et al. 2005). Interestingly, although one of the earliest events after ligand binding is phosphorylation of its tyrosine residues, ITAM-receptors and adapters are devoid of any intrinsic activity and their signal transduction capacity is entirely dependent on the association with various cytoplasmic enzymes (Reth 1989). However, its two tyrosine residues are both necessary and sufficient for the induction of downstream intracellular signals. Upon

receptor stimulation, non-receptor phosphotyrosine kinases (PTKs) transiently or constitutively associate with ITAM-containing receptor subunits and lead to their phosphorylation (Samelson, Patel et al. 1986; Baniyash, Garcia-Morales et al. 1988; Reth 1989; Gold, Matsuuchi et al. 1991), a prerequisite for the docking of receptor-proximal Src homology 2 (SH2) domain-containing proteins (Reth 1989; Irving and Weiss 1991; Clark, Johnson et al. 1994; Bu, Shaw et al. 1995; Isakov, Wange et al. 1995). In classical immunoreceptor-signaling, ITAM-ligation has been shown to be dependent on Src-PTKs and to activate the tyrosine kinase Syk, thereby connecting receptor engagement to common signaling pathways, eliciting cell activation, endocytosis and phagocytosis (Daeron 1997).

1.4.2 Molecular events following CEACAM3 receptor engagement

Like ITAM-signaling observed in the opsonin-dependent phagocytosis via the FcγRIIA receptor on phagocytes, the opsonin-independent phagocytosis via CEACAM3 involves the activation of Src-family kinases, such as Fgr and Hck, and the downstream activation of the small GTPase Rac (Daeron 1997; Billker, Popp et al. 2000; Hauck, Grassme et al. 2000; Booth, Telio et al. 2003; McCaw, Schneider et al. 2003; Schmitter, Agerer et al. 2004; Pils, Gerrard et al. 2008). It has also been demonstrated that the integrity of the ITAM-like sequence of CEACAM3 is indispensable for the efficient internalization and elimination of bacteria. Consequently, disabling the ITAM-like in the cytoplasmic tail of CEACAM3 by replacement of the critical tyrosines by phenylalanine heavily impairs CEACAM3-responses. Several groups could demonstrate that even a single exchange of one of the tyrosines significantly reduces the bacterial internalization. This effect is increased by replacement of both tyrosines or deletion of the cytoplasmic domain altogether, which nearly abrogates bacterial internalization (Chen, Bolland et al. 2001; Billker, Popp et al. 2002; McCaw, Schneider et al. 2003; Schmitter, Agerer et al. 2004). Interestingly, the Syk-kinase, critically involved in the events following Fcγ-receptor engagement, seems to be dispensable for internalization via CEACAM3 (Hauck, Meyer et al. 1998; Sarantis and Gray-Owen 2007). Moreover, Schmitter et. al. observed that

the guanine-nucleotide exchange factor (GEF) Vav promotes the opsonin-independent phagocytosis of Opa_{CEA}-expressing gonococci by connecting phosporylated CEACAM3 to Rac-activation (Schmitter, Pils et al. 2007) (Fig. 1.7).

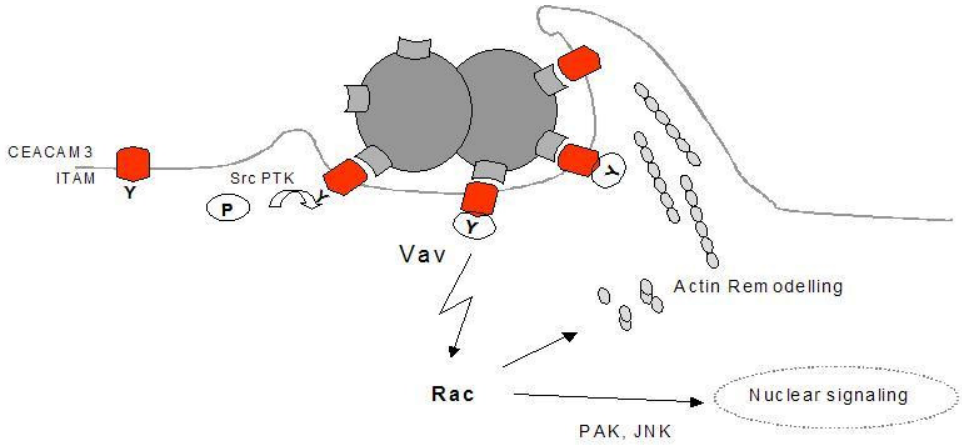


Fig. 1.7 **Current model of CEACAM3-signaling events.**
CEACAM molecules are engaged by Opa_{CEA}-expressing gonococci, leading to tyrosine phosphorylation of the ITAM-like by Src-PTKs. This triggers a signaling cascade leading to the activation of the small GTPase Rac, which is loaded by interaction with the Rac GEF Vav. Subsequently, Rac leads to extensive actin remodeling, bacterial engulfment and internalization and stimulates its effectors p21-activated kinase and Jun-N-terminal kinase, thereby triggering nuclear signaling.

Recently, it has been revealed that the functional differences observed for epithelial CEACAMs and granulocytic CEACAM3 are mirrored by distinct molecular requirements (McCaw, Liao et al. 2004). Src PTKs, while indispensable for bacterial internalization via CEACAM3, are not required for CEACAM6-initiated uptake, while cholesterol depletion impairs bacterial uptake by epithelial CEACAMs but does not affect CEACAM3-mediated phagocytosis (Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008). Moreover, the cytoplasmic domain of CEACAM1 seems to be altogether dispensable for bacterial internalization triggered by this receptor (Muenzner, Bachmann et al. 2008). Interestingly, CEA and CEACAM6 are GPI-anchored family members and CEACAM1, 6 and CEA, other than CEACAM3, have been found to partition into a low-density Triton-insoluble membrane fraction upon clustering

(Muenzner, Bachmann et al. 2008). Taken together, these data point to lipid raft association of these CEACAM members and to mechanistically distinct molecular processes. Despite coexpression of CEACAM1, 6 and 3 on human PMNs, bacterial uptake in these cells is only but severely reduced by the inhibition of Src-kinase-dependent Rac-mediated cytoskeletal reorganization while cholesterol-depletion has no effect, pointing to the dominant role of CEACAM3 in this cell type (Schmitter, Pils et al. 2007). It seems to be the transmembrane domain of CEACAM1 which directs these molecules to the cholesterol-rich microdomains and promotes bacterial uptake into CEACAM1-carrying cells. Interestingly, lipid rafts, which are found in most cell types, have been implicated in numerous cellular processes including vesicle trafficking (Helms and Zurzolo 2004) and cellular signaling (Simons and Toomre 2000). It is thus feasible that CEACAM1 and 6, which predominate CEACAM-mediated endocytosis in epithelial cells are exploited by gonococci to access epithelial cells and for subsequent traversal, while CEACAM3 on granulocytes outcompetes coexpressed family members by the direct and fast opsonin-independent bacterial recognition and phagocytic internalization (Wang, Gray-Owen et al. 1998; Schmitter, Pils et al. 2007).

Although much light has already been shed on the molecular pathways involved in the interaction of *N.gonorrhoeae* with human granulocytes, the early events after receptor ligation remain elusive and require further attention. A conclusive model linking receptor phosphorylation, recruitment of Rac, PAK and the activation of the nuclear signaling events, still needs to be established.

1.5 Aims and objectives

In previous studies the ITAM had been proposed as the key feature of CEACAM3 responsible for subsequent signaling events. However, neither the binding partners nor the exact sequence of signaling events had been identified. The objective of this thesis was to look for the missing link between CEACAM3, downstream stimulation of the small GTPase Rac and cytoskeletal rearrangement.

- The ITAM-like, being a tyrosine-containing conserved signaling motif, strongly suggests a phosphotyrosine-mediated pathway. Phosphotyrosine dependent signaling pathways create binding sites for molecules containing SH2 domains by receptor clustering and subsequent receptor phosporylation. We planned to screen for potential binding partners of CEACAM3 using the GST-pull down method, chosing a range of candidate SH2-domain-containing molecules of signal transduction. Since CEACAM3 engagement ultimately leads to extensive cytoskeletal rearrangement, these proteins should also be implicated - directly or indirectly- in actin remodelling.

- Employing a mutational approach we planned to pinpoint interaction of the putative interaction partner and CEACAM3 to the SH2-domain and the ITAM-motif.

- Since Src-PTK-dependent phosphorylation of the ITAM seems to be a crucial step in ITAM signaling, we wanted to investigate whether the putative interaction is phosphotyrosine-dependent.

- CEACAM3 has been proposed to be a novel phagocytic receptor, triggering bacterial internalization and destruction by human PMNs. As studies on human granulocytes are limited by their vulnerability and coexpression of several CEACAM-molecules, we took advantage of a 293T-cell system lacking an endogenous CEACAM background established by Schmitter et al. Making use of this sytem and laser-scanning confocal microscopy we intended to visualise in vivo colocalisation of signaling molecule and receptor upon infection.

- Using the same cell system we explored the effect of a dominant negative signaling construct on CEACAM3-mediated bacterial uptake.

Ultimately, we were hoping to add another piece to the complex puzzle of cellular signaling in the opsonin-independent phagocytosis of *Neisseria* and shed more light onto the intricate workings of the human innate immune system in the combat of bacterial infection.

2 Materials

2.1 Bacteria

2.1.1 Neisseria

N280 (MS11-F3)	PilEF3, P+, Opa30-, opaC::cat, (CmR))
N302 (MS11-B1)	PilEB1(S), Opa30-, opaC::cat, (CmR), pTH6a (TetR), (ErmR)
N309 (MS11-B2.1)	PilEB1(S), Opa30-, opaC::cat, (CmR), pTH6a::opa52 (pEMK62), (TetR), (ErmR)

2.1.2 E.coli

BL21 (DE3)	F-, ompT. hsdSB,(rB-mB-), gal, dcm (DE3), (Novagen)
NovaBlue	endA1, hsdR17(rk12-mk12+), supE44, th-1, recA1, gyrA96, relA1, lac[F' proA+B+ lacIqZΔN15::Tn10(tetR)], (Novagen)
DH5α	F' Phi80dlacZ, DeltaM15, Delta(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rK-mK+)phoA, supE44 lambda- thi-1 (Novagen)

2.2 Cell lines

293T	human embryonic kidney cell line (fibroblastoid, adherent)

2.3 Culture media

2.3.1 Bacterial culture

LB-medium	10g Bacto-Trypton, 5g yeast extract, 5g NaCl, pH 7,0, ad 1l a. bidest.
LB-plates	10g Bacto-Trypton, 5g yeast extract, 5g NaCl, 10ml MgCl2 (1M),1g Agar-Agar, ph 7,0, ad a. bidest.
GC-plates	36g GC-agar, ad1l a. bidest., 10ml vitamine mixture
BHI-agar	31,5g BHI-agar (Becton Dickinson), ad 600ml a. bidest., 1:50 hemin, NAD 1:1000 (Applichem), 6µg/ml ampicillin
BHI-medium	37g BHI-medium ad 1000 ml a. bidest. (Becton Dickinson)
Freezing medium	600µl LB-medium, 600µl glycerol (50%)

Hemin	1mg/ml Hemin, 1mg/ml Histidine, 4%Triethanolamine (Applichem)
Vitamin mixture	20g dextrose, 2g L-glutamine, 3,2g L-cysteine, 0,02g cocarboxylase, 0,004g Fe(No)3, 0,0006g thiamine-HCL, 0,05g NAD, 0,002g vitamin B12, 0,03g L-arginine, 0,0026g p-aminobenzoate, 0,22g L-cystine, 0,2g adenine, 0,1g uracil,0,006g guanine, pH 3.5. ad 200ml a. bidest., sterile filtration (10ml to 1l GC-agar)
Antibiotics	ampicillin 100µg/ml chloramphenicol 30µg/ml in LB-Medium, 10µg/ml in GC-Agar erythromycin 7µg/ml kanamycin 30µg/ml penicillin 10mg/ml (PAA Laboratories) streptomycin 10mg/ml (PAA Laboratories) gentamicin 50µg/ml (PAA Laboratories)

2.3.2 Cell culture

DMEM	Synthetic Cell Culture Medim L-glutamine (PAA Laboratories)
FCS	fetal calf serum (PAA Laboratories)
CS	calf serum (PAA Laboratories)
Hi CS	heat inactivated calf serum
Additives	gentamicin 50 µg/ml (PAA Laboratories) cytochalasin D 1µg/ml (Calbiochem)

2.4 Antibodies

2.4.1 Anti-CEACAM

D14HD11	anti-CD66 (CEA;human), monoclonal, mouse (Genovac)
Col1	anti-CD66 (CEACAM3, -5), monoclonal, mouse (Zymed)
Kat4c	anti-CD66 (CEACAM1, -5, -6) monoclonal, mouse (DAKO)

2.4.2 Anti-N.gonorrhoeae

Ak92 anti-*N.gonorrhoeae*, polyclonal, rabbit, 1:200 (Prof. Meyer)

2.4.3 Other

12CA5 anti-HA-epitope, monoclonal, mouse (Hybridoma)

B-14 anti-GST, monoclonal, mouse (Santa Cruz)

ab290 anti-GFP, polyclonal, rabbit (Abcam)

9E10 anti-myc-epitope, monoclonal, mouse (Santa Cruz)

P-Tyr anti-phosphotyrosine, monoclonal, mouse (Millipore)

Nck anti-human-Nck (aa 279-377), monoclonal, mouse (BD)

Grb2 anti-human-Grb2

Pi3N anti-human-Pi3N

Pi3C anti-human-Pi3C

Hck anti-human-Hck, polyclonal, rabbit

anti-c-Src (human), monoclonal, mouse (Hybridoma)

v-src anti-avian Src, monoclonal, mouse (Millipore)

2.4.4 Secondary antibodies

Cy5 anti-mouse, polyclonal, goat-anti-mouse, Cy5-conjugated (Sigma)

2.5 Enzymes and proteins

2.5.1 Enzymes

Taq-DNA-Polymerase (BioLabs), Sal I (BioLabs), Hind III (BioLabs), Trypsin (PAALaboratories), Vent-DNA-polymerase (BioLabs), Cre-Recombinase (Bio-Labs), In-Fusion Enzyme (BD-Bioscience), Protein A/G-Plus sepharose (Santa Cruz Biotechnology), lysozyme (Serva)

2.5.2 Proteins

PI3N SH2-GST, PI3C SH2-GST, SrcSH2-GST fusion proteins were kindly provided by David Schlaepfer (Dept. of Immunology, Scripps Research Institute, La Jolla, CA)

2.6 Plasmids and oligonucleotides

2.6.1 Plasmids

pBS Sk (+) CEACAM3 WT HA (AmpR)

pBS Sk (+) CEACAM3 delta CT HA (AmpR)

pBS Sk (+) CEACAM3 Y241F HA (AmpR)

pBS Sk (+) CEACAM3 Y230F HA (AmpR)

pBS Sk (+) CEACAM3 Y230/241F HA (AmpR)

pDNR-Dual *oripUC, loxP*, MCS, 6xHN-tag, (CamR), *SacB* (BD Bioscience)

pLPS-3'EGFP *oripUC, loxP*, MCS, SA, EGFP, (KanR), (NeoR) (BD Bioscience)

pLP-CMV-Myc *oripUC, loxP*, MCS, SA, c-Myc-tag, (AmpR) (BD Bioscience)

pGEX4Ti oriBR322, MCS, GST-tag, lacI, (AmpR) (Amersham Phamacia)

pDSRedN1 CEACAM3 WTRed (KanR)

pRC/CMV v-src

pOTB7 hNck (CamR)

pGex4Ti loxP HckSH2 (AmpR)

2.6.2 Oligonucleotides

Name	Sequence
CEACAM3 IF sense	AAGTTATCAGTCGATACCATGGGGCCCCCCTCAGCC
HA CEACAM3 IFanti	ATGGTCTAGAAAGCTTGCAGCGTAATCTGGAACGTCATATGG
pDNR lib-anti	CCAAACGAATGGTCTAGAAAGC
Check1-sense	GCTCACCGTCTTTCATTGCC
Check2-antisense	TCCGCTCATGAGACAATAACC
Grb2-SH2-IF-sense	GAAGTTATCAGTCGACCCGTGGTTTTTTGGCAAAATCC
Grb2-SH2-IF-anti	ATGGTCTAGAAAGCTTTATCTGTGATAATCCACCAGCTC
hNck1-IF-sense	GAAGTTATCAGTCGACATGGCAGAAGAAGTGGTGGTAG
hNck1-IF-anti	TGGTCTAGAAAGCTTCAGCAGTATCATGATAAATGCTTGAC
hNck1-SH2-IF-sense	GAAGTTATCAGTCGACAAGTTTGCTGGCAATCCTTGG
Nck-R308K-sense	GAAGGGGATTTCCTTATAAAAGATAGTGAATCTTCGCCAAATG
Nck-R308K-anti	GAAGATTCACTATCTTTTATAAGGAAATCCCCTTCATGTCC

Oligonucleotides were obtained from MWG-Biotech

2.7 Buffers and solutions

2.7.1 For eukaryotic cells

PBS (1x)	24g NaCl, 0,6g KCl, 3,45g Na2HPO4*7H2O, 0,6g KH2PO4, pH 7,4, ad 1l with a.bidest.
PBS++	1xPBS, 0,35mM CaCl2, 0,25mM MgCl2
Paraformaldehyde	solve 4g PFA in 80ml warm a.bidest., add 1N NaOH until clear, titrate pH 7,4 by adding 1N HCl, add 10ml 10xPBS, ad 100ml with a.bidest.
Saponine solution	solve 1% saponine in 1xPBS
CaCl2-solution	2,5M CaCl2
2x HBS	16,4g NaCl, 11,9g Hepes, 0,21g Na2HPO4 in 1l a.bidest., pH 7,05; sterile filtration
RIPA-buffer	1% Triton X-100, 50mM Hepes, 10% glycerol, 150mM NaCl, 1mM EGTA, 1,5mM MgCl2, 10mM sodiumpyrophosphate, 100mM fodiumfluoride, 0,1% sodiumdodecylsulfate (SDS), 1% deoxycholate, 1mM sodiumorthovanadate, 10µg/ml leupeptine, 10µg/ml aprotinine, 10µg/ml Pefabloc, 10µg/ml Pepstatine, 10µM benzamidine; ad 250ml with a.bidest.
Triton-buffer	25mM HEPES (pH 7,4), 1% Triton X-100, 150mM NaCl, 20mM MgCl2, 10% glycerol, 10mM sodiumphosphate, 100mM NaF, 1mM NaVO4, 10µg/ml Leupeptine, 10µg/ml aprotinine, 10µg/ml Pefabloc, 10µg/ml Pepstatine, ad 250ml with a. bidest.
MgCl2	10% glycerol, 10mM sodiumphosphate, 100mM NaF, 1mM
PVA/NaCl	1% PVA (Applichem), 0,9% NaCl ad 0,5l with a. bidest
Citrate solution	3,8% Na-citrate ad 0,2l with a. bidest

2.7.2 Molecular biology

TAE-buffer (1x)	4,84g Tris-Base, 1mM EDTA, 1,14ml glacial acetic acid, ad 1l with a.bidest.
TE-buffer	10mM Tris-HCl, 1mM EDTA, pH 8

GEBS	50mM EDTA, 20% (v/v) glycerol, 0,5% (w/v) sarcosyl, 0,05% (w/v) brome phenole blue
dNTPs	1,25mM dNTPs in TE-buffer, pH 8
P1-buffer	50mM Tris-HCl, 10mM EDTA, 100µg/ml RNAse A, pH 8,0
P2-buffer	200mM NaOH, 1% (w/v) SDS, RT
P3-buffer	3M potassiumacetate, pH 4,8, RT
1kb DNA Ladder	Mass Ruler DNA Ladder (Fermentas)

2.7.3 SDS-PAGE, Coomassie stain, Western blot

2xSDS buffer	125mM Tris-HCl (pH 6,8), 10% (v/v) β-mercapto-ethanol, 5% (w/v) SDS, 0,1% (w/v) brome phenole blue, 20% (v/v) glycerol
SDS-PAGE Standard	1% (w/v) lysozym, 1% (w/v) soybean-trypsin-inhibitor, 1% (w/v) peroxidase, 1% (w/v) BSA, 1% (w/v) lipoxidase, 5ml Triton-buffer, 5ml 2x SDS buffer
Acrylamide solution	40% polyacrylamide
Stacking-gel-buffer	0,5M Tris-HCl pH 6,8
Resolving-gel-buffer	1,5M Tris-HCl pH 8,8
APS	10% ammoniumperoxodisulphate
TEMED	0,1% N,N,N',N'-tetramethyldiamine
SDS	20% (w/v) SDS
Running buffer	25mM Tris-HCl, 192mM glycine, 0,1% (w/v) SDS
Staining solution	25% (v/v) isopropanole, 10% (v/v) glacial acetic acid, 0,03% (w/v) Coomassie Brillant Blue R250
Destaining solution	10% (v/v) isopropanole, 10% (v/v) glacial acetic acid
Kathode-buffer (5x) pH 9,4	125mM Tris-Base, 200mM 6-aminohexanoic acid,
Anode-buffer (5x)	125mM Tris-Base, pH 10,4
Transfer buffer	6,0g Tris-Base, 28,8g glycine, 430ml methanol, 0,1% (w/v) SDS, ad 2l with a.bidest.
TBS	25mM Tris (pH 7,5), 125mM NaCl
TBS/Tween	25mM Tris (pH 7,5), 125mM NaCl, 0,1% Tween-20

Blocking solution	2% (w/v) BSA in TBST, 0,05% (w/v) NaN3
ECL	0,225mM p-coumaric acid, 1,25mM luminol, 0,1M Tris-base pH 8,5
H_20_2-solution	30% (v/v) hydrogen peroxide
Stripping-buffer	0,8% (w/v) SDS, 0,8% (v/v)-mercaptoethanol, 80mM Tris-HCl, pH 6,8

2.7.4 Protein purification

IPTG	100mM isopropyl-beta-D thiogalactopyranoside
T-buffer	100mM Tris (pH8,0), 5mM EDTA
Lysis-buffer	50mM Tris (pH8,0), 10 mM EDTA, 10% glycerol, 2,5mM DTT, 1mM PMSF (Applichem)
10%TritonX-100	TritonX-100 (v/v) in a. bidest.
Elution buffer	50mM Tris-HCl (pH8,0), 10mM reduced glutathione
Dialysis buffer	20mM Hepes, 150mM NaCl, 1mM EDTA, 10%(v/v) glycerol

2.8 Kits and reagents

Acrylamide (Roth), Agar-Agar (Roth), Agarose (Roth), Ammoniumsulfate (AppliChem), Antibiotics (Roth, AppliChem), APS (Roth), BSA (Roth), Cytochalasin D (Calbiochem), Ethanol (AppliChem), Ficoll Paque Plus (Amersham Pharmacia); GCAgar (Difco), Glycerol (Roth), Glycine (AppliChem), Glutathione Fast4Flow (Amersham Pharmacia), Carbamide/Urea (Riedel-De-Häen), Yeast extract (Roth), Hemin (Fluka), Imidazole (Upstate), Methanol (AppliChem), mounting Medium (Sigma), NAD (Applichem), Sodiumchloride (AppliChem), Nucleotide (BioLabs), Paraformaldehyde (AppliChem), Polyvinyl alcohol (Applichem), Saccharose (Roth), Saponin (Roth), TEMED (Roth), Triethanolamine (Applichem), Tris-Base (AppliChem), Triton-X-100 (Roth), Trypan blue (Sigma), Trypton (Roth), Tween-20 (Roth), Mercaptoethanol (Roth), Qiaprep Spin Miniprep Kit (Qiagen), Qiaquick Gel Extraction Kit (Qiagen), In-Fusion PCR Cloning Kit (BD Bioscience), BD Creator DNA Cloning Kit (BD Bioscience)

2.9 Laboratory tools

Autoklav (Varioklav), Incubators (Heraeus, Memmert), Object slides/Cover slips (Knittel), Electroblot-apparatus (Bio-Rad, Schleicher & Schuell), Developer (Agfa), Fluorescence Microscopy (Nikon), FPLC (Bio Rad), Apperatus for Polyacrylamide Gel Electrophoresis (Bio-Rad), Gel documentation (Bio-Rad), Agarose gel chambers (Bio-Rad), Glassware (Schott, VWR Brand), Heating block (Roth, Grant Boekel), Laser scanning confocal microscope (Zeiss), Fridges and Freezers (Privileg), Magnetic stirrer (Ika), Master Cycler Gradient (Eppendorf), Microwave (Electronia), pH-Meter (Beckman), Photometer (Hach), Pipettes (Gilson), Plasticware (Eppendorf, Greiner, Costar), PVDF Membranes (Millipore), photographic films (Retina), Shaker (Bühler), Bench (Heraeus), Shaking incubator (Eppendorf), Vivaspin concentrator 50K (Vivascience), Vortex mixer (Ika), Scales (Kern, Scaltec), Water bath (Memmert, Julabo), Counting chamber (Neubauer), Centrifuges (Heraeus, Sorvall)

3 Methods

3.1 Handling of bacteria

3.1.1 Bacterial culture

Variants of *Neisseria gonorrhoeae* strain MS11 (Opa_{CEA}-expressing (Opa_{52}, N309), non-opaque (N302)) were kindly provided by Thomas Meyer, Berlin, Germany. *Neisseriae* were grown on GC-agar (Gibco BRL, Paisley, UK) supplemented with vitamins at 37°C, 5% CO2 and subcultured daily.

E.coli and expression strains were cultured on plates of LB-agar, supplemented with the respective antibiotics for selection. For overnight culture, 2-10ml LB-medium were inoculated with a single colony taken from the selection plate by using a sterile tooth pick. Subsequently, the medium was kept on a shaking incubator at 37°C and 200-250rpm overnight.

For gentamicin protection assays, overnight grown bacteria were taken directly from vitamin-supplemented GC-plates.

3.1.2 Storage

Half the bacterial harvest of a thickly-grown agar-plate was resuspended in 600µl LB-medium and equal amounts of 50% glycerol in eppendorf tubes and kept at -80°C.

3.1.3 Selection

For selection of Opa_{CEA}-expressing bacteria, *Neisseria gonorrhoeae* were plated on erythromycin/chloramphenicol-containing GC plates 2 days prior to the infection experiment (our strains carried an E/Ch-resistance on the Opa_{CEA}-carrying plasmid). After 24h, Opa_{CEA}-expressing bacteria were selected optically (light-microscope) and subcultured on regular GC-plates to obtain sufficient numbers of bacteria. For gentamicin protection assays, *Neisseria* were taken from GC-plates and suspended in DMEM+0,5% h.i.CS.

3.1.4 Rendering bacteria competent

N.gonorrhoeae were naturally competent. *E.coli* were prepared for transformation using the calciumchloride method. A 10ml culture of *E.coli* was left to grow in regular LB-culture medium overnight, and 4ml of this suspension used subsequently to inoculate 200ml of fresh LB-medium. The bacteria were cultured on a shaking incubator at 37°C until an OD (optical density) of 0,6-0,8 was reached. The bacterial suspension was then reduced in an iced 0,1M CaCl2-solution to a volume of 1,25 ml and kept on ice in a cold room (4°C) for an hour. Subsequently, 520µl glycerol were added to the suspension, and aliquots of competent bacteria kept at -80°C until use.

3.1.5 Transformation of bacteria

Bacteria were gently defrosted on ice. 0,2-1µg DNA of the ligation- or recombination preparation were added to 100µl competent *E.coli*, mixed thoroughly and incubated on ice for 30min. The preparation was subsequently heated to 42°C for 75sec in a waterbath and immediately cooled down on ice. 1000µl of iced LB-medium were added and the mixture kept on a shaking incubator at 37°C for one hour. Bacteria were then pelleted by centrifugation for 3min at 3000rpm and all but 100µl supernatant discarded. The pellet was resuspended in the remaining supernatant and plated on selective LB-agar.

3.2 Handling of eukaryotic cells

3.2.1 Cell culture

Human embryonic kidney epithelial cells (293T cells) were grown in DMEM/10% calf serum (CS) at 37°C, 5% CO2. Confluent cultures were subcultured every 3 – 4 days by detachment with 2ml trypsin solution, centrifugation at 600rpm for 3min and dilution of the pellet in fresh medium 1:5 or 1:10. A day prior to analysis by immunofluorescence studies, $2x10^5$ cells/well were seeded in poly-L-lysine coated (20µg/ml) 24-well plates in DMEM/10% CS (gentamicin protection assay and immunofluorescence staining) or 1 x 10^6 cells/dish were seeded in poly-L-lysine coated 6-cm dishes and serum-starved for 20 h in DMEM containing 0.5% CS (cell lysates).

3.2.2 Storage of eukaryotic cells

Semiconfluent dishes were trypsinised and pelleted at 600rpm for 3min (~$5x10^6$ cells). The pellet was carefully resuspended in 1ml DMEM/20%CS/10%DMSO and transferred into cryotubes. They were kept frozen at –80°C in a styrofoam box and until transferral into liquid nitrogen (-180°C). For thawing, the cryotubes were transferred as quickly as possible into a waterbath at 37°C. The suspension was mixed with fresh DMEM and centrifuged at 600rpm for 3min. The pellet was resuspended in 10ml DMEM/10%CS and cells incubated in a culture dish for 24-48h, after which they could be subcultured.

3.2.3 Cell count

To ensure standard conditions, the number of cells in 10µl cell suspension was quantified using a conventional light microscope and a counting chamber with *Neubauer* ruling. Cell numbers were calculated according to the following formula: Cells per large quadrant x 10^4 = cells/ml (Vol. of large quadrant = 10^{-4})

3.2.4 Transfection of cells

We employed the standard calcium-phosphate co-precipitation method to transfect 293T cells. One to ten micrograms DNA, CEACAM constructs or empty control vector per 10cm cell culture dish were employed. In all samples total DNA was adjusted to 10µg using the empty control vector. To 500µl sterile A.bidest, DNA constructs and 500µl cool 2xHBS were added. Under constant vortexing, 50µl of sterile 2,5$MgCl_2$ were slowly added to trigger DNA-cristallisation and the resulting preparation incubated at room temperature for 10-15min. Cells culture dishes were prepared with 25mM Chloroquin (1/1000) and dishes kept in the incubator. The transfection mixture was carefully spread onto cultured cells and the dishes kept at 37°C and 5% CO_2 for 6-8 hours. The medium was exchanged for fresh culture medium and cells incubated for another 12-20 hours. Cells were employed in infection experiments 48h after the transfection.

3.2.5 Cell lysates

293Tcells were transfected with the respective receptor constructs and cells cultured in 10cm cell culture dishes as described under *Transfection of cells* until confluent. Protein expression levels were controlled visually for GFP-fused or RFP-fused receptor-protein. At sufficient expression levels, cell culture medium was removed in a cold room (4°C), dishes washed carefully with PBS and cells lysed with 1ml RIPA buffer per dish. The preliminary lysate was transferred with cell scraper and syringe into an eppendorf tube. To shear DNA mechanically, a regular sterile canula was used. Cell debris and DNA were bound by the addition of 100µl sepharose in Triton buffer and incubation on an inverter for at minimum 5min. The solution was then centrifuged for 15min and 800µl of the supernatant transferred into 2 (400µl each) fresh 1,5ml eppendorf tubes into which equal parts of 2xsample buffer were added. Such a lysate was ready for immediate use or frozen at –80°C. The remaining 100-200µl lysate in 2xsample buffer were employed for control of protein expression levels.

3.2.6 Isolation of primary human granulocytes

Primary human granulocytes were purified from freshly-drawn blood by Ficoll Paque gradient separation. Three mililitres of 3,8% Na-Citrate were directly added to 20-25ml freshly-drawn human blood to prevent clotting. Equal amounts of 1xPBS were added and 15ml Ficoll Paque Plus covered by 10ml of the PBS-diluted fresh blood. Subsequently, the tubes were centrifuged for 30min at 20°C and 1000rpm, leading to density-dependent separation of blood constituents. Granulocytes and erythrocytes migrate to the bottommost phase and were therefore won by removing the top layers. To separate granulocytes from erythrocytes, the cellular suspension was incubated for 45min with PVA/NaCl in which erythrocytes sink to the bottom of the tube while neutrophils remain in the supernatant. The supernatant was pelleted by centrifugation for 4min at 1000rpm. Any remaining erythrocytes were lysed osmotically by incubation with A.bidest for 30sec and stopped by the addition of 5xPBS to give a physiological solution. Purified granulocytes were again pelleted by centrifugation (3min, 1000rpm) and resuspended in

1xPBS/10mMGlucose/1%h.i.Serum. To prevent unspecific activation, cells were kept on ice until processing. On average 2-4x10^7 viable primary human granulocytes were harvested.

3.3 Protein handling

3.3.1 Denaturing sodiumdodecylsulfate-polyacrylamide gel electrophoresis

Gels were composed by mixing appropriate volumes of 40% bis-acrylamide with a.bidest (10% gel: 2,5ml + 5ml; 12,5% gel: 3,1ml+ 4,3ml) and 2,5ml 1,5M Tris-buffer pH 8,8. Gas bubbles were removed by vacuum pump. Subsequently, 50µl of SDS were added and polymerisation started with 30µl of 10% ammonium persulphate and 15µl TEMED (Tetramethyl-ethylene-diamine). The gel polymerised between two glass sheets. After polymerisation, the stacking gel was produced similarly from 1,25 acrylamide, 6,15 A.bidest and 2,5ml 0,5M Tris-buffer pH6,8 and cast over the resolving gel with a spacer, to render gel pockets for protein samples. Electrophoresis was performed in an electrophoretic chamber containing appropriate buffer solution at 100mV for 1,5-2 hours. Protein probes were denatured by heating to 90°C in sample buffer for 5min.

3.3.2 Western blotting

To analyse the proteins separated by SDS-PAGE, Western-blotting was employed. To this purpose, a PVDF-membrane pretreated with methanol and water was mounted onto the gel and placed in an electrophoresis chamber. The transfer from gel to membrane was achieved by application of an electric current of 30V in direction of the membrane for 12-14 hours. The membrane was stained with Coomassie stain to mark the molecular weight ladder, subsequently destained and unspecific binding sites of the membrane blocked by incubation in a BSA-solution for 2h. The primary antibody was applied to the membrane and left to incubate for 4hours at 4°C. After removal of the primary antibody solution, the membrane was washed thrice in TBST/Tween for 15min and incbated with HRP-coupled Protein G (for monoclonal primary antibody, 3µl antibody in 15ml TBST) or HRP-coupled protein A (for polyclonal primary

antibody). The membrane was washed again and developed for a minute with 10ml ECL and 3µl H2O2 or the Chemiglow-system.
When detection of different proteins within the same Western-blot was desired, membranes were stripped of the bound antibody by incubation in a SDS – ß-mercaptoethanol at 75°C for 5-10min. The membranes could then be processed as before.

3.3.3 Coomassie staining

Gels and PVDF-membranes were stained in Coomassie-staining buffer for 5-15 min. and destained in methanol-glacial-acetic-acid.

3.3.4 Protein purification

E.coli were transformed with the construct-carrying expression vector pGEX 4Ti carrying an inducible prokaryotic promotor and a DNA-sequence coding for an N-terminal Glutathione-S-transferase. Cloning was performed as described under *DNA-cloning* and bacteria transformed as described under *Transformation of E.coli*. A 2l culture medium was inoculated with the transformed expression strains and incubated until an OD (optical density) of 0,8 – 1 was achieved. Protein biosynthesis was induced by addition of IPTG to a final concentration of 0,1M in the exponential growth phase and the medium incubated for another 4-6 hours on a shaker. Bacteria were pelleted by centrifugation of 500ml suspension each at 4°C and 6000rpm for 15min and the the pellets resuspended in 20ml T-buffer. This suspension was incubated with lysozyme at 25mg/ml for 30min and subsequently sonicated in an ice-bath to complete bacterial lysis. To bind the generated protein, sepharose-solution was added to the suspension and cell debris pelleted at 16000rpm at 4°C for 20min. Thus cleared, the supernatant was mixed with Glutathione-sepharose washed in T-buffer and incubated on a rocker at 4°C for 2-12 hours. The sepharose was the washed 3 times with 1xPBS at 1000 rpm and protein expression confirmed by SDS-PAGE. Recominant protein was either directly employed by using the sepharose-bound protein, or also eluted by incubation with 10mM glutathione

and dialysed against a physiological buffer. The resulting protein solution was stored at -80°C.

3.4 Handling of DNA

3.4.1 Agarose gel electrophoresis

In this work, agarose gel electrophoresis was mainly used for analytical purposes but incidentally also as a preparative technique. Gels were made from purified agarose, dissolved in boiling 1x TAE by using a microwave. Concentrations ranging from 0,7 – 1,4% were employed. The warm gel was poured into a suitable gel cassette, provided with a spacer to form loading pockets and left to cool. When set, the gel was placed into the electrophoretic appliance which was filled with 1xTAE as a running buffer. DNA samples were mixed with equal amounts GEBS-buffer. Analytical gels were used at concentrations of 0,7 or 1,4 and samples separated at 50-90V, while preparative gels were used at 1,0 or 1,4% and a low voltage applied for separation. The DNA was visualized by using ethidiumbromide (0,3%) and UV-light. Analytical gels were simply photographed while DNA bands in preparative gels were removed under visual control and processed according to *DNA preparation*.

3.4.2 DNA preparation from agarose gels

DNA was reisolated from agarose gel by using a commercial kit (*Qiaquick gel extraction, Quiagen*®). According to the manufacturer´s instructions, the piece of gel containing the DNA sample was weighed and thrice the volume of QG-buffer added (100mg=100µl). This mixture was heated to 50°C until completely dissolved. PH variations needed to be adusted by the addition of NaCOOH pH5 for DNA fragments smaller than 500bp or larger than 4000bp, isopropanol was added at equal amounts to increase the yield. The dissolved and buffered gel was loaded onto specific columns provided with the kit and centrifuged at 13000rpm, the flow discarded. The column was washed with 500µl QG-buffer and incubated with 750µl PE-buffer for 2-3min. The column was centrifuged

twice, the flow discarded. DNA was eluted from the column with 50µl EB-buffer or water.

3.4.3 DNA preparation from bacteria (Miniprep, Midiprep)

Miniprep - An inoculating loop was loaded with transformed *E.coli*, which were dissolved in 300µl P1 solution by vortexing. The resulting suspension was mixed well with 300µl P2 solution and incubated for 5min at room temperature. Next, 300µl of P3 were added and left to incubate for another 10min at room temperature. The resulting mixture was centrifuged at 4°C for 30 min at 13000 rpm and 800µl of the supernatant transferred into a fresh eppendorf. To precipitate the plasmid DNA, 560µl of isopropanol (0,7%vol) were added, mixed well by vortexing and incubated on ice for 2-3min. DNA was pelleted by centrifugation for 30min at 13000rpm, washed with 300µl ethanol (70%) and again spun at 13000 for 5min. The supernatant was carefully removed and the pellet left to dry at 37°C (~10min). The dry DNA was resuspended in 40µl sterile A.bidest or TE-buffer.

Midiprep - A commercial kit (NucleoBond™, Clonetech/Becton Dickinson) was used according to the manufacturer´s instructions. The desired bacterial clone was grown on LB-agar containing a suitable antibiotic at 37°C overnight. A single colony was used to inoculate 1ml LB-medium containing a suitable antibiotic and cultured for 6 hours at 37°C on a shaking incubator. 200µl of this pre-culture were transferred into 200ml of the same medium and incubated under similar conditions overnight. The resulting culture was spun at 4500-6000g for 15 min at 4°C and the bacterial pellet resuspended in 4ml S1+RnaseA buffer. Subsequently, 4ml S2 buffer were added, mixed well and incubated for 5min. at room temperature. After the addition of 4ml S3 buffer the suspension was incubated on ice for 5min and centrifuged at 4°C for at least 25min at 10000g. The NucleoBond™ column was equilibrated with 2,5ml N2 buffer in the meantime. The supernatant was added onto the column and the flow discarded. The column was washed with 10ml N3 buffer, the DNA eluted with 5ml N5 buffer and, for DNA-precipitation, mixed with 3,5ml isopropanol.

The solution was then spun at 4000rpm at 4°C for an hour. The resulting pellet was washed with 2ml 70% ethanol and again centrifuged for 10min, the alcohol removed with a pipette. The pellet was dried and dissolved in 200-400µl TE-buffer. DNA-purity and concentration were determined by photometry.

3.4.4 Polymerase chain reaction

Quantitative PCR for cloning was performed by using a mixture of the two DNA-polymerases Taq and Vent. For more flexibility, enzyme and reaction mix were prepared separately. A typical PCR was performed in a thermocycler using:

Reaction mix:		Enzyme mix:	
10xbuffer	4µl (-$MgCl_2$)	H_2O	8 µl –
$MgCl_2$ 25mM	1,5µl	10xPuffer	1µl (-$MgCl_2$)
Primer1	1µl (10pmol)	Taq-Polymerase	0,5µl
Primer2	1µl (10pmol)	Vent-Polymerase	0,5µl
Template	1µl (~100ng)		
dNTP-Mix	1µl		
H_2O	30,5µl		

For new primers, samples were amplified using a programme with a temperature gradient, as outlined below.

PCR programme:

1 - 94 °C		hot start
2 - 94 °C	for 20sec	denaturing
3 - 52-60 °C	for 20sec	annealing
4 - 72 °C	for 60sec per 1kbp	elongation
30 cycles of	step 1-4	amplification
5 - 72 °C	for 5-10min	elongation
6 - 8°C		stop

For analytical purposes, only Taq-polymerase was used and no temperature gradient employed:

Reaction mix:		Enzyme mix:	
10xbuffer	1,5µl (-$MgCl_2$)	H_2O	4,3µl
$MgCl_2$ 25mM	1,3µl	10xPuffer	0,5µl (-$MgCl_2$)
Primer1	0,4µl (10pmol)	Taq-Polymerase	0,2µl
Primer2	0,4µl (10pmol)		
Template	0,2µl (~20ng)		
dNTP-Mix	0,2µl		
H_2O	11µl		

PCR programme:

1 - 94 °C		hot start
2 - 94 °C	for 20sec	denaturing
3 - 55 °C	for 20sec	annealing
4 - 72 °C	for 60sec per 1kbp	elongation
20 cycles of	step 2-4	amplification
5 - 72 °C	for 5-10min	elongation
6 - 8°C		stop

All PCR results were checked on agarose gels (adjusted to fragment length) as described under *Agarose Gel Electrophoresis*. Correct PCR-products for cloning purposes were isolated as described under *DNA Preparation from Agarose Gels.*

3.4.5 PCR-Cloning using the InFusion-Reaction™

To generate bacterial clones carrying a specific DNA-sequence for transfection of eukaryotic cells or recombination into prokaryotic expression vectors, we employed an efficient restriction-enzyme and ligase-independent cloning method. This system was purchased from the manufacturer (BD, In-Fusion PCR Cloning Kit™) and works with an InFusion (IF) DNA sequence which is

integrated into the PCR primers for the fragment in question and recognized by the so-called InFusion-enzyme, as well as a specific pre-linearised vector (pDNR-Dual) that contains DNA-sequences complementary to the IF-primer sequences. In a first step, oligonucleotide primers complementary to the DNA-fragment to be amplified were designed. Additionally, the 15b IF sequence was added to the 5´ end of the primer. PCR was performed as described under PCR and the PCR products purified by a preparative gel electrophoresis and checked by an analytical gel electrophoresis. In a second step, the actual InFusion reaction was induced by adding appropriate amounts of InFusion enzyme and linearised pDNR-Dual vector in a reaction mix:

Reaction mix:

50-100ng	PCR product
1µl	linearised pDNR-Dual vector (100ng/µl)
1µl	1:10 diluted BD InFusion enzyme (20U/µl)
1µl	10xBD InFusion reaction buffer
1µl	10xBSA
xµl	A.bidest to adjust to 10µl reaction mix

Incubation for 30min at room temperature. After incubation, the reaction mix was placed on ice and competent *E.coli* were transformed as described in *Transformation of E.coli*. Successfully transformed clones of *E.coli* were selected on LB-X-Gal/IPTG/Amp-agar, subcultured overnight on fresh plates and plasmids isolated from bacteria as described under *Plasmid Mini-preparation* for analysis by PCR and gel-electrophoresis.

3.4.6 Cloning using the Cre-loxP Site-Specific Recombination™

By using this commercial system, also purchased from the manufacturer (BD, Creator DNA Cloning Kit™), target sequences can be transferred directly from pDNR-Dual donor vectors into a multitude of expression vectors. It is based on enzymatic recombination of donor and acceptor vector by Cre-Recombinase is recognizing so-called loxP-sites. The transferred DNA fragment contains a

chloramphenicol resistance gene which, after successful recombination, comes under control of a prokaryotic promotor in the acceptor vector and is therefore suited for selection of successfully recombined clones. The donor vector carries the SacB gene, coding for the sucrase of B. subtilis, which inhibts bacterial growth on sucrose-containing media. By recombination an artificial intron in the eukaryotic expression vector is created, containing the Cam resistance with its promotor and a lox-P site, removed at RNA-level by the eukaryotic splicosome.
Donor vector is the pDNR-Dual vector containing the DNA fragment in question (see InFusion reaction), acceptor vector were the prokaryotic expression vector pET-28a-loxP and the eukaryotic expression vector pLPS-3´EGFP. For successful recombination, concentrations of donor and acceptor vectors need to be adjusted to a 1:2 ratio in the reaction mix:

Reaction mix:

100ng	donor vector
200ng	acceptor vector
0,5 µl	Cre-recombinase
1µl	10x Cre-reaction buffer
1µl	10xBSA
xµl	A.bidest to adjust to 10µl reaction mix

After exactly 15min incubation at room temperature, Cre-recombinase was heat-inactivated at 70°C for 5min. Prolonged heating periods lead to undesired recombination products and diminuished yield of correctly recombined plasmids. Straight after inactivation of recombinase the reaction mix was used to transform 100µl competent *E.coli* Nova-Blue using 5µl reaction mix, and successfully transformed bacteria selected overnight on LB-agar containing 7% sucrose and 30µg/ml chloramphenicol at 37°C. Colonies grown under these selective conditions were subcultured for another 24h, plasmids isolated by mini-preparation and analysed by gel-electrophoresis for size and subsequently by PCR with specific oligonucleotides. To exclude sequence errors, products were in a final step analysed by restriction and sequencing. Correct clones were

subcultured and aliquots stored at –80°C, or plasmids harvested by midi-preparation and used for transfection of eukaryotic cells.

3.4.7 In vitro site-specific mutagenesis

Mutagenesis was conducted by overlapping PCR with primers containing the appropriate mutated site and restriction sites for PSI I:

Nck-R308K-sense:

5´-GAA GGG GAT TTC CTT ATA AAA GAT AGT GAA TCT TCG CCA AAT G-3´

Nck-R308K-anti:

5´-CGA AGA TTC ACT ATC TTT TAT AAG GAA ATC CCC TTC ATG TCC-3´

By changing the base sequence at position 308 from CGT/GCA to AAA/TTT, the amino acid Arginine was exchanged for Lysine in the protein product. The creation of a new restriction site (PSI I) did not change the amino acid sequence.

Reaction mix:		Enzyme mix:	
10xbuffer	4µl (+$MgSO_4$)	10xPuffer	4,5µl (+$MgSO_4$)
Primer1	1µl (10pmol)	Pfu-Polymerase	4µl (2,5U/µl)
Primer2	1µl (10pmol)	H_2O	36,5µl
Template	1µl (~50-100ng)		
dNTP-Mix	0,5µl		
H_2O	32,5µl		

PCR programme:

1 - 94 °C	for 30sec	denaturing
2 – 52, 56, 60 °C	for 45sec	annealing
3 - 68 °C	for 6min (1min/1kbp)	elongation
20 cycles of step 1-3		amplification/termination
4 - 68 °C	for 7min	final elongation

PCR was performed using the Pfu-polymerase, which stops polymerization just before the 5´ending of the primer. To remove the template DNA, Dpn I, an enzyme which digests specifically methylated DNA, e.g. the nonmutated parental template, was employed. The PCR-product was incubated with Dpn I for 2hours at 37°C. and subsequently used to transform *E.coli* NovaBlue. The resulting colonies were then checked by miniprep and restriction digest with Psi I, see *Restriction Digest*. Correct clones were subsequently subcultured and stored as described under *Storage of Bacteria*.

3.4.8 Restriction digest

DNA was extracted with a miniprep and subsequently digested with restriction enzymes. The digest was performed as follows:

Reaction mix:

4U	Restriction enzyme (Psi I)
1µl	Buffer
1-3µl (0,5-1µg)	DNA
xµl	H_2O to a total of 10µl

Incubation at 37°C for 1-2 hours.

The DNA-digest was mixed with GEBS-sample buffer, the resulting fragments visualised by agarose gel electrophoresis and the restriction pattern analysed.

3.4.9 DNA-sequencing

Fragments were sequenced using an Applied Biosystems AbiPrism 310™ single capillary electrophoresis instrument for automated DNA sequencing and DNA fragment analysis and the AmpliTaq-Polymerase™ (FS) and BigDye-terminator sequencing kit. This technology employs the chain termination method for sequencing. Forward and reverse primers identical to the PCR primers were used for the sequencing reaction in the following reaction mix:

Reaction mix:

4µl	Premix (polymerase, BigDye terminators, dNTPs)
0,1- 0,5µg	Plasmid DNA
50pmol	Forward and Reverse Primer
xµl	A. bidest to give 10µl in total

For amplification in the thermocycler the following reaction cycle was used:

PCR programme:

1 - 96 °C	for 2min	hot start
2 - 96 °C	for 30sec	denaturing
3 - 45-60 °C	for 15sec	annealing
4 - 60 °C	for 4min	elongation
25 cycles of step 2-4		amplification/termination
5 - 60 °C	for 10min	final elongation

The resulting PCR-products were precipitated in an ethanol-mixture to remove salts, primers and polymerase (90µl A.bidest, 10µl 3M sodiumacetate (pH 4,6) and 250µl of 100% ethanol). The suspension was then spun at 15000rpm for 15min and the supernatant discarded. The pellet was dissolved in 250µl ethanol 70% and again centrifuged at 15000rpm for 5min, subsequently dried in a Speed-Vac™, denatured at 95°C for 2min and redissolved in 25µl TSR-buffer. Subsequently, the DNA sequence fragments were analysed by single capillary electrophoresis with the AbiPrism 310T^{TM} Standard system.

3.5 In vitro protein interaction studies

3.5.1 GST-pull down assay

GST-fusion protein was produced as a recombinant protein fused to an N-terminal glutathione-S-transferase as described in *Transformation of E.coli* and *Protein purification*. To allow interaction, an 800µl aliquot of cell-lysate containing the prey protein was incubated with 5-10µg purified GST-fusion-protein-sepharose suspension at 4°C for 1-3 hours under continuous inversion.

Where eluted GST-protein was employed, the lysate needed to be incubated subsequently with glutathione sepharose for another 2 hours. Afterwards, samples were shortly centrifuged at °4C and 13000rpm and washed three times with Triton-buffer. Finally, 2xsample buffer was added for analysis by SDS-PAGE and Western-blotting.

3.5.2 Co-immunoprecipitation

Cells were co-transfected with recombinant constructs of the putative interaction partners and cultured till confluent. Cell lysates were obtained as described under *Cell Lysis*. An 800µl aliquot was incubated with 3µl of a suitable antibody for 4 hours at 4°C on an inverter. Subsequently, 25µl of a protein A/G sepharose suspension were added and incubated for another hour at 4°C to facilitate binding of antibody/antigen complex to the sepharose. The suspension was centrifuged shortly at 10000rpm (20sec), the supernatant carefully removed and the pellet washed 2-3 times. Finally, the washing medium was removed to leave only 40µl and 15µl 4xsample buffer added. The sample was mixed thoroughly and processed as described in *SDS-PAGE*.

3.6 Infection studies

3.6.1 Gentamicin protection assay

For gentamicin protection assays, cells were transfected with the respective constructs as described and distributed at 6-8 x 10^5 293T cells/well into 24-well plates. Prior to seeding of cells into the wells, the plates were coated overnight with fibronectin (4µg/ml) in PBS at 4°C. For expression control, 1x10^6 cells were seeded into 10cm cell culture dish. Plates were incubated over night at 37°C and 5% CO_2. Opa_{CEA}-expressing and non-opaque *Neisseria* were selected as described and resuspended as described. The bacterial content was determined by measuring the optical density at 550 nmand an MOI of 20 bacteria/cell calculated. Cells were infected for 60min at 37°C and 5% CO_2. Following infection, the medium was replaced with DMEM+10%CS containing 50 µg/ml gentamicin. After 45 min incubation the medium was removed and cells were lysed by the addition of exactly 1ml sterile 1% saponin in PBS for 15

min and mechanical disruption using the pipette. Suitable dilution preparations of a dilution series were plated in duplicates onto GC-plates to determine the number of recovered intracellular bacteria by the number of formed colonies.

3.6.2 Immunofluorescence studies

Cells for immunofluorescence labeling were cultured on acid-washed glass cover slips coated with fibronectin (4µg/ml) and poly-L-lysin (20µg/ml) in 24 well plates. $5x10^4$ – $1x\ 10^5$ cells were seeded initially into the wells and incubated in DMEM+10% CS at 37°C and 5%CO2 overnight. After incubation, cells were infected with bacteria at an MOI of 10-40 and left for 30-90min. Subsequently, the medium was removed and cells washed with PBS++, then fixed in 300-400µl 4% PFA for 20min at room temperature and again washed thrice with PBS++. To label extracellular bacteria for microscopy, a rabbit-anti-*N. gonorrhoeae* (anti-Opa) antibody was added, which in turn was labeled with goat-anti-rabbit-Cy5 antibody, both in 20µl blocking buffer (PBS, 5% FCS, 0,2% Saponin) for 45min in a dark and moist environment. The resulting samples were washed three times with PBS++, endued with mounting medium and placed upside-down on a slide. The sides were sealed with nail varnish and kept at 4°C.

3.6.3 Confocal laser-scanning microscopy

The immunofluorescence preparations, as described under *Immunofluorescence Staining*, were viewed with a confocal argon/crypton laser microscope (Zeiss LSM). A transmission filter allowed separate adjustment of the intensity of the emitted wave lengths. Optical images were achieved by using 40x/oil and 60x/oil objectives and the data processed with the specified software (Zeiss LSM), which assigned defined pseudocolours to the emitted light signals (GFP-green, Cy3-red, Cy5-blue). Pictures were edited with LSM Image Browser (Zeiss) and Adobe Photoshop 6.0 (Adobe Systems, Mountain View, Canada) software.

3.6.4 Oxidative burst

We followed a protocol based on the methodologic paper by John de la Harpe and Carl F. Nathan (De la Harpe and Nathan 1985). Human peripheral blood neutrophils were obtained as described in *Isolation of Primary Cells*, taken up in Krebs-Ringer buffer (KRB) and kept on ice just before use. The cell population was checked for viability by conventional light microscopy, then seeded in triplets at $2x10^5$ cells/50µl/well into a 96well plate which had been coated with PBS 0,25%BSA at 37°C for 3-4h. Primary human granulocytes were infected with Opa-negative *N.gonorrhoeae*, Opa_{CEA}-expressing gonococci or left uninfected. Bacteria were gained as described in *Selection of Opa_{CEA}-expressing bacteria*, washed 3x with PBS and kept at a concentration of $1x10^9$ bac/ml in PBS.

<u>2xAssay Mix</u>

30µl	Scopoletin 10mM
13µl	NaN_3 5%
100µl	Horseradish Peroxidase
50µl	Glucose 1M
4807µl	Krebs-Ringer-Buffer to give a total of 5ml 2x AM

<u>Krebs-Ringer-Buffer</u>

580µl	NaCl 5M
456µ	$NaPO_4$ 0,25M
194,4µl	KCl 0,5M
4,32µl	$CaCl_2$ 2,5M
244µl	$MgSO_4$ 0,1M
18,5ml	H_2O_2 to give a total of 20ml KRP

Infection was started with an MOI of 50, i.e. $1x10^7$ bacteria in 10µl per well just after the addition of 50µl 2x assay mix (AM) to the cells in KRP. Fluorescence was measured using a photometer at time points 0min till 90min after infection at 10min intervals. In between, cells were kept at 37°C. Each measurement was

recorded in triplets. The amount of H_2O_2 produced per 90min was calculated as follows:

$$H_2O_2 \text{ released in nmol /90min} = \frac{(F_0\text{-}E) - (F_{90}\text{-}E) \times (S_0\text{-}E)/(S_{90}\text{-}E)}{(S_0\text{-}E)} \times \text{Scop}$$

F_0	–	Fluorescence at timepoint 0min
F_{90}	–	Fluorescence at timepoint 90min
E	–	Fluorescence reading of an empty well (background fluorescence)
S_0	–	Fluorescence in the cell-free standard at timepoint 0min
S_{90}	–	Fluorescence in the cell-free standard at timepoint 90min
Scop	–	Scopoletin in nmol in each well at timepoint 0min

Prior to calculation of the oxidative response for each bacterium and the uninfected control, the mean value of the triplets was determined. The results were displayed graphically in a bar diagram with their standard deviance.

4 Results

CEACAM3 has a potential to induce the opsonin-independent elimination of CEACAM-binding bacteria by human granulocytes (Hauck, Meyer et al. 1998; Schmitter, Agerer et al. 2004; Kuespert, Pils et al. 2006; Schmitter, Pils et al. 2007). In this process, association of Opa_{CEA}-expressing bacteria with CEACAM3 triggers an intracellular signaling cascade, followed by cytoskeletal rearrangement and an oxidative response. Phosphorylation of an ITAM-like sequence in the cytoplasmic tail of CEACAM3 by Src-family-PTKs and stimulation of the small GTPase Rac have been shown to be crucial events in this process (Abedi, Dawes et al. 1995; Gray-Owen, Dehio et al. 1997; Hauck, Meyer et al. 1998; Billker, Popp et al. 2002; McCaw, Schneider et al. 2003; Schmitter, Agerer et al. 2004; Schmitter, Pils et al. 2007). However, the immediate, membrane-proximal molecular events after receptor ligation leading to Rac activation, have not yet been fully characterised. To explore these molecular signaling events of CEACAM3, we took advantage of 293T cells, derived from a human embryonic kidney cell line. These cells are stable, easily transfectable and importantly, endogenously express no member of the CEACAM family. Furthermore, when transfected with CEACAM molecules these receptors are expressed on the cell surface, conferring the ability to internalise Opa_{CEA} gonococci onto these cells. They thus enable, in contrast to primary human granulocytes, isolated studies on distinct CEACAM-members (Schmitter, Agerer et al. 2004).

To study putative interaction partners of CEACAM3, we employed recombinant SH2 domains of various signaling molecules in interaction assays and as a dominant-negative protein in a phagocytic assay. By employing laser scanning confocal microscopy we wanted to visualize this interaction in vivo and link it to infection with Opa_{CEA} expressing bacteria. Using a Gentamicin-protection assay, the role of individual signaling molecules in the process of internalization was analysed. Additionally, we stimulated primary human PMNs with Opa-expressing bacteria and monitored the oxidative response.

4.1 Src-SH2 binds specifically to the phosporylated cytoplasmic domain of CEACAM3 but not CEACAM6

Phosphorylation of tyrosine residues is a key relay signal in many cell signaling pathways that mediate the internalization of bacteria (Bliska and Falkow 1993; Bliska, Galan et al. 1993). Hauck et al. first demonstrated that infection of in vitro-differentiated human myelomonocytic JOSK-M cells with Opa_{CEA} expressing *Neisseria* leads to extensive tyrosine phosphorylation of several cellular proteins. This phosphorylation is dependent on Src family PTKs, as it can be abrogated by Src-family-kinase-inhibitors, and initiates bacterial internalization (Hauck, Meyer et al. 1998). Since CEACAM3 has been shown to be the phagocytic receptor responsible for phagocytosis of Opa_{CEA} -expressing *Neisseria*, these processes in human PMNs should involve Src-family PTK protein phosphorylation (Schmitter, Agerer et al. 2004; Pils, Gerrard et al. 2008). Accordingly, CEACAM3, in its most common splice variant, is a transmembrane protein with a cytoplasmic domain containing a characteristic tyrosine-based activation motif (ITAM-like), likes of which are responsible for phagocytic signaling in the Fc-receptor-mediated uptake. With the exception of CEACAM4, other CEACAM family members do not carry such a tyrosine phosphorylation motif. In ITAM-initiated signaling events, receptor engagement and clustering is followed by Src-PTK tyrosine phosphorylation. The thus created phosphotyrosines facilitate the docking of proteins that contain Src homology 2 (SH2) domains (Iwashima, Irving et al. 1994; Chan, Dalton et al. 1995; Isakov 1997; Isakov 1997; Aderem and Underhill 1999; McCaw, Liao et al. 2004)

To test whether CEACAM3 supports the binding of the Src SH2-domain in contrast to other family members lacking tyrosine-based signaling motifs, we designed a preliminary experiment using the GST-fusion protein pull-down strategy, a simple and effective method to detect protein interaction by exploiting the high affinity of gluthathione-S-transferase (GST) to its substrate glutathione. A recombinant c-SrcSH2-fusion protein was already available in the laboratory and was subsequently employed as bait protein by incubation with

CEACAM protein-containing cell lysates. CEACAM family members were expressed in 293T cells, to ensure correct folding and glycosylation and minimize artefacts in their binding behaviour. 293T cells were co-transfected with v-src to constitutively phosphorylate the receptor in vivo, independently of bacterial infection. On day 2 after transfection, when maximum levels of protein expression could be expected, the cells were lysed and the whole cell lysates employed in the interaction studies.

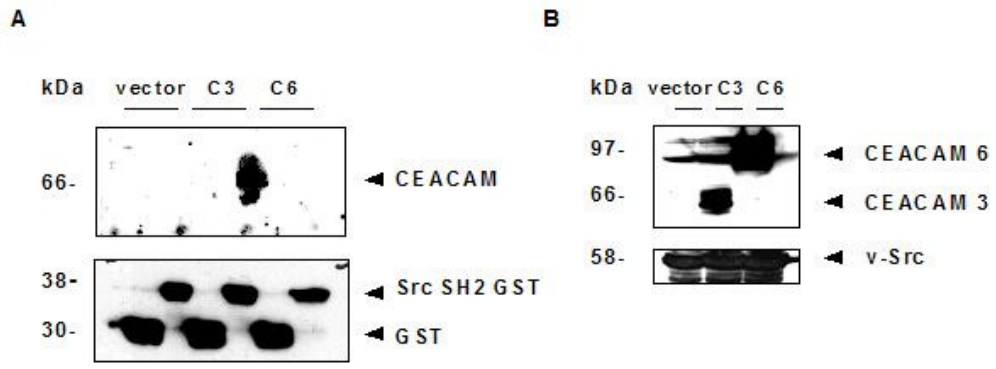


Fig. 4.1 **Src-SH2 binds specifically to the phosporylated cytoplasmic domain of CEACAM3.**
293T cells were cotransfected with the empty control vector (pcDNA), CEACAM3, or CEACAM6 and constitutively active Src (v-Src). (A) Cell lysates were employed in pull-down assays with the isolated Src-SH2 domain fused to GST or GST alone. Precipitates were analyzed by Western blotting with monoclonal anti-CEACAM antibody (top) and then stripped and reprobed with a monoclonal anti-GST antibody (bottom). (B) On a separate blot whole cell lysates were analysed for CEACAM (top) and v-src expression (bottom).

Cells were either transfected with the empty control vector, CEACAM3 or CEACAM6 and v-Src. The resulting lysates were incubated with GST-c-Src SH2 or GST as a negative control. After precipitation with gluthathione-sepharose, the samples were analysed by western blotting with an α-CEACAM antibody. Importantly, specific interaction with c-Src SH2 could only be detected for the ITAM-containing CEACAM3, but not for the GPI-anchored CEACAM6 or GST alone (Fig. 4.1 A and B).

To rule out stoichiometric effects, we checked that equal amounts of fusion protein were employed in the assays (lower blot). Our findings imply, that the efficient CEACAM3-dependent internalization of gonococci by PMNs requires

direct interaction with kinases of the Src-family in contrast to CEACAM6, for which this interaction seems to be dispensable.

4.2 The small adapter protein Nck binds directly to the cytoplasmic domain of CEACAM3 after receptor activation

In tyrosine phosphorylation-dependent signaling pathways, receptor clustering and subsequent phosphorylation by tyrosine-specific PTKs creates binding sites for SH2 domain-containing molecules. In Fcγ-mediated phagocytosis, Syk family kinases are the first to be recruited to the activated receptor (Darby, Geahlen et al. 1994; Greenberg, Chang et al. 1996). Interestingly, Syk has been shown to be dispensable in early ITAM-signaling in CEACAM3-mediated phagocytosis (Hauck, Meyer et al. 1998; Humphrey, Lanier et al. 2005; Cougoule, Hoshino et al. 2006; Sarantis and Gray-Owen 2007; Schmitter, Pils et al. 2007). To explore the early molecular interaction events after activation of CEACAM3, we again employed the GST-fusion protein pull-down strategy to screen a panel of SH2-containing signaling molecules. In a first step, the SH2-domain encoding regions of several molecules were genetically fused to GST, expressed in *E.coli* and the resulting fusion proteins affinity purified on a glutathione sepharose matrix. Cell lysates containing constitutively phosphorylated and HA-tagged CEACAM3 were probed in a GST pull-down format.

Interestingly, of the screened molecules of signal transduction, only the SH2 domains of Nck and Src showed reproducibly significant association with CEACAM3 (Fig. 4.2 A and B), implying a specific interaction between CEACAM3 and NckSH2. Again, the amount of GST-fusion protein in each assay was carefully titrated, so that a distortion of results on the basis of quantity can be ruled out.

Nck, unlike the kinase Syk, is a small adapter protein lacking intrinsic enzymatic activity and has not been described in ITAM-signaling so far. It has however been described in various processes of actin dynamics where it serves as an

adaptor protein which recruits components of the actin polymerization machinery to the plasma membrane (Li and She 2000; Li, Fan et al. 2001; Buday, Wunderlich et al. 2002). This activity allows local membrane reshaping and podocyte formation, consistent with the processes of engulfment and internalization in CEACAM3-mediated phagocytosis.

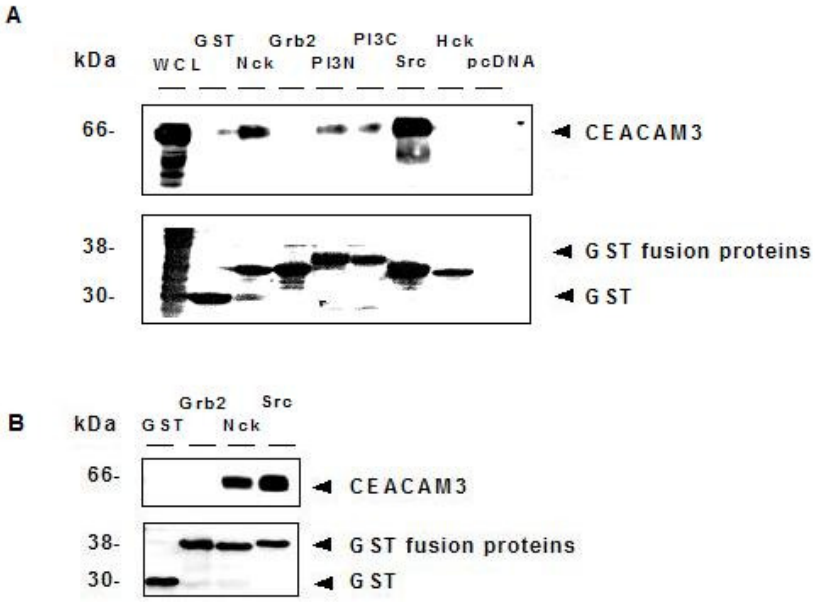


Fig. 4.2 **The small adapter protein Nck binds directly to the cytoplasmic domain of CEACAM3 after receptor activation.**
293T cells were cotransfected with CEACAM3-HA and constitutively active Src (v-Src). (A) and (B) Cell lysates were probed with the isolated SH2 domains of various molecules of cell signaling fused to GST or with GST alone in a GST pull-down format. Precipitates were analyzed by Western blotting with monoclonal anti-HA antibody (top) and then stripped and reprobed with a monoclonal anti-GST antibody (bottom).

To test the hypothesis that Nck is a binding partner of CEACAM3 after receptor activation, the following experiments were designed.

4.2.1 Interaction between NckSH2 and CEACAM3 requires the cytoplasmic domain

The members of the CEACAM family vary greatly in their molecular structure. While CEACAM3 encompasses a cytoplasmic domain with an ITAM-motif,

CEACAM1, while likening CEACAM3 in its extracellular portion, contains a functional ITIM. GPI-anchored CEACAMs, like CEACAM6 lack a cytoplasmic portion altogether, and these structural differences also seem to determine their association with membrane microdomains. All CEACAM receptors however, have been found to be capable of mediating uptake of Opa_{CEA}-expressing bacteria into various cell types. Yet, CEACAM1 and 6 only show a minor contribution to the opsonin-independent phagocytosis by human PMNs (Schmitter, Agerer et al. 2004; Schmitter, Pils et al. 2007; Pils, Gerrard et al. 2008). This differential behaviour of CEACAM molecules is probably due to distinct signaling cues, dictated by the molecular structures of the respective CEACAM member. It has already been demonstrated that the ITAM is indispensable for the phagocytic processes induced by neisserial interaction with CEACAM3, requiring phosphorylation by kinases of the Src-family and recruitmet of SH2-domain containing effector proteins. With this in mind, we postulate that NckSH2 binding to CEACAM3 takes place at the cytoplasmic domain and is dependent on tyrosine phosphorylation. To prove our hypothesis, we generated a CEACAM3 deletion mutant lacking the cytoplasmic domain, specified as CEACAM3ΔCT (Fig. 4.3 A), to abrogate NckSH2 binding.

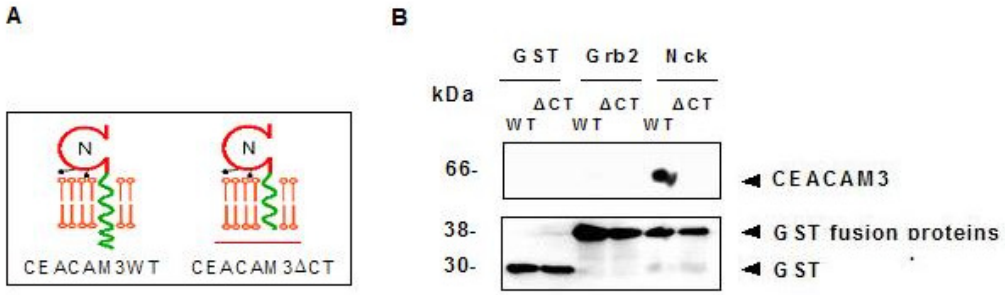


Fig. 4.3 **Interaction between NckSH2 and CEACAM3 requires the cytoplasmic domain of CEACAM3.**
(A) A specific deletion mutant of CEACAM3 (CEACAM3_CT) was created, lacking the cytoplasmic domain. (B) The deletion mutant of CEACAM3HA (_CT) and wildtype CEACAM3HA (WT) were expressed in 293T cells and phosphorylated by cotransfection with v-Src.. Cell lysates were probed with NckSH2-GST, Grb2SH2-GST and GST alone. Precipitates were analyzed by Western blotting with a monoclonal anti-HA antibody (top) and then stripped and reprobed with a monoclonal anti-GST antibody (bottom).

Consistent with our hypothesis, no interaction was detectable in GST-pull-down assays between CEACAM3ΔCT and the SH2-domain of Nck, while wild-type CEACAM3 strongly associated with NckSH2 (Fig. 4.3 B). Importantly, the introduction of the HA-tag, which like the cytoplasmic domain contains tyrosine residues, did not bias the interaction as C3ΔCT-HA reproducibly showed no association with GST-NckSH2.

4.2.2 Interaction between NckSH2 and CEACAM3 requires an intact ITAM and specific tyrosine phosphorylation

To investigate the role of tyrosine phosphorylatin for binding of NckSH2, we designed a set of experiments with phosphorylated versus unphosphorylated WT-CEACAM3. Phosphorylation was achieved by co-transfection of cells with v-Src. We checked that the addition of v-Src did not bias the expression of our construct. At equal levels of GST-fusion protein and even slightly higher levels of unphosphorylated CEACAM3, absolutely no association of unphosphorylated CEACAM3 and NckSH2 was observed, while association of NckSH2 with phosphorylated CEACAM3 occured as before (Fig. 4.4).

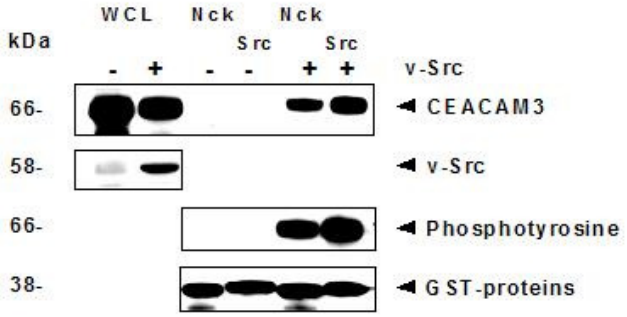


Fig. 4.4 **Interaction between NckSH2 and CEACAM3 requires specific tyrosine phosphorylation of the ITAM tyrosine residues.**
CEACAM3 wild type was expressed in 293Tcells and either phosphorylated by cotransfection with v-src (v-Src +) or left unphosphorylated (v-Src -). The lysates were probed with the SH2 domains of Nck and Src in a GST pull-down format. Precipitates were analyzed by Western blotting with monoclonal anti-HA antibody (top), then stripped and reprobed with an anti-phosphotyrosine antibody (third from top). GST fusion proteins were detected with a monoclonal anti-GST antibody (bottom). Whole cell lysates (very left) were analyzed for receptor expression (top left) and v-Src expression (bottom left).

To determine the role of the ITAM-tyrosines in this process, a specific CEACAM3 mutant (CEACAM3 Y230/241F) deficient of both tyrosine residues was engineered (Fig. 4.5 A). Importantly, replacement of both tyrosines lead to severely reduced binding of NckSH2 to the receptor (Fig. 4.5 B).

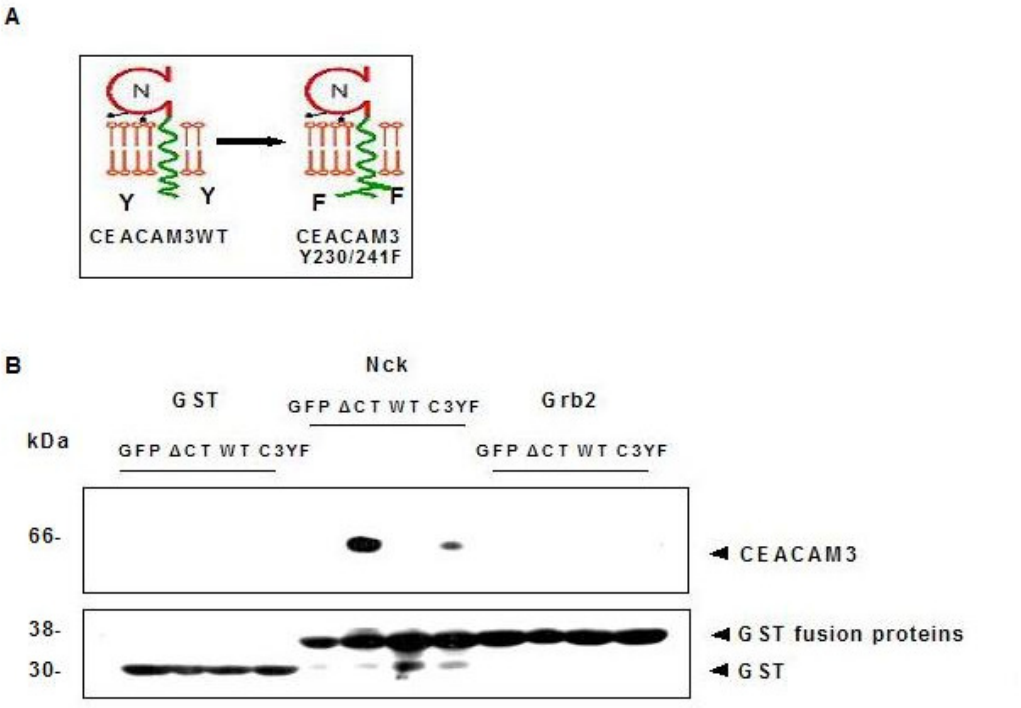


Fig. 4.5 **Interaction between NckSH2 and CEACAM3 requires an intact ITAM-like motif.**
(A) A double mutant of CEACAM3 (CEACAM3 Y230/241F) was created, in which the conserved tyrosine residues are replaced by phenylalanine. (B) the double mutant of CEACAM3HA (C3YF), the deletion mutant of CEACAM3HA (ΔCT), wild type CEACAM3HA (WT) and the empty transfection vector (GFP) were expressed in 293T cells and phosphorylated by cotransfection with v-Src. Cell lysates were probed with NckSH2-GST, Grb2SH2-GST and GST alone. Precipitates were analyzed by Western blotting with monoclonal anti-HA antibody (top) and then stripped and reprobed with a monoclonal anti-GST antibody (bottom).

These studies consolidate the significance of the ITAM motif for association with downstream mediators of cell signaling. The remaining activity of the double mutant CEACAM3 Y230/241F suggests, that the overall sequence of the ITAM contributes to binding specificity.Taken together, these data support the view that binding specificity of NckSH2 to CEACAM3 is a function of receptor

phosphorylation and that its binding domain is located within the CEACAM3 ITAM-like sequence.

4.2.3 Interaction of NckSH2 and CEACAM3 is dependent on the functional integrity of the SH2-domain

Our previous observations demonstrate that the isolated SH2 domain of Nck is capable of interacting with the phosphorylated wild-type receptor. However, we were seeking to confirm that the observed NckSH2 association with CEACAM3 is a specific function of the SH2-domain and not simply due to the overall amino acid composition of our construct. To prove this point, we took advantage of the fact that the functional integrity of Nck-SH2 relies on the amino acid Arginine at position 308. When substituted by Lysine, interaction between Nck and phosphotyrosine-motifs is abrogated (Pawson and Gish 1992; Tanaka, Gupta et al. 1995; Li, Meriane et al. 2002). We achieved site-specific mutagenesis of our NckSH2-GST construct by overlapping PCR with specific primers containing appropriate mutated sites. The resulting mutant NckSH2 R308K was employed as a probe in our pull down experiments (Fig. 4.6).

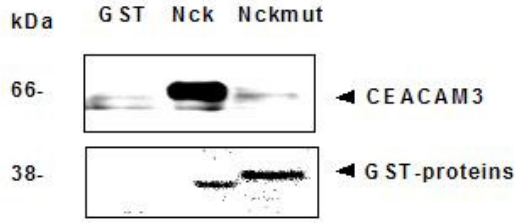


Fig. 4.6: **Interaction of NckSH2 and CEACAM3 is dependent on the functional integrity of the SH2-domain.**
Wild type CEACAM3HA was expressed in 293T cells and phosphorylated by otransfection with v-Src. Cell lysates were probed with wild type NckSH2-GST (Nck), mutant NckSH2-GST (Nckmut) and GST alone. Precipitates were analyzed by Western blotting with monoclonal anti-HA antibody (top) and then stripped and reprobed with a monoclonal anti-GST antibody (bottom).

Interestingly, we saw an important decrease of CEACAM3-NckSH2 interaction when NckSH2 carried the mutation (NckSH2 R308K), suggesting that the interaction is indeed mediated by SH2 domain binding to phosphotyrosine residues.

Concluding, we can state that

- Src-family kinases are capable of associating with and phosphorylating CEACAM3
- the adaptor molecule Nck has a potential to interact with the granulocyte receptor CEACAM3
- the interaction requires the cytoplasmic domain of the receptor
- and phosphorylation of its ITAM-like sequence by Src-kinases
- the interaction is dependent on a functionally intact SH2-domain

These in-vitro data strongly suggest a role for Nck in the CEACAM3-mediated uptake of opaque gonococci by human granulocytes. Furthermore, they imply that the ITAM-like sequence of the receptor plays a central role in this process, since the interaction is dependent on tyrosine-phosphorylation of CEACAM3 and functional integrity of NckSH2 is indispensable.

4.3 Human granulocytes specifically eliminate Opa_{CEA}-expressing gonococci

The interaction of CEACAM molecules with Opa_{CEA}-carrying bacteria in the course of infection induces a strong cellular and humoral innate immune response. In the course of this inflammatory response, gonococci are found associated with and located within human PMNs. Recently, evidence has been accumulating that internalized gonococci are subjected to oxygen-dependent phagocytic activity, indicated by a strong respiratory burst (Virji and Heckels 1986; Fischer and Rest 1988; Hauck, Lorenzen et al. 1997; Schmitter, Agerer et al. 2004; Sarantis and Gray-Owen 2007). Internalization and the respiratory burst are key events in phagocytosis by professional phagocytes. However, although it has been shown that CEACAM3 is a phagocytic receptor mediating efficient internalization and elimination of Opa_{CEA}-carrying *N.gonorrhoeae* in the absence of specific antibodies or complement, it is not clear to which extent the respiratory burst observed in response to neisserial infection is also a function of the CEACAM receptor.

Phagocytic activity is roughly proportional to the oxidative burst which can be measured in vivo by chemiluminescence. To investigate whether the respiratory response observed after gonococcal internalization is linked to CEACAM-receptor engagement in vivo, we designed a chemiluminescence assay with which the oxidative activity of primary human granulocytes after infection with *Neisseria gonorrhoeae* could be quantified. Primary human granulocytes were isolated from freshly-drawn human blood by Ficoll gradient. These cells were counted and subsequently seeded at equal numbers into a 48-well plate. Luminol was added at a concentration of 0,1 mM and cells infected with Opa-negative or Opa_{CEA}-expressing gonococci and incubated at 37°C for 60´. Our negative control was left uninfected (Fig. 4.7).

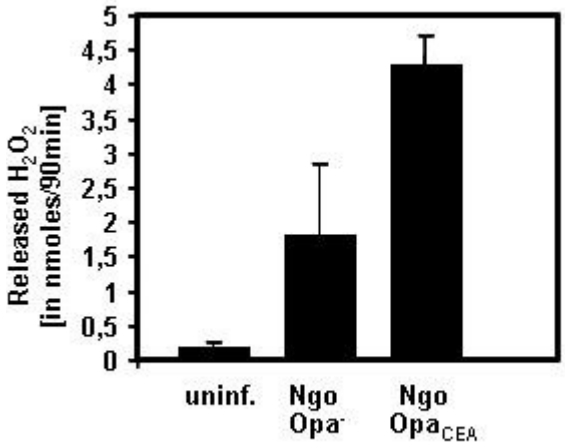


Fig. 4.7 : **Human granulocytes specifically eliminate Opa_{CEA}-expressing gonococci.** Oxidative burst induced in primary human granulocytes after infection with opaque (Ngo Opa_{CEA}) versus non-opaque (Ngo Opa-) *Neisseria gonorrhoeae*. Opaque bacteria induce a significantly higher respiratory burst i.e. phagocytic activity than non-opaque bacteria, that are unable to interact with the phagocytic receptor CEACAM3 on granulocytes. Uninfected cells show no oxidative activity.

Our results show a strong stimulation of the oxidative burst by Opa_{CEA}-expressing bacteria, compared to a significantly weaker stimulation by non-opaque *Neisseria*. From this data we can project, that it is the specific interaction of Opa_{CEA}-adhesin with members of the CEACAM family triggering

an oxidative response, since Opa_{CEA}-negative bacteria do not provoke such a reaction. Moreover, the oxidative burst observed in this experimental assay was independent of prior opsonization by specific antibody or complement factors, as is CEACAM3-dependent bacterial internalization. In this context, it is interesting to note that Nck is a key player in the assembly of cytoskeletal building blocks and can be linked to the activation of the NADPH-oxidase, and both events are indispensable for phagocytosis. It is therefore feasible that CEACAM3-mediated Nck-recruitment and Rac-activation integrate the molecular events essential for an efficient phagocytosis and killing of bacteria.

4.4 Full-length Nck and phosphorylated CEACAM3 associate in vivo

In a subsequent set of experiments, we wanted to analyse the biological relevance of the NckSH2-CEACAM3 interaction. To rule out that we had simply observed an artificial in vitro association, CEACAM3-HA and full-length Nck-myc were coexpressed in transiently transfected 293T-cells. CEACAM3 was manipulated such as to contain a GFP for easy assessment of expression levels and cells were again additionally transfected with v-Src. At maximum expression levels, measured visually by GFP-fluorescence, cells were lysed at 4°C in buffer containing 1% Triton X-100 and the lysate was fractioned by centrifugation. CEACAM3-GFP-HA was immunoprecipitated from the whole-cell lysates with an anti-HA antibody, and after resolution by SDS-PAGE, immunoprecipitates were examined by Western blotting for associated Nck-myc. Whole cell lysates were analysed for equal expression levels of receptor protein on a separate blot with an anti-HA antibody.

Consistent with our in vitro pull-down experiments, CEACAM3-GFP-HA associated with Nck-myc only, when CEACAM3 was phosphorylated by expression of v-src. No relevant levels of Nck-myc could be detected in cells transfected with the empty transfection vector and Nck-myc or when transfected with CEACAM3 alone (Fig. 4.8), ruling out any cross-reacting protein species in our cell lysates. We can therefore confidently conclude that not just the isolated SH2-domain of Nck but the full-length molecule has a binding affinity to

phospho-CEACAM3 and both molecules seem to associate in vivo upon phosphorylation.

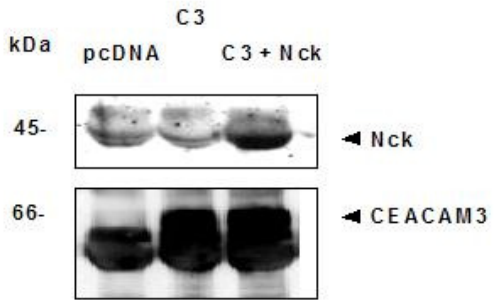


Fig. 4.8: **Full-length Nck and phosphorylated CEACAM3 associate in vivo.**
Wild type CEACAM3HA (C3) and full length Nckmyc (Nck) were coexpressed in 293T cells and the receptor phosphorylated by cotransfection with v-Src. The CEACAM receptor was immunoprecipitated from the whole cell lysates with an anti HA antibody. Precipitates were analyzed for coprecipitation by Western blotting with monoclonal anti-myc antibody (top) and then stripped and analyzed for receptor expression with a monoclonal anti-HA antibody (bottom).

4.5 Full-length Nck and phosphorylated CEACAM3 colocalize at the site of bacterial attachment

Infection of PMNs with gonococci leads to extensive tyrosine-phosphorylation of host proteins and an opsonin-independent phagocytosis of the pathogen, involving the action of the small GTPase Rac (Schmitter, Agerer et al. 2004; Schmitter, Pils et al. 2007; Pils, Gerrard et al. 2008). Phagocytosis involves marked cytoskeletal rearrangement, morphologically characterised by lamellipodia-like membrane protrusions which surround and enclose foreign particles. This act of particle inclusion can be visualised microscopically and constitutes a hallmark of phagocytosis (Greenberg 1999). Lamellipodia formation requires extensive, energy dependent cytoskeletal rearrangement. (Hall 1998; Kaibuchi, Kuroda et al. 1999; Chimini and Chavrier 2000; Dinauer 2003). The identification of the Nck-CEACAM3 interaction is consistent with our governing hypothesis that CEACAM3-dependent signaling serves to coordinate cytoskeletal dynamics, since Nck is known to associate with proteins of the actin polymerization complex and can be linked to the NADPH-oxidase. Plasma

membrane recruitment of Nck to several known tyrosine-phosphorylated transmembrane proteins results in concomitant recruitment of components of the actin polymerization complex and induction of localized actin polymerization (Chen, She et al. 2000; Barda-Saad, Braiman et al. 2005) involving the small GTPase Rac.

To test the hypothesis that recruitment of Nck to CEACAM3 at the plasma membrane induces localized actin polymerization and lamellipodia formation, we once again took advantage of the human embryonic kidney cell-line 293T. Importantly, recruitment of Rac to the site of infection and lamellipodia formation has been demonstrated in this cellular system (Schmitter, Agerer et al. 2004). For our purpose, 293T cells were co-transfected with plasmids encoding red-fluorescent CEACAM3-RFP, and green-fluorescent Nck-GFP. In this setup, *no* artificial phosphorylation was employed which, according to our hypothesis, should be achieved by infection with Opa_{CEA} *N.gonorrhoeae*. Laser-scanning confocal microscopy was employed to visualize the recruitment of Nck-GFP to CEACAM3-RFP after infection with either Opa_{CEA}-expressing gonococci (N309) or Opa_{CEA}-negative *Neisseria* (N302). At peak protein expression, measured by conventional fluorescence microscopy, cells were infected with the respective strain for 30mins, samples fixed in 4% PFA and bacteria stained with rabbit-anti-*N.gonorrhoeae* and goat-anti-rabbit-Cy5 prior to analysis by indirect immunofluorescence microscopy.

293T cells transfected with CEACAM3 that have been infected with Opa_{CEA} *Neisseria gonorrhoeae* for 30 minutes characteristically display lamellipodia-like membrane protrusions around the infecting microorganisms, indicative of phagocytic activity. CEACAM3 can be found in high concentrations along a fine line at the basal aspect of the lamellipodia-like membrane-protrusion. Importantly, Nck accumulates in high concentrations at the site of bacterial infection and the overlay studies show an intense colocalization of bacterium, CEACAM3 and Nck at the nascent phagosome (Fig. 4.9 top and bottom).

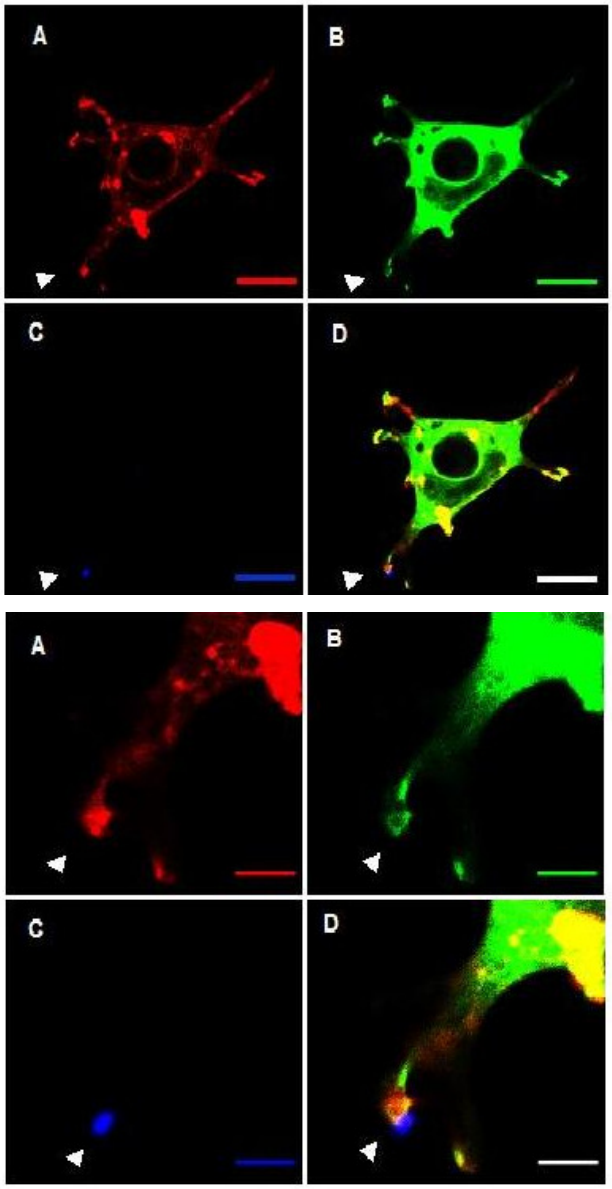


Fig. 4.9: **Full-length Nck and CEACAM3 associate in vivo and can be localised to the site of bacterial attachment.**
(Top) Laser scanning confocal microscopy of 293T cells coexpressing wild type CEACAM3RFP (red, A) and full length NckGFP (green, B). Cells were infected with Opa- expressing Ngo N309 for 30min, then fixed in 4% PFA and bacteria stained blue with cy5 (blue, C). The overlay (D) shows areas of colocalisation in white (white arrow).
(Bottom) Detail of (a): Lamellipodia-like protrusions form around attached bacteria (C). Colocalization of receptor and Nck can be detected at the site of bacterial attachment (D). There was no artificial phosporylation.

Bacterial engulfment is a function of Opa-CEACAM3 interaction, since Opa_{CEA}-negative *Neisseria* (N302) show neither spatial association with CEACAM3 nor

Nck and do not induce receptor clustering nor lamellipodia formation after 30´ or 60´ (Fig. 4.10 top and bottom).

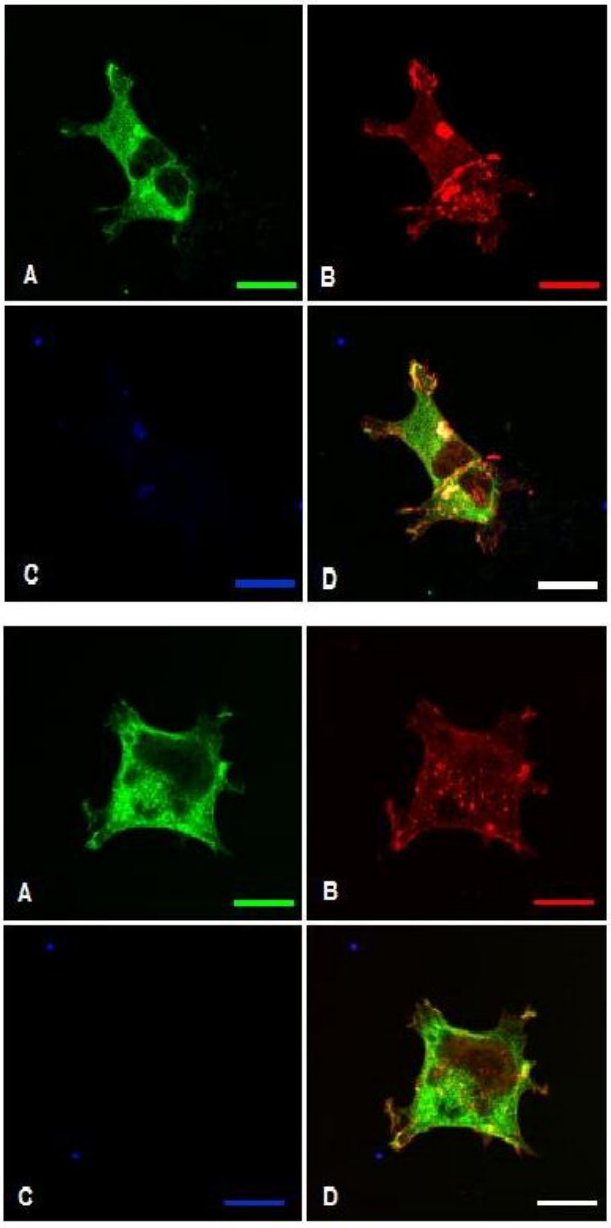


Fig. 4.10 **No membrane attachment of Ngo nor recruitment of Nck to CEACAM3 can be detected in cells infected with nonopaque Ngo N302 after 30´ or 60´.** (Top and Bottom) Laser scanning confocal microscopy of 293T cells coexpressing full length NckGFP (A) and wild type CEACAM3RFP (B). Cells were infected with Opa- Ngo N302 for 30 and 60min., then fixed in 4% PFA and bacteria stained blue with Cy5 (C). Nck is evenly distributed in the cytosol and no spatial association of bacteria and 293T cells can be observed.

According to our hypothesis, infection of CEACAM3-transfected cells with Opa_{CEA} expressing Ngo induced strong receptor phosphorylation at the site of bacterial attachment (Fig. 4.11 top and bottom). This was visualized with an anti-phosphotyrosine-Cy5 antibody on a second set of cells infected according to the same protocol.

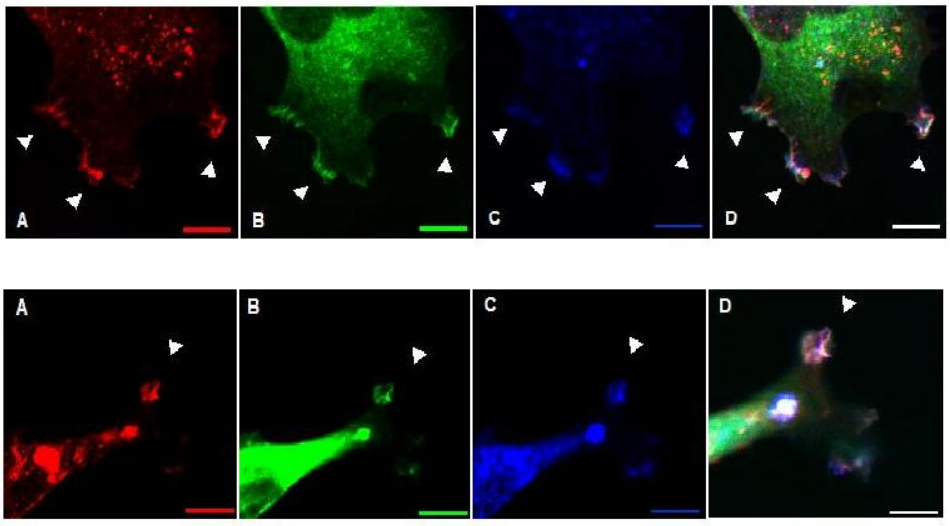


Fig. 4.11: CEACAM3 shows strong phosphorylation at the edge of lamellipodia formation.
(Top and Bottom) Laser scanning confocal microscopy of 293T cells coexpressing wild type CEACAM3RFP (A) and full length NckGFP (B). Cells were infected with Opa_{CEA}-expressing Ngo N309 for 30min., then fixed in 4% PFA and phosphotyrosines labelled with a Cy5-anti phosphotyrosine antibody (C). There was no artificial phosphorylation. The overlay shows areas of colocalisation in white (D). Intense tyrosine phosphorylation can be detected at the edge of lamellipodia formation.

4.6 Nck–SH2 interaction with CEACAM3 is required for an efficient internalization of Opa_{CEA}-expressing bacteria

To confirm the functional role of Nck in the uptake of opaque gonococci by CEACAM3-expressing cells, we employed the isolated NckSH2 domain as a dominant negative construct. As has been demonstrated by previous studies, CEACAM protein expression in 293T cells confers the ability to internalize Opa_{CEA}-expressing *Neisseria* onto these cells. However, 293T cells are lacking the oxidative apparatus of professional phagocytes, resulting in an uptake, yet no killing of bacteria, which can be quantified by a simple, yet efficient assay:

the Gentamicin protection assay. Using this assay, we quantified bacterial internalization by CEACAM3-expressing cells, either in the presence of the isolated NckSH2-domain or its dominant-negative version (NckSH2R308K) after infection with Opa_{CEA}-expressing cells. CEACAM3-negative cells and CEACAM3-positive cells infected with non-opaque gonococci served as negative control. After incubation with the strictly extracellular antibiotic gentamicin, bacteria were released from 293T cells by lysis and plated onto agar-plates. By a dilution procedure resulting in countable single bacterial colonies, their relative quantity in different experimental setups was assessed as a measure of internalization capacity.

Assuming a crucial role for Nck in the process of CEACAM3-mediated uptake of gonococci, overexpression of the isolated Nck SH2-domain in these CEACAM3-expressing cells should inhibit uptake by uncoupling receptor binding from the recruitment of Nck-SH3-domain associated effectors.

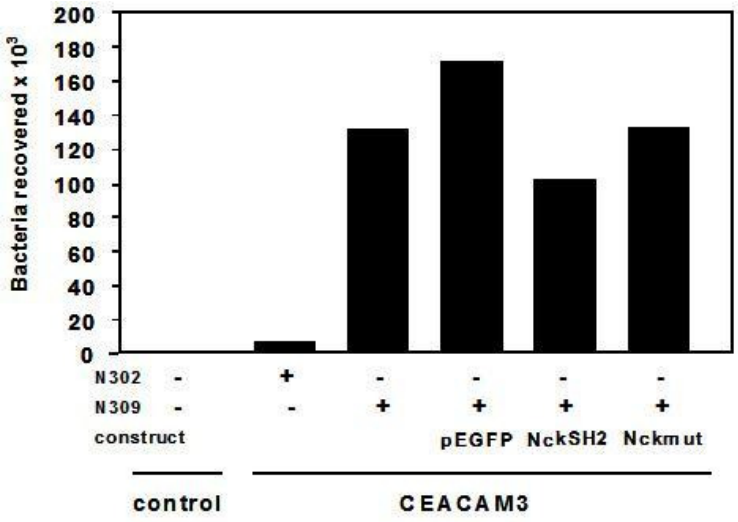


Fig. 4.12: Nck–SH2 interaction with CEACAM3 is required for an efficient internalization of Opa_{CEA}-expressing bacteria.
293T-cells were cotransfected with the phagocytic receptor CEACAM3 and the isolated NckSH2 domain (NckSH2), the mutant NckSH2 domain (Nckmut), the empty control vector (pEGFP) or no additional construct. Transfected cells were then infected either with the non opaque strain N302 or the opa_{CEA} expressing strain N309 for 60mins. The numbers of internalized bacteria were analyzed in a Gentamicin-protection assay. Opa-carrying bacteria are internalized in contrast to non-opaque Ngo. Expression of the isolated SH2-domain of Nck (NckSH2) decreases uptake significantly while the mutant NckSH2 (Nckmut) does not influence uptake.

Indeed, overexpression of NckSH2 decreased bacterial uptake by up to 22%, despite our observation that NckSH2 was rapidly degraded after transfection. Importantly, the dominant-negative construct NckSH2 R308K failed to confer an inhibitory effect on bacterial uptake by 293T cells. Neither CEACAM3-negative 293T cells infected with Opa_{CEA} gonococci nor CEACAM3-expressing 293T cells infected with non-opaque gonococci showed internalization of bacteria (Fig. 4.12).

However, the rapid degradation of the isolated NckSH2-domain made it difficult to ensure equal quantities of NckSH2 and NckR308K and might explain the only partial reduction of bacterial uptake, since the inhibitory effect depends on the presence of large amounts of the isolated SH2 domain to outcompete endogenous adaptor protein. We thus speculate, that increased intracellular levels of the isolated Nck SH2 domain would result in a stronger reduction of bacterial uptake.

Summing up, we have provided experimental evidence that:

- primary human PMNs are capable of phagocytosis of Opa_{CEA}-expressing *N.gonorrhoeae*
- Nck and CEACAM3 colocalize during bacterial internalization in vivo
- Opa_{CEA}-expressing gonococci induce receptor phosphorylation and Nck recruitment to the site of infection
- Nck plays a functional role in the internalization of pathogenic bacteria into CEACAM3-expressing cells

5 Discussion

Pathogens known to bind to CEACAMs are members of the genera *Haemophilus*, *Moraxella* and *Neisseria*. These microbes exploit CEACAMs to contact their human host cells. In particular, several CEACAM family members expressed on the apical aspect of polarized epithelial cells, such as CEACAM1, CEA, and CEACAM6 provide the bacteria with an exposed structure to anchor themselves to the mucosal surface. Additional functions have been proposed for bacteria-CEACAM1 interaction such as the blockage of epithelial exfoliation or the interference with T-cell proliferation, both processes that could facilitate bacterial colonization of their host and undermine its immune responses (Boulton and Gray-Owen 2002; Muenzner, Rohde et al. 2005; Kuespert, Pils et al. 2006; Lee, Boulton et al. 2007; Muenzner, Bachmann et al. 2008).

Recently, it has been reported that bacterial engagement of CEACAM3, a member of the CEACAM family restricted to human granulocytes, initiates phagocyte effector mechanisms leading to bacterial elimination (McCaw, Schneider et al. 2003; Schmitter, Agerer et al. 2004; Pils, Gerrard et al. 2008). This finding implies that bacterial engagement of CEACAM3 does not provide a benefit for the microorganism, but rather that CEACAM3-mediated recognition by granulocytes results in phagocytic clearance of the pathogens. It has therefore been suggested that granulocyte CEACAM3 is a phagocytic receptor of the innate immune system mediating recognition and elimination of human-specific pathogens (Schmitter, Agerer et al. 2004; Pils, Gerrard et al. 2008).

CEACAM3, although itself restricted to human granulocytes, is not the only member of its family expressed on these cells. Both CEACAM1 and CEACAM6 can be found on human PMNs and the ability to efficiently internalize *Neisseria* has been attributed to all three receptors (Kuespert, Pils et al. 2006). Recent data suggests, that the different members of the CEACAM family initiate bacterial uptake in mechanistically distinct pathways (Billker, Popp et al. 2002). It has been found that the interaction with the small GTPase Rac, a critical

regulator of actin polymerization and the oxidative burst in human phagocytes, is indispensable for CEACAM3-initiated bacterial uptake. The epithelial CEACAMs 1 and 6 however, seem to associate with lipid rafts and bacterial uptake by these receptors is impaired by cholesterol depletion (Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008). What then are the specific signaling cues connecting CEACAM3 to an efficient elimination machinery while other CEACAM-members serve to promote bacterial colonization?

In comparison to the process of `classical´ phagocytosis via Fcγ- or complement receptors there is an important difference to CEACAM3-mediated internalization: whereas the former requires prior opsonization either by components of the complement system or specific antibodies, CEACAM3-mediated uptake takes place in the absence of such opsonins. In this regard, we are looking at a direct receptor-particle interaction, bypassing the otherwise essential steps of particle demarcation by the immune system. It is not clear, however, whether the CEACAM3-mediated opsonin-*independent* uptake also differs in its signaling pathways from those observed in opsonin-*dependent* phagocytosis or if it is only the trigger which differs, while the phagocytic signaling cues rely on stereotypical signaling events.

As the process of phagocytosis is essentially a process of particle binding, membrane remodeling, phagosome formation and production of reactive oxygen intermediates (Greenberg 1995; Greenberg 1999; Cox, Dale et al. 2001; Dinauer 2003; Forsberg, Druid et al. 2003), precisely regulated actin polymerization and cytoskeletal reorganization mediated by Rho-family GTPases must be central to this process. Consistently, bacterial internalization by CEACAM3 is closely linked to its ability to stimulate the small GTPase Rac (Greenberg 1999; Dinauer 2003; Forsberg, Druid et al. 2003; Schmitter, Agerer et al. 2004). However, the role of individual signaling molecules involved in this opsonin-independent phagocytic process has only in part been characterised.

In this study, we provide evidence that bacterial internalization mediated by CEACAM3 involves the small adapter molecule Nck. Capable of modulating actin cytoskeleton dynamics by linking proline-rich effector molecules to tyrosine kinases (Frese, Schubert et al. 2006), Nck molecules are essential SH2-SH3 domain-containing adapters in cellular signaling processes. Using CEACAM3 transfected 293T cells and recombinant GST-fusion proteins, we were able to demonstrate a direct protein/protein interaction between the isolated SH2-domain of the adapter Nck and the cytoplasmic domain of CEACAM3. This interaction depends on tyrosine phosphorylation of CEACAM3 and requires an intact binding interface of Nck´s SH2 domain. Indeed, site-specific mutagenesis of the Nck SH2 domain (NckSH2R308K) results in severely reduced binding. Most importantly, immunofluorescence studies localize Nck to the nascent CEACAM3-initiated phagosome upon bacterial infection and overexpression of the isolated Nck SH2 domain reduces bacterial internalization via CEACAM3. Therefore, we propose that the phosphotyrosine-dependent association with the SH2 domain of Nck transiently couples CEACAM3 to several intracellular effector pathways via its three SH3 domains. In line with our hypothesis, the known cellular binding partners of Nck SH3 domains are closely linked to functions implicated in phagocytosis, including cytoskeletal rearrangement and the generation of reactive oxygen species by the NADPH oxidase (Diekmann, Abo et al. 1994; Chen, She et al. 2000; Dinauer 2003).

Nck, recognized as a key player in actin remodeling, has been shown to interact with a number of receptor tyrosine kinases upon their activation (McCarty 1998; Li and She 2000). In a growing number of studies, Nck has been implicated in actin dynamics-dependent processes, including the mammalian EGF, PDGF, and T-cell receptor complexes (Chen, She et al. 2000; Barda-Saad, Braiman et al. 2005; Yamaguchi, Lorenz et al. 2005; Verma, Kovari et al. 2006). Moreover, several bacterial and viral pathogens exploit mammalian Nck-mediated cytoskeletal dynamics, such as actin pedestal formation triggered by the Tir/Intimin receptor/ligand pair of enteropathogenic *E.coli* or Vaccinia virus´ A36R protein-mediated motility (Frischknecht, Cudmore et al. 1999;

Frischknecht, Moreau et al. 1999; Gruenheid, DeVinney et al. 2001; Gruenheid and Finlay 2003). Most significantly, a landmark paper by Eden and colleagues in 2002 followed by a publication in 2004 by Innocenti et al identified Nck-binding proteins Nap1 and PIR121 as part of a multiprotein complex crucial for Rac1-mediated actin nucleation by the WAVE-Arp2/3 complex (Eden, Rohatgi et al. 2002; Innocenti, Zucconi et al. 2004; Steffen, Rottner et al. 2004). Consequently, our finding that Nck associates with phosphorylated CEACAM3 is consistent with the hypothesis that CEACAM3 is an immunoreceptor serving to recognize and eliminate pathogenic bacteria by opsonin-independent phagocytosis. In this process, CEACAM3-bound Nck may interact with the Nck-binding proteins of the WAVE activation complex via one of its SH3 domains, thus relocating it to induce site-directed actin polymerization.

Not surprisingly, we observed that the single SH2 domain of Nck associated with the cytoplasmic tail of CEACAM3. This tail carries a characteristic amino acid motif with resemblance to the ITAM, a structural feature found in several immunoreceptors critically involved in the opsonin-*dependent* phagocytosis via the Fcγ-receptor (Reth 1989; Cambier 1995; Cox, Dale et al. 2001; Fodor, Jakus et al. 2006; Underhill and Goodridge 2007) (Fig. 5.1).

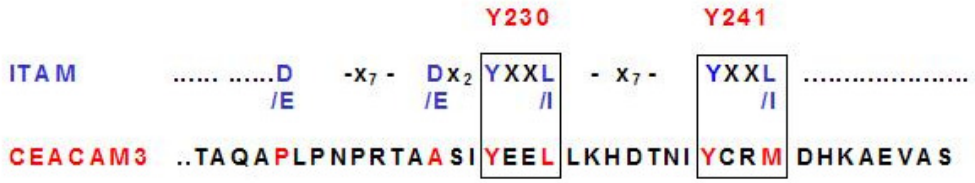


Fig. 5.1 **Sequence comparison of ITAM-like sequence of CEACAM3 and the canonical ITAM.**

In analogy to classical ITAM-signaling, which is initiated by receptor clustering and Src-PTK-dependent phosphorylation (Thomas and Brugge 1997; Greenberg 1999; Schmitter, Pils et al. 2007), several publications have shown

CEACAM3-mediated bacterial internalization to be dependent on the phosphorylation of its intracellular tyrosine residues (Chen, Bolland et al. 2001; Billker, Popp et al. 2002; McCaw, Schneider et al. 2003; Schmitter, Agerer et al. 2004; Sarantis and Gray-Owen 2007; Schmitter, Pils et al. 2007; Schmitter, Pils et al. 2007). This has been demonstrated by disabling the phosphorylation of the ITAM-like by substituting tyrosines with phenylalanine. Consistently, we observed that the interaction of NckSH2 and CEACAM3 did not take place with truncated (CEACAM3ΔCT) or unphosphorylated receptor. Importantly, NckSH2 binding to our specifically engineered double mutant CEACAM3Y230/241F was significantly lower than that to wildtype CEACAM3, indicating that Nck-binding to CEACAM3 also seems to rely on these phosphotyrosines.

In contrast to studies by Chen et al. who proposed that CEACAM3-triggered phagocytosis of Opa_{CEA}-carrying bacteria relies upon the same intracellular signaling machinery as classical ITAM-signaling (Fig. 5.2), the data presented herein suggest that the SH2-SH3 adapter protein Nck *directly* integrates CEACAM3 activation with downstream cytoskeletal dynamics and, possibly, the oxidative burst in human neutrophils (Chen, Bolland et al. 2001). Our findings are in line with previous reports which demonstrated that the tyrosine-kinase Syk, critically involved in Fcγ-mediated opsonin-dependent phagocytosis (Humphrey, Lanier et al. 2005; Fodor, Jakus et al. 2006; Underhill and Goodridge 2007), is not activated in the early signaling events after CEACAM-engagement in human phagocytic cells (Hauck, Meyer et al. 1998; Sarantis and Gray-Owen 2007). Accordingly, we could show, that Nck co-precipitates with CEACAM3 from cells co-transfected with v-Src but not unstimulated cells. No additional adapter molecules or kinase activity were required, and importantly, Syk-activity was dispensable.

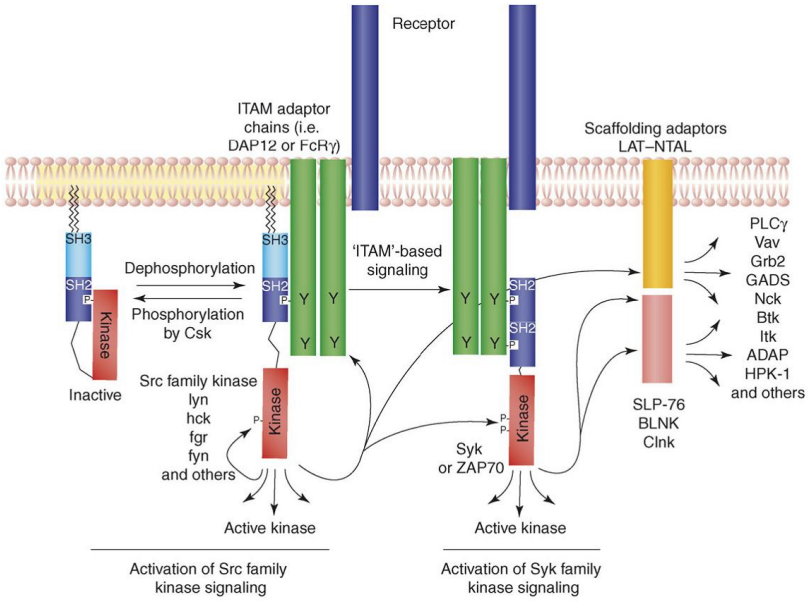


Fig. 5.2 **ITAM-based signaling.**
A typical ITAM-signaling cascade by ITAM-containing receptors or adapter molecules is illustrated. 1. Src-family kinases are activated by dephosphorylation of a C-terminal tyrosine in the kinase domain. 2. Receptor engagement and subsequent clustering enables activated Src-kinases to phosphorylate tyrosine residues on the ITAM. 3. Phosphorylated ITAMs interact with a) Src PTKs to prolong their activation and recruit b) Syk family kinases via their dual SH2 domains. 4. Such recruited, Src PTKs also act on Syk kinases to phosphorylate and thus ativate them. 5. Activated Syk kinases drive signaling by phosphorylation of key scaffolding adapters, including BLNK, SLP-76 and Clnk.
(from: `The many faces of ITAMs´ (Underhill and Goodridge 2007))

Interestingly, a recent publication describes another such direct interaction for the guanosine nucleotide exchange factor (GEF) Vav with tyrosine 230 in CEACAM3. Rac, a master regulator of actin cytoskeleton dynamics and the oxidative response in human PMNs, has been shown to play an indispensable role in CEACAM3-mediated phagocytosis. Vav, acting as a Rac-GEF, is critically required for GTP-loading of the small GTPase. Interference with Vav significamtly reduced granulocyte uptake by CEACAM-binding bacteria. Just as the direct association between CEACAM3 and Vav bypasses the usually multi-

factorial signaling pathway via Syk family kinases and SLP76/SLP65 found in canonical ITAM-signaling, the Nck pathway seems to short-wire communication with Rac effectors involved in actin cytoskeleton dynamics and NADPH oxidase regulation (Schmitter, Agerer et al. 2004; Schmitter, Pils et al. 2007). It is feasible that Vav may serve local Rac-activation while simultaneously, Nck recruits to the membrane locus the Rac-effector complex. Both activators and effectors critical for cytoskeletal rearrangement would thus be recruited directly and without intermediates to the site of bacterial attachment, thereby promoting fast and efficient phagocytic responses.

Apart from the immediate membrane-proximal events orchestrated by Nck-WAVE interactions, CEACAM3-mediated events include an oxidative response to the bacteria. We and others were able to show that the oxidative response of human granulocytes to infection with Opa_{CEA}-expressing gonococci is significantly stronger than that to non-opaque gonococci, suggesting that the engagement of CEACAMs results in a stimulation of the oxidative response. Interestingly, the second SH3 domain of Nck has been found to associate with the p21-activated kinase PAK (Kitamura, Kitamura et al. 1996; Kitamura, Kitamura et al. 1997; Li, Fan et al. 2001). PAK, or more precisely PAKs, represent a family of Ser/Thr kinases capable of interacting with activated Rac and Cdc42. The two main endpoints of PAK signaling are cytoskeletal dynamics and nuclear events influencing gene expression (Knaus and Bokoch 1998; Bagrodia and Cerione 1999). Experimental evidence demonstrates, that Nck recruits PAKs to membranes in response to growth-factor receptor tyrosine kinase activation where it participates in membrane remodeling, a process regulated by Rac or Cdc42 (Galisteo, Chernoff et al. 1996; Lu, Katz et al. 1997; Bagrodia, Bailey et al. 1999; Bagrodia and Cerione 1999). Significantly, PAK has also been found to phosphorylate the cytosolic component of the NADPH-oxidase p47phox, implying a regulatory role in the oxidative burst of granulocytes (Knaus and Bokoch 1995; Knaus and Bokoch 1998; Forsberg, Druid et al. 2003). Moreover, Izadi et al. established a direct connection

between Nck-Pak interaction and the Fcy-receptor-mediated activation of the respiratory burst in myeloid cells (Izadi, Erdreich-Epstein et al. 1998).

Secondly, PAKs are involved in nuclear signaling via the JNK/p38 subgroup of nuclear MAPKs and the Erk pathway, thereby regulating gene expression (Poitras, Jean et al. 2003). Studies by Hauck et. al. showed that the signaling cascade induced by the CEACAM-Opa interaction leads to activation of PAK and subsequently Jun-N-terminal kinase (Hauck, Meyer et al. 1998) in a Rac-dependent manner. Experimental data demonstrate a scaffold-like Nck-PAK-JNK interaction, linking tyrosine kinase receptors to the MLK-JNK cascade (Poitras, Jean et al. 2003). Thus, Nck would also provide the means for nuclear signaling via the MAPK-JNK pathway, leading to altered gene expression patterns which dictate the ensuing inflammatory response.

Next to its three SH3-domains which enable Nck to bind various proline-rich effector molecules and thus act as a scaffold for the recruitment of the essential components for cytoskeletal remodeling, NADPH-oxidase activation and nuclear signaling, Nck possesses only a single SH2 domain. In Fcγ-mediated phagocytosis, Syk, with its tandem SH2 domain structure preferentially engages both ITAM-phosphotyrosines simultaneously. There are two possible interaction modi for Nck with the CEACAM3-ITAM, either one molecule of Nck each may bind to tyrosines 230 and 241. Alternatively, Nck, according to its preferred binding sequence, engages only one of the two phosphotyrosine motifs while the other is left to bind different effectors. The preferred phosphopeptide binding sequence of Nck1SH2 has already in a 1993 *Cell* publication been described as being a pYDE(P/D/V) sequence (Songyang, Shoelson et al. 1993). Frese et al. recently published binding data for the SH2 domains of Nck1 and 2*,* extending this motif to include additional adjacent residues (Frese, Schubert et al. 2006). Out of the two tyrosine motifs in CEACAM3, the YEEL sequence around residue Y230 seems to be a candidate for Nck binding. Despite slight differences of the core sequence from the established consensus sequence, it displays an overall sequence similarity of more than 90%. Bearing in mind that

essentially all positions from –2 to +6 participate in forming a specific interaction, our data strongly suggest that the Nck SH2 domain supports specific binding to Y230 of the CEACAM3 receptor. In support of our data, evidence is accumulating on other ITAM-like sequences that vary from the consensus, yet specifically trigger ITAM-dependent pathways, for example Dectin-1, CLEC2 and RhoH in TCR signaling (Humphrey, Lanier et al. 2005; Underhill and Goodridge 2007).

Although ITAM with its conserved structure seems predestined for engagement by tandem SH2 adapters such as Syk, it is interesting to note that recent studies demonstrated effector molecules to be recruited without tandem phosphorylated tyrosine residues. Accordingly, the second tyrosine residue (Y241) in CEACAM3 is embedded in a context which diverges significantly from the consensus binding sequence for NckSH2, displaying a methionine residue at position +3 (YxxM). With this YCRM sequence, tyrosine 241 seems a highly unlikely candidate for NckSH2-binding. Therefore, rather than amplifying the same signal, it seems that the two phosphotyrosines activate distinct pathways, and such differential signaling capacities of ITAM-tyrosines have already been demonstrated (Chen, Bolland et al. 2001; Kim, Pan et al. 2001; Humphrey, Lanier et al. 2005; Underhill and Goodridge 2007). An interesting candidate for binding to Y241 is the p85 subunit of phosphatidylinositol-3' kinase (Piccione, Case et al. 1993; Schmitter, Agerer et al. 2004). Not only have PI3Ks been implicated in bacterial invasion of epithelial cells as well as Fc-mediated phagocytosis by macrophages, but Booth et al reported them to be involved in the CEACAM3-mediated internalization of *Neisseria gonorrhoeae* (Booth, Telio et al. 2003). It is feasible, that recruitment of PI3K is involved in the local activation of Rac at the membrane/receptor complex to supply the colocalized Nck/WAVE activation complex with GTP-loaded Rac for actin nucleation. Summed up, these data support a model in which Nck-mediated recruitment of the actin polymerization machinery and PI3K-product formation occur simultaneously via binding to distinct tyrosine residues of the ITAM-like motif. PI3K products in turn have been shown to activate a number of Rac GEFs,

such as Vav and SWAP70 (Bustelo 2000; Shinohara, Terada et al. 2002) which are essential for the activation of Rac in the macromolecular WAVE-signaling complex (Fig. 5.3).

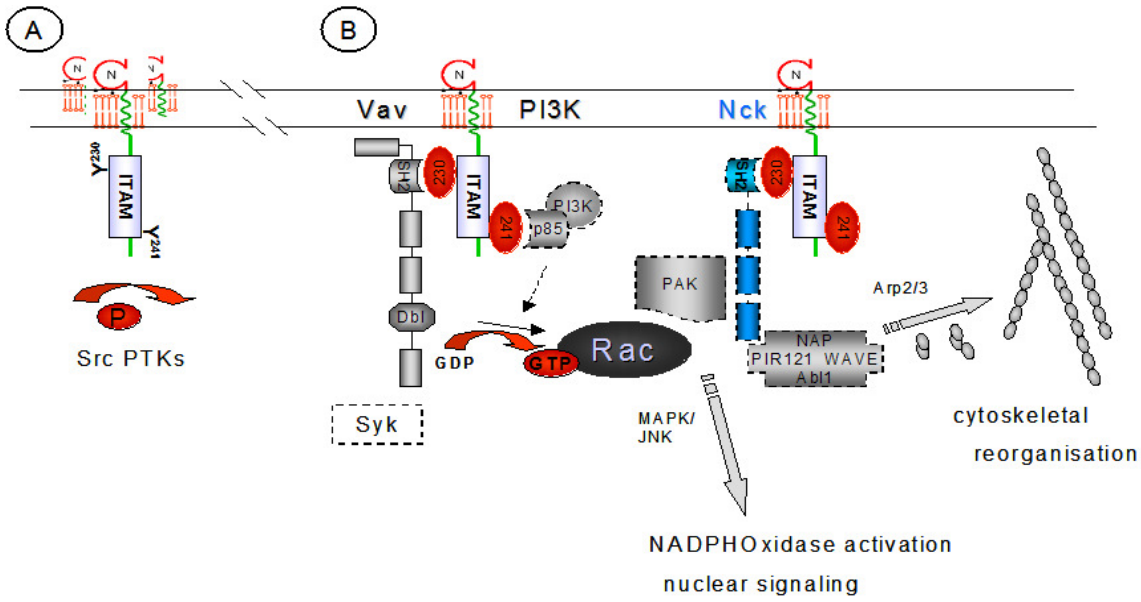


Fig. 5.3 **Proposed model of the signaling pathway triggered by engagement of granulocytic CEACAM3**
(A) Receptor engagement by opaque *Neisseriae* and subsequent clustering leads to receptor-ITAM phosphorylation by Src-kinases.
(B) The multivalent bacteria recruit numerous receptors in a tight local space. Nck and Vav bind to Y230 on different receptor molecules and act in a concerted fashion to recruit the actin polymerization machinery and activate it via GTP-loading of Rac. The Rac GEF in turn may be activated by PI3K products, formed by PI3K bound to the Y241 of CEACAM3. Nck may also recruit PAK which participates in nuclear signaling and activation of the NADPH-oxidase. The role of Syk is not clear, it may however have a part in later, membrane distal events.

Interestingly, the SH2 domain of Vav appears connected to the same phosphorylated tyrosine residue, tyrosine 230, within the CEACAM3 ITAM as Nck. Likewise, the SH2 domains of several Src family kinases, such as Hck and c-Src, have been shown to associate with the cytoplasmic domain of CEACAM3 (Schmitter, Pils et al. 2007). Rather than questioning individual binding properties, we propose a model wherein the multivalent bacteria aggregate numerous CEACAM3 molecules in a small area of the granulocyte plasma membrane. The local concentrations of Src family kinases, Nck, and Vav, as

well as their respective binding affinities to phosphorylated tyrosines will dictate the composition of the CEACAM3 complexes. The exact spatiotemporal sequence of molecular events, however, has not yet been resolved. To gain more insight into the molecular dynamics in time and space, further studies need to be conducted. These could include live cell video microscopy using fluorescently labelled proteins of interest, which enable visual tracing of CEACAM3´s interaction partners after infection with opaque *Neisseria*. Valuable information about the specificity and intensity of a molecular interaction may also be gained by either microscopic or flow-cytometric FRET analysis (fluorescence resonance energy transfer) using spectral variants of GFP in living cells.

Our functional data, although intriguing, leave a few open questions. In our CEACAM3-dependent phagocytosis assay with 293T cells we observed diminuished, yet not abrogated phagocytosis by overexpression of the isolated Nck SH2-domain as a dominant-negative protein. Although strengthened by the inability of the mutated Nck-SH2 domain to confer such an inhibitory effect on bacterial uptake, the partial reduction of bacterial uptake by 293T cells suggests, that additional, possibly redundant mechanisms come into play. From these results in 293T cells, it is difficult to establish the significance of Nck in CEACAM3-mediated internalization of Opa_{CEA}-bacteria. Possibly, 293T cells may draw on other signaling components to mediate bacterial uptake via CEACAM3, thus circumventing the Nck-mediated pathway. Therefore, it will be important to interfere with Nck in primary human granulocytes and to test, if a more dominant phenotype with regard to CEACAM3-mediated phagocytosis is observed in this cell type. One promising approach will be to perform experiments with TAT-mediated protein transduction of primary human granulocytes to test the role of Nck in granulocyte signaling.

Nevertheless, the data accumulated is compelling, that Nck with its links to actin remodeling, regulation of the oxidative burst and nuclear signaling, is a suitable relay for the signaling events following CEACAM3 engagement. Its structure

with one SH2 and three SH3 domains provides the basis for simultaneous activation of distinct pathways, while the CEACAM3-ITAM-like may represent an evolutionary variant supporting dual effector signaling. Moreover, receptor clustering by the usually multivalent bacteria at their site of attachment enables the simultaneous binding of distinct effector molecules to phosphotyrosine motifs. We therefore propose a novel phagocytic signaling pathway that circumvents conventional Syk-kinase signaling by short-wiring CEACAM3-engagement and Rac-activation via Vav and Nck, thus propagating fast signal propagation. An evolutionary rationale for this type of shortwiring lies in the competitive nature of CEACAM-expression: CEACAM 1, which carries an immunosuppressive ITIM-motif is coexpressed with CEACAM3 on granulocytes. The shortcut taken by Vav and Nck to stimulate the GTPase Rac seems suited to outcompete conflicting signals emanating from the ITIM harboured by CEACAM1. Reduction of intermediate steps in the signaling cascade of CEACAM3 would thus represent an optimization of CEACAM3 signaling, promoting bacterial phagocytosis before inhibitory/ immunosuppressive signals may interfere.

6 Abstract

Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are exploited by human-specific pathogens to anchor themselves to or invade host cells. Interestingly, human granulocytes express a specific isoform, CEACAM3, that can direct efficient, opsonin-independent phagocytosis of CEACAM-binding *Neisseria*, *Moraxella* and *Haemophilus* species. As opsonin-independent phagocytosis of CEACAM-binding *Neisseria* depends on Src-family protein tyrosine kinase (PTK) phosphorylation of the CEACAM3 cytoplasmic domain, we hypothesized that an SH2-containing protein might be involved in CEACAM3-initiated, phagocytosis-promoting signals. Accordingly, we screened glutathione-S-transferase (GST) fusion proteins containing SH2 domains derived from a panel of signaling and adapter molecules for their ability to associate with CEACAM3. In vitro pull-down assays demonstrated that the SH2 domain of the adapter molecule Nck (GST-Nck SH2), but not other SH2 domains such as the Grb2 SH2 domain, interact with CEACAM3 in a phosphotyrosine-dependent manner. Either deletion of the cytoplasmic tail of CEACAM3, or point-mutation of a critical arginine residue in the SH2 domain of Nck (GST-NckSH2R308K) that disrupts phosphotyrosine binding, both abolished CEACAM3-Nck-SH2 interaction. Upon infection of human cells with CEACAM-binding *Neisseria*, full-length Nck comprising an SH2 and three SH3 domains co-localized with tyrosine phosphorylated CEACAM3 and associated bacteria as analyzed by immunofluorescence staining and confocal microscopy. In addition, Nck could be detected in CEACAM3 immunoprecipitates confirming the interaction in vivo. Importantly, overexpression of a GFP-fusion protein of the isolated Nck SH2 domain (GFP-Nck-SH2), but not GFP or GFP-Nck SH2 R308K reduced CEACAM3-mediated phagocytosis of CEACAM-binding *Neisseria* suggesting that the adaptor molecule Nck plays an important role in CEACAM3-initiated signaling leading to internalization and elimination of human-specific pathogens.

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Curriculum Vitae

Name	**Lisa Peterson**
Geboren am	13. März 1978
Geboren in	Hannover

Schulbildung

1984 - 1990	Grundschule Horst und Orientierungsstufe Garbsen
1990 - 1997	Johannes- Kepler- Gymnasium Garbsen, ● Leistungsfächer: Biologie / Englisch, ● Abschluss: Allgemeine Hochschulreife
1994 - 1995	Ringwood Secondary College Melbourne, Australien

Berufliche Ausbildung

1997 - 2000	Studium der Humanmedizin an der Universität Rostock, ● 1. Staatsexamen
2000 - 2002	Studium Cell and Molecular Biology an der Anglia Polytechnic University Cambridge, ● Thesis: `SNP-detection in the MHCIII region by DHPLC´ ● Abschluss: Bachelor of Science with Honours
2002 - 2005	Studium der Humanmedizin an der Universität Würzburg, ● 2. Staatsexamen ● 3. Staatsexamen ● Abschluss: Ärztliche Prüfung

Wissenschaftliche Arbeit

07/2001 - 12/2001	Thesis am Human Genome Mapping Project-Resource Centre, Cambridge, Dr. G. Ribas-Despuig, `Single Nucleotide Polymorphism detection in the MHCIII region by DHPLC´
03/2003 - 02/2004	Promotion am Institut für Molekulare Infektionsbiologie/Zentrum für Infektionsforschung, Würzburg, Prof. Dr. Dr. h. c. mult. J. Hacker/ Betreuer Prof. Dr. rer. nat. C. R. Hauck, `CEACAM3-mediated phagocytosis of human-specific bacterial pathogens involves the adapter molecule Nck´ 1. Platz Promomed-Kongress Würzburg 2004
seit 06/2008	Mitglied der Arbeitsgruppe `Klinische Infektiologie´ an der Medizinischen Klinik und Poliklinik III der Ludwig-Maximilians-Universität München, Prof. Dr. med. H. Ostermann

Beruflicher Werdegang

01/2006 – 12/2006	Assistenzärztin für Neurologie Neurologische Klinik und Poliklinik der Julius-Maximilians-Universität Würzburg
Seit 15.02.2007	Assistenzärztin für Hämato-Onkologie Medizinische Klinik und Poliklinik III der Ludwig-Maximilians-Universität München

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