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A Role for TLRs in *Moraxella*-superantigen induced polyclonal B cell activation

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1. ABSTRACT

A number of microorganisms are capable of binding immunoglobulins (Igs) in a manner, which excludes binding to conventional antigen binding sites. Interaction of such bacterial proteins with surface immunoglobulins leads to polyclonal activation of B-lymphocytes. A recent example is *Moraxella catarrhalis* that binds to B lymphocytes in an IgD-dependent manner and induces proliferation and differentiation of B lymphocytes leading to the production of unspecific Igs. The activation is mediated by *Moraxella* IgD binding protein (MID), which specifically binds to both soluble IgD and the IgD B cell receptor (BCR). Besides cross-linking the BCR, whole *Moraxella* and outer membrane vesicles (OMVs) engage Toll like receptors (TLRs) to further increase the response. TLR activation leads to initiation of signaling pathways, which evoke a proinflammatory response against the invading microbes. Polyclonal B cell activation has in general been implicated in various phenomenons that are detrimental for the host but beneficial for pathogens, for example, autoimmune manifestations and redirection of the immune system.

2. INTRODUCTION

Moraxella catarrhalis is a Gram-negative human specific pathogen of the respiratory tract. For a long time, the potential pathogenicity of *M. catarrhalis* was ignored and the species was mainly recognised as a commensal. However, *M. catarrhalis* is now acknowledged as a causative agent of respiratory tract infections in children and adults leading to a considerable morbidity. *M. catarrhalis* is the third most important pathogen causing otitis media in children and is responsible for up to 20 % of cases with a majority of the children experiencing at least one episode of otitis media by the age of 3 years (1, 2). Other respiratory tract infections in children associated with *M. catarrhalis* include sinusitis, conjunctivitis, bronchitis and pneumonia (1, 3). The carriage rate of *M. catarrhalis* is low in healthy adults and if infected, adults are usually predisposed to lower respiratory tract infections with *M. catarrhalis* in case of compromised situations such as chronic obstructive pulmonary disease (COPD) and previous episodes of pneumonia.

Colonization of the nasopharynx by *M. catarrhalis* and other predominant respiratory tract

pathogens such as non-typeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae* begins at a very early age in the life and at least 50 % of children are colonized by the age of 2 years (4-6). Colonization not only provides the potential pathogens an entry point to various associated tissues including the ear, sinuses, larynx and lower respiratory tract but is also means of spreading in the community via nasal secretions.

To establish as a successful pathogen, *M. catarrhalis* like other pathogens, employs multiple ways not only to evade the immune response but also to dampen it. These bacterial strategies result in successful colonization in a normal host environment, and eventually invasion when the host conditions are altered. Attachment to the nasopharyngeal epithelium and various host extracellular matrix proteins, evasion of the complement system and redirection of the immune system are amongst some of these strategies (7). Examples of characterized virulence factors of *M. catarrhalis* comprise multifunctional adhesins such as the adherence protein (McaP), family of ubiquitous surface protein A (UspAs) including UspA1, UspA2 and UspA2H, and *Moraxella* immunoglobulin-D (IgD) binding protein/Hemagglutinin (MID/Hag) (7-10). McaP increases bacterial binding to several respiratory tract epithelial cell lines and in addition confers the bacterium with additional lipolytic activity (11). UspA1 interacts with epithelial cells including those of the oropharynx and lower respiratory tracts via binding to human carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (12). In addition, the UspA family members harbour binding sites for various extracellular matrix proteins such as fibronectin, laminin and vitronectin (13-15), and thus facilitate the colonization. Similar to McaP, MID also confers binding to several epithelial cell lines including ciliated bronchial epithelial cells (16).

An altered host state or an increased microbial load allows the established microbes to invade the initial epithelial barriers. This invasion evokes a strong inflammatory response followed by an adaptive immune recognition, which counteracts the presence of pathogens. On the other hand, invading microbes for their benefit try to either dampen or alter this response. The focus of this review is to discuss one such characterized mechanism employed by *M. catarrhalis*, that is to redirect the immune system by inducing polyclonal B cell activation.

Polyclonal B cell activation can be induced by many different microorganisms and by means of different classes of molecules such as secretory/soluble proteins from parasites, i.e. *Trypanosoma cruzi*, soluble proteins from *Schistosoma japonicum*, cell surface proteins from bacteria including fibronectin binding protein I (SfbI) of *Streptococcus pyogenes*, secreted membrane *Staphylococcal* protein A (SpA), *Peptostreptococcus* protein L (PpL) and, finally, viral envelop protein HIV-1 glycoprotein gp120 (17-22). These activators are referred to as "B-cell superantigens" and recognize conserved regions of Igs in a non-immune manner. Non-immune recognition of microbial molecules by Igs is defined as recognition regardless of the antigenic specificity. Cross-linking surface B cell receptor (BCR) leads to activation of intricate signaling pathways culminating in B cell activation. Depending on the B cell subset targeted and the Ig class recognized, superantigens induce an activating response in a large pool of B cells, unlike that induced by conventional antigens, which react with a small population of antigen specific naïve B cells (23). For example, *Peptostreptococcus* PpL, binds to the framework region of k-light chain variable domain, which is present in two third of Igs (24, 25).

In addition to the BCR-mediated polyclonal B cell activation, proliferation of B cells can also be induced by triggering the Toll like receptor (TLR) signaling pathways (26). Non-protein microbial products such as lipopolysaccharide (LPS) and non-methylated DNA induce polyclonal B cell activation by stimulating the TLRs, which are the innate recognition receptors and respond to conserved microbial products (27). TLRs are one of the most studied and characterized pathogen recognition receptors (PRRs), and evolutionary well conserved amongst vertebrates (28, 29). As a part of innate immune response, PRRs recognize various microbial features called pathogen associated molecular patterns (PAMPs), which are not unique to a certain type of microbe, instead are common amongst several classes of microbes. TLRs are germ line encoded and recognize conserved features of microorganisms (30, 31). At present 13 members of the TLR family have been reported in mammals. They are proposed to belong to six families on the basis of amino acid sequence similarities and the kind of molecular pattern recognized as ligands (28).

M. catarrhalis by means of the IgD-binding protein MID, induces a polyclonal B cell activation to promote a response, which is characterized by B cell proliferation and unspecific Ig production. This interaction of bacteria with B cells is achieved by binding of MID in a non-immune manner to the BCR, i.e. membrane-bound IgD (mIgD) (Figure 1). *M. catarrhalis* encounters B lymphocytes in the pharyngeal lymphoid tissues surrounding the nasopharynx where bacteria are co-localized with immune cells (32). Approximately 50 % of the B lymphocyte population in the pharyngeal lymphoid tissues and 60 % of peripheral blood B cells are represented by naïve IgD^+ cells, rendering a huge B cell pool susceptible to *Moraxella* mediated modulation (33). The IgD^+ B cell population represents the naïve mature B cells with expected low antigenic specificity, that is crucial in initiating the primary immune response. These IgD^+ B cells contribute to the B cell population, which is the source of natural antibodies, where these antibodies provide an initial antibody-mediated response, including opsonization and thus complement mediated clearence.

3. *MORAXELLA* IgD-BINDING PROTEIN (MID)

3.1. MID: structure and function

MID was discovered by us in 2001 and the knowledge on MID has expanded ever since (34). MID is a 200-kDa multifunctional outer membrane protein with a specific and unique affinity for human IgD. It belongs to the autotransporter protein family with sequence

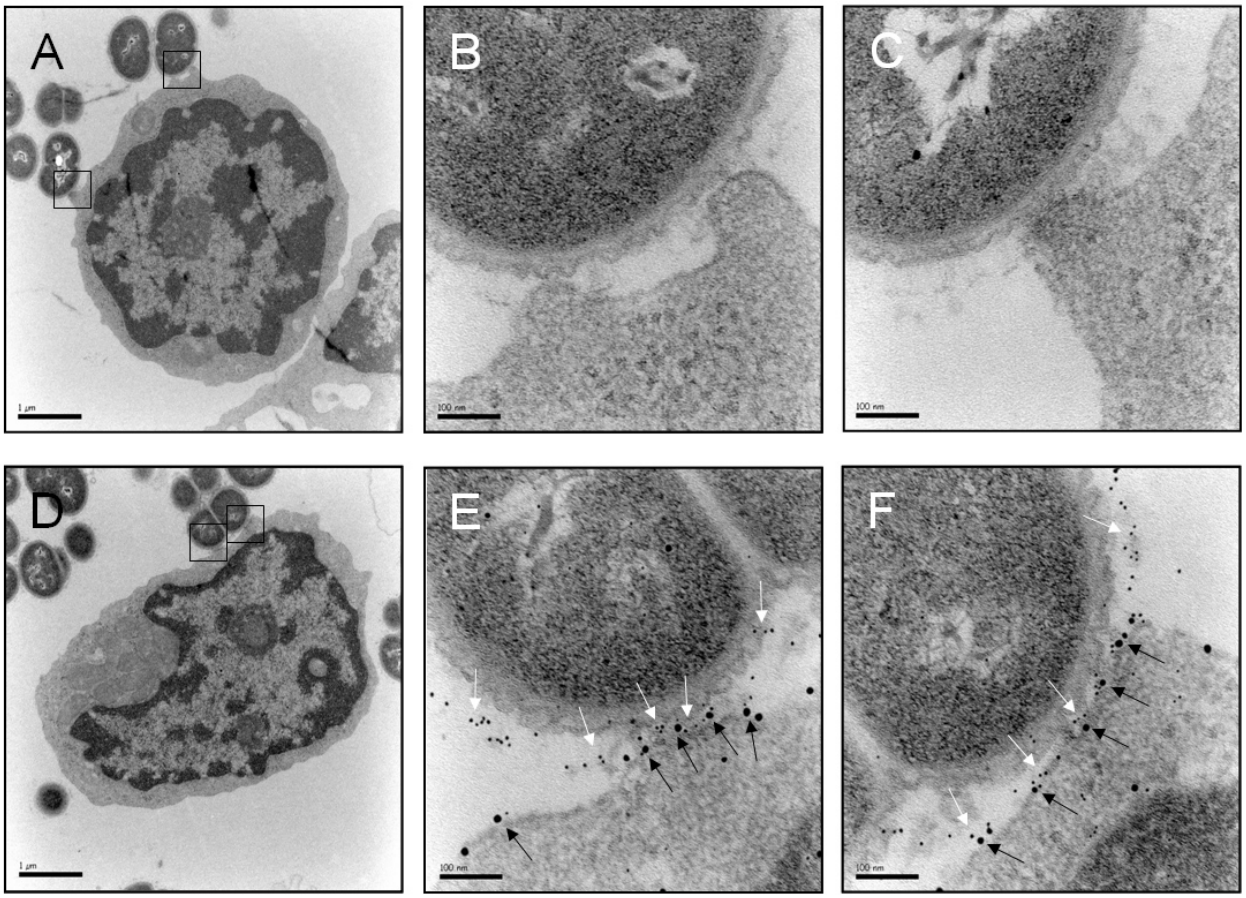

Figure 1. *M. catarrhalis* binds B cells via IgD BCR. MID expressing *M. catarrhalis* (overnight grown culture) were incubated with B cells and the binding was analyzed by Transmission electron microscopy (TEM). Magnified areas in A and D are indicated with squares. D-F: Binding of bacteria to IgD BCR via MID protein was confirmed using gold-labeled Abs directed against IgD (large granules, black arrows) or the IgD-binding part of MID (small granules, white arrows). The scale in A and D represents 1 µm, and in the magnified pictures B, C, E and F 100 nm. Figure reproduced with permission from ref. 43.

similarities to trimeric autotransporters such as *Yersinia enterocolitica* outer membrane protein (YadA) and the *Haemophilus* adhesin (Hia) (35). Autotransporter proteins are in general organised into three characteristic domains, including an N-terminal signal peptide, an internal passenger domain, and a C-terminal translocator domain (36). MID migrates as an oligomer in SDS-PAGE, which is related to its trimeric structure. In contrast to conventional autotransporters, MID molecules fold back on themselves building up a trimer and exposing the central globular region at the tip. The exposed parts comprise of both the IgD-binding and adhesive domains of MID. The last 210 amino acids (aa) of the C-terminal region translocates MID through the outer membrane and the rest of the protein forms an approximately 120 nm long, fibrillar structure (35) (Figure 2). The smallest fragment of the MID protein with preserved IgD-binding capacity consists of 238 aa (MID962-1200) (37). The native recombinant MID aa 962-1200 fragment ($MID^{962-1200}$) is a trimer and binds IgD more efficiently than the monomeric form. This shows the importance of the trimeric structure for optimal IgD-binding (37). The adhesive part of MID is located upstream of the IgD-binding site. The residues between aa 385 and 745 of MID display adhesive properties for human lung and middle ear epithelial cells, whereas residues 706 to 1194 are necessary for binding to type IV collagen (38). MID is present in at least 84 % of the clinical strains and is regulated by phase variation due to modulation of the number of G's in a poly(G) tract located in the N-terminal region of the open reading frame (ORF) (39). By means of phase variation, bacteria can alter their phenotype leading to an "on" and "off" expression of genes in a reversible manner in response to stress or changes in the environment.

3.2. MID as a B-cell superantigen

Owing to the fact that the IgD-binding property of MID is well preserved within $MID^{962-1200}$, this shorter protein has been used in most functional studies in order to increase the understanding of the B-cell superantigen functions of this protein. The truncated $MID^{962-1200}$ specifically recognises a stretch of 198-206 aa in the C_H1 region of soluble recombinant IgD but not the same region in IgG subclass 1 antibodies (40). The exact binding site in the IgD C_H1 region was delineated by constructing a series

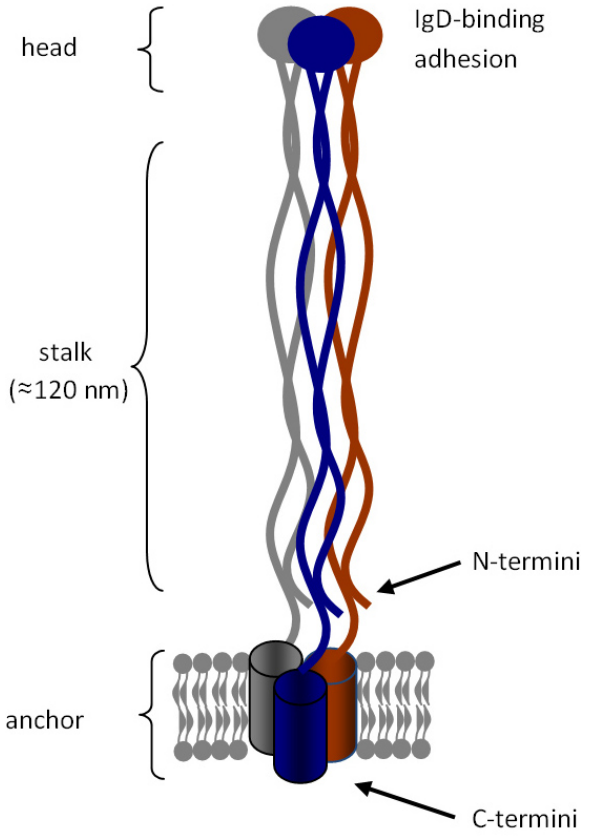

Figure 2. A model of the MID filaments on the bacterial surface. The C-terminal translocator domain anchors the multimeric autotransporter MID in the outer membrane. The rest of the protein forms a ca. 100 nm long fibrillar stalk as each MID monomer folds back on itself and comprises of alpha-helical strands. Each MID monomer is suggested to contribute its 10 beta-strands to form a single large pore containing 30 beta-strands with one central channel. Folding back of MID structure brings the N-termini in close proximity to the membrane spanning C-terminal region. The membrane distal, central stalk regions of the MID monomers cluster together to form a globular head domain that is involved in functions such as IgD-binding and adhesion. Figure reproduced with permission from ref. 35.

of chimeric IgD antibodies with substitutions of corresponding IgG sequences in the C_H1 region. Furthermore, the bivalent conformation of IgD was suggested to be necessary for MID binding as the F(ab´) IgD fragment showed diminished binding as compared to the F(ab´)2. Moreover, in experiments where binding of purified MID was checked with IgD^+ IgM^+ B cells, a positive signal could only be detected to surface IgD and not to surface IgM, confirming the specificity of MID towards IgD (41).

Another proof of evidence of MID as a B-cell superantigen, is that purified MID and $MID^{962\text{-}1200}$ stimulates B cell proliferation via mIgD in a T cell independent (TI) manner (42). An augmented response requires, however, the presence of interleukins (ILs) such as IL2 and IL4 or the T cell derived CD40L (41). In order to mimic the natural conformation of MID on the bacterial surface, MID conjugated to Sepharose beads or coated on a surface is required (42, 43). Moreover, after stimulation with MID, purified B cells need additional signals (IL-4, IL-10, CD40L) for production of IgM and class switching to IgA and IgG. We also showed that IgD production induced by MID is dependent on the particular donor in addition to costimulatory molecules, whereas IgE secretion could not at all be detected (41). Taken together, the ability of MID to strongly cross-link the BCR and thus activate B cells in the absence of cognate T cell help, but its dependency on additional stimulants makes MID to be considered as a TI-2 antigen (44).

The potential of *Moraxella* to induce B cell activation has been known for several years and it is now clear that MID is the only protein component in *Moraxella* responsible for this stimulation (43, 45). MID-expressing *Moraxella* induces B cell proliferation and upregulation of early activation cell surface markers including CD54, CD86, CD69, HLA-DR and CD40 (46). In contrast, an isogenic mutant devoid of *mid*, completely lacks the stimulatory capacity (42). Interestingly, in the absence of added cytokines, the mitogenic capacity of purified MID as conjugated/coated and MID protein expressed on the cell surface are quite different, the latter inducing a very strong effect (43). It is important to consider this scenario, because in the complex host situation, it is highly probable that MID is encountered by the immune system as a part of whole bacteria. In fact, whole *Moraxella* utilizes an additional mechanism besides the MID-dependent BCR ligation, to exert a stronger cumulative mitogenic effect (43). When whole cell lysates and purified $MID^{962\text{-}1200}$ were combined, a B cell stimulatory effect similar to that induced by whole bacteria could be mimicked, which suggested the role of additional soluble bacterial factors in B cell activation. As discussed in detail below, we found that other bacterial factors are recognised as ligands for TLR and the synergistic effect of BCR ligation and TLR stimulation led to strong mitogenic effects on B cells (43).

4. B CELL RECEPTOR (BCR) CROSS-LINKING AND TOLL-LIKE RECEPTOR (TLR) STIMULATION: A SYNERGISTIC APPROACH TO ACTIVATE B CELLS

The synergistic effect of TLR and BCR-dependent stimulation are accounted by the overlapping set of the transcription factors such as NFkB and MAPK that are activated in the cell (47). As shown in Figure 3, whole *M. catarrhalis* as well as derived outer membrane vesicles (OMVs, described in detail below) induce crosslinking of the BCR. The BCR complex consists of the surface Ig molecule and the Ig-alpha/beta heterodimer(s), each of both Ig-alpha and Ig-beta contains the signaling motif ITAM (Ig [superfamily] tyrosine-based activation motif) in their cytoplasmic tails. Antigen binding to the BCR complex leads to segregation of the antigen-receptor complex in the specialized membrane region called lipid rafts. Cross-linking of BCR leads to recruitment of kinases in the lipid

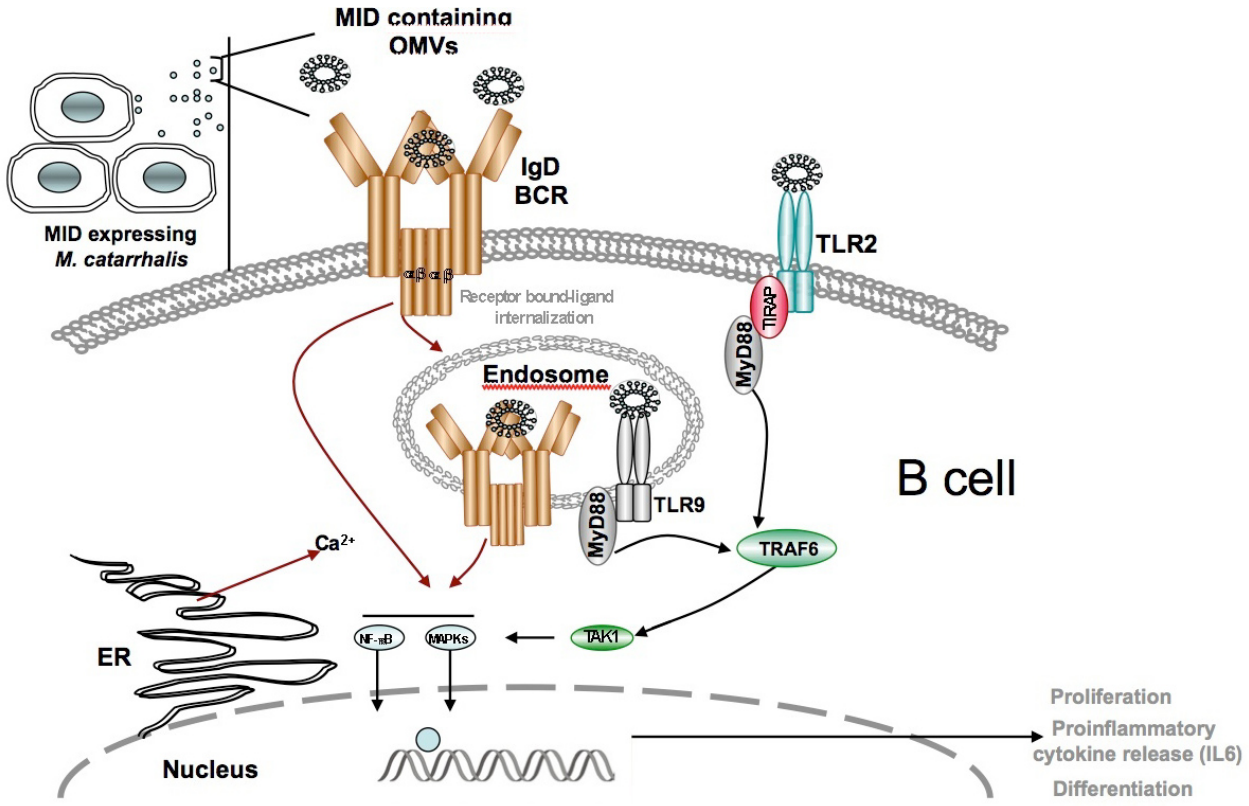

Figure 3. The proposed model for *Moraxella* mediated B cell activation by BCR cross-linking and TLR stimulation. BCR engagement, here exemplified by outer membrane vesicles (OMVs) that are released by the bacteria, leads to phosphorylation of the cytoplasmic tail of Ig-alpha and Ig-beta on the ITAM motifs and enhanced recruitment of Src-family tyrosine kinases (SFTKs) and/or Syk. These protein tyrosine kinases (PTKs) mediate downstream events such as increased intracellular Ca^{2+} influx, which results in activation of multiple signaling pathways including MAPK and NFkB (48). TLR recognise conserved microbial patterns and upon ligand binding dimerizes into functional receptors. Engagement of TLRs leads to recruitment of signalling adaptors such as MyD88, which transmits the signal via TRAF 6 and TAK-1 to the activation of NFkB and MAPK pathways (29). Overlapping activation of NFkB and MAPK pathways promotes synergictic and enhanced activation of transcription factors involved in proinflammatory cytokine release and B cell activation. Whole *M. catarrhalis* cells and OMVs, engage IgD BCR and TLRs such as TLR2 and mainly TLR9, leading to strong polyclonal B cell activation.

rafts, phosphorylation and altered configuration of ITAMs. These events result in recruitment of additional molecules such as tyrosine kinase Syk and its autophosphorylation. BCR ligation leading to phosphorylation of SyK facilitates a number of downstream signaling pathways. For instance, activation of lipid metabolizing enzymes such as PI3-K and PLCgamma2, which are involved in generation of secondary messengers, Ca^{2+} mobilization and in addition to the initiation of kinase relays, all these leading to NFkB- and MAPK-dependent pathways (48).

Ligand binding leads to dimerization of TLRs, which leads to intracellular recruitment of signaling adaptors. MyD88 (myeloid differentiation 88) is one of the adaptor proteins of the TIR (Toll/interleukin 1 receptor) family and is employed by majority of TLR receptors. Post recruitment of MyD88, IL1 receptor-associated kinases (IRAKs) are assembled at the signaling complex, where IRAK-1 is recruited and activated by IRAK-4 by multiple phosphorylation events. This signaling complex is further connected to downstream events by tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). The interaction of TRAF-6 with IRAK-1 leads to its activation and oligomerization by ubiquitin conjugating enzymes. In its polyubiquinated form, TRAF-6 interacts with (TGF)-beta-activated kinase 1 (TAK1) and the TAK1-binding proteins and TAK-1 links the receptor mediated pro-inflammatory response via NFkB and MAPK pathways (49). All TLRs, except for TLR3 employ MyD88 dependent pathway where TLR2 and TLR4 utilize an additional TIR-domain containing adaptor, TIRAP/Mal (TIR-domain containing adaptor protein/MyD88-adaptor-like protein) which acts as a bridging adaptor between MyD88 and the downstream factors (50). Activation of these signaling pathways by BCR engagement and TLR stimulation (NFkB and MAPK) eventually leads to enhanced production of pro-inflammatory cytokines and thus synergistic activation of B lymphocytes.

TLR expression is observed in almost all cell types including non-immune cells such as epithelial, endothelial, glial cells, fibroblasts and immune cells such as

dendritic cells, macrophages and lymphocytes (51-53). However, to achieve a targeted, increased and highly regulated response, expression of TLRs is often spatially restricted, selectively up or down regulated by various mechanisms (54). The presence of TLRs in immune cells including lymphocytes establishes a unique bridge between innate and adaptive immunity. In naïve B-lymphocytes, TLRs are implicated to be critical for inducing a sustained activation and differentiation in response to both T cell dependent (TD) and TI antigens (55). During conventional TD antigen recognition, naïve B cells bind antigens with their BCR. This BCR cross-linking triggers a series of signaling cascades leading to efficient internalization of the antigens, targeting them into endocytic compartment, upregulation and assembly of MHC class II molecules and export them on the cell surface for recognition by specific T cells (56). In addition, BCR engagement leads to upregulation of various co-stimulatory surface receptors and release of cytokines. B cell activation is further supported by specific T-cell costimulation via the CD40-CD40L interaction and released cytokines. Finally, for efficient maturation, differentiation and isotype switch, stimulation of TLR is required. Activation via TLR can either be via direct stimulation of TLR expressed by B cells or indirectly via released cytokines such as IL6 and IL12, due to TLR stimulation of other cells, such as dendritic cells (57). Moreover, TLR signaling is found to be essential for primary development of T_H1 cells by B cells as was shown in mice in response to *Salmonella enterica* infection (58, 59). TI antigens, including B-cell superantigens are not dependent on physical contact with T cells and are endowed with extensive B cell activating capacity as compared to a conventional antigen. TLR stimulation provides the additional signal for cell proliferation and differentiation in response to these antigens (44, 60, 61). The interpretation of requirement of TLR stimulation, besides BCR ligation, is suggested as to introduce an additional specificity step in recognition of antigens so that only foreign antigens with PAMPs induce a stimulatory response.

Amongst the mammals, humans and mice share the expression of TLR1-9. Humans further express TLR10, which is absent in mice. On the other hand, mice express three additional TLRs, namely 11, 12 and 13 (27, 62). Based on their cellular location, TLRs can be either localized on the cell surface or present in the endosomes. As cell surface receptors, TLR1, TLR 2, TLR4, TLR6, TLR5, and TLR10 are expressed on the cell membrane and recognize PAMPs exposed on the surface of pathogens thus mounting a cognate response at the initial detection of pathogen. TLR2 recognizes various structurally diverse PAMPs including, peptidoglycans (PG), lipopeptides and lipoprotein moieties in Gram-positive and Gram-negative bacteria, lipoteichoic acid (LTA) in Gram-positive bacteria, Lipoarabinomannan and Lipomannan from *Mycoplasma* and fungal polysaccharides such as zymogen (63-65). TLR4 detects LPS from Gram-negative bacteria as its main ligand, whereas TLR5 is mainly responsible for sensing the presence of flagellin as a PAMP (66). Unlike other TLRs, TLR10 has not been assigned a specific ligand (67). Recently a homology-modeling approach was used to speculate the ligand-binding site of the TLR10 homo/heterodimer (68). Endosomal TLRs include TLR3, TLR7, TLR8 and TLR9 and the pre-requisite for detection by these TLRs is the co-occurance of PAMPs in the endosomal compartment expressing these PRRs. Endosomal TLRs detect nucleic acid as their ligand, which is usually a product of degradation mediated by acidic environment and proteolytic activity of various enzymes within the intracellular compartment such as endosomes and lysosomes. TLR9 recognizes ssDNA containing a specific dinucleotide motif (CpG) in which the cytosine is unmethylated (69-77).

As compared to naïve human B cells, human memory B cells express detectable levels of TLR1, TLR2, TLR6, TLR7, TLR9 and TLR10 and are more responsive to TLRs. When stimulated by the cognate ligand such as CpG for TLR9 stimulation, they proliferate and differentiate into antibody producing cells (78). Naïve human B cells, on the other hand, are considered rather unresponsive to TLR ligands. However, in the presence of BCR cross-linking, naïve B cells are reported to either be sensitized to TLR ligands or upregulate TLR expression to be more responsive towards the presence of the ligand (78, 79). Murine B cells are reported to express TLR1, TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9, though differential expression has been observed for some of the TLRs in different B cell subsets (80). It is plausible, that this two-way relationship between BCR ligation and TLR stimulation is a strategic way to minimize deleterious activation levels of B cells. However, the fact that *Moraxella* employs both the pathways to be a mitogen only represents a clear example of pathogen-mediated exploitation of the host immune system.

4.1. *Moraxella*- dependent BCR cross-linking and activation via TLRs

We have recently demonstrated that *Moraxella* activates B cells in a BCR- and TLR-dependent manner (43). When synthetic ligands for TLR2/6, TLR1/6, TLR7 and TLR9 were added in addition to purified $MID^{962-1200}$, the strongest response with IgD^+ tonsillar B cells was elicited in the presence of the TLR9 ligand CpG2006. As reported for naïve B cells, CpG2006 or bacterial DNA alone did not induce any activation unless the primary signal was induced via the BCR cross-linking MID962-1200. TLR9 is mainly localized in endosomes and access to bacterial DNA is provided by BCR-mediated uptake of MID-expressing *Moraxella*. This is in accordance with the observations that binding of B-cell superantigens to BCR, like conventional antigens, leads to receptor downregulation and ligand inter nalization (81). $MID^{962-1200}$ and MID-containing *Moraxella* cause downregulation of surface IgD. Upon internalization whole bacteria can be detected in the intracellular endosomal-like compartments, and this provides the connection to the TLR9 containing endosomes (Figure 4). In a recent work, it was shown that unlike synthetic CpG oligodeoxynucleotides (ODNs), which are often used to study TLR9-mediated activation, bacterial dsDNA is not taken up efficiently by splenic B cells. Interestingly, only a physical association with the BCR ligand increases the uptake of bacterial DNA (82). It is proposed that BCR cross-linking recruits TLR9 from

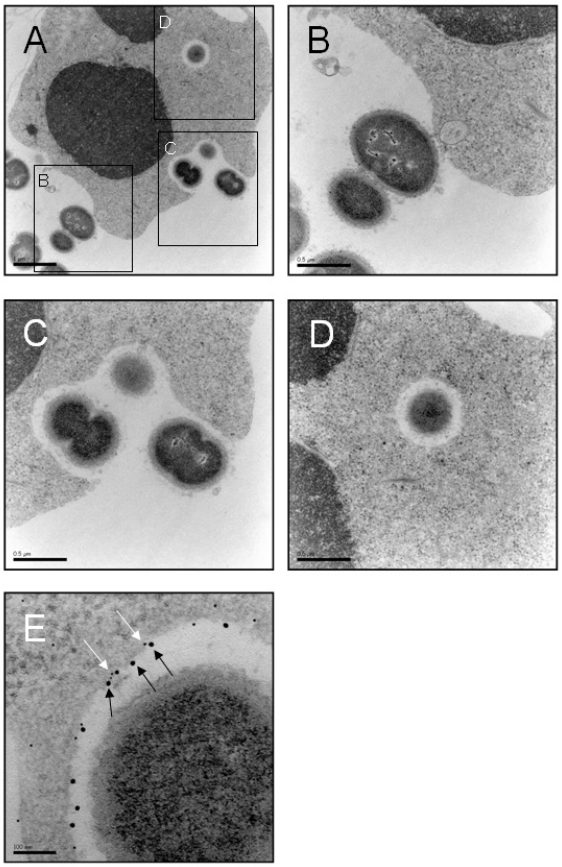

Figure 4. Internalisation of *M. catarrhalis* by B cells. Live *M. catarrhalis* were incubated with B cells for 2 hours and internalisation was analyzed by TEM. The scale in A represents 1 μm, and in the enlarged panels, B–D, 0.5 μm. (E) Cells were incubated with *M. catarrhalis* and analyzed by TEM using gold-labeled Abs directed against IgD (large granules, black arrows) or TLR9 (small granules, white arrows). The scale in E represents 100 nm. Figure reproduced with permission from ref. 43.

their resting steady state intracelullar location to BCR-antigen containing autophagosomal compartments and in these compartments optimal conditions for TLR9 activation and ligand recognition are met (83).

The ability of B cells to take up whole *Moraxella* contradicts the general perception of B cells as non-phagocytic cells. B lymphocytes are conventionally considered to engulf particulate shedded bacterial proteins or extracted protein antigens from the surface of dendritic cells by receptor mediated endocytosis (84). However, it is now well established that B cells has the capacity to take up large size molecules and exhibit ligand-selective phagocytosis. This was also shown in a more recent study, where *Salmonella* coated with beads fused with anti-IgM that target BCR were internalized by B cells in a BCR-mediated manner (85).

Synergistic BCR and TLR stimulation has also been shown for B cell activation by *Staphylococcal* protein A (SpA) (78). The B cell response is triggered in a T cell-independent manner via SpA and the TLR2 ligand peptidoglycan, a component of the bacterial cell wall. Moreover, BCR cross-linking by SpA sensitizes B cells for TLR ligand stimulation. However, no Ig synthesis is induced during co-stimulation of B cells by SpA and TLR2, unless an additional stimulatory signal such as IL-2 is provided. This report highlighted not only the qualitative differences in the B cell response that can arise from engagement of different TLRs but also that Ig synthesis might be a feature unique to nucleic acid detection via TLR9 by B cells (79).

4. 2. *Moraxella* outer membrane vesicles (OMVs) and B cell activation

Outer membrane vesicles are long-distance delivery vehicles of the pathogenic bacteria, which transport diverse virulence factors and allow pathogens to interact with the host without close contact (for a review see 86). Bacterial OMVs are bilayered, spherical structures released from the Gram-negative bacterial surface. They are 50-250 nm membranous vesicles containing adhesins, invasins, LPS and other virulence factors. *M. catarrhalis* produces OMVs *in vitro* as well as *in vivo* during infection (86). Besides other virulence factors, *Moraxella* OMVs carry MID and bacterial DNA. MID-BCR interaction results in the uptake of OMVs by BCR internalization (Figure 5) and upregulation of surface activation markers in addition to IL-6 and IgM secretion (87). MID-containing *Moraxella* OMVs induce Ca^{2+} mobilization and receptor clustering in lipid raft motifs, which are crucial for the recruitment of receptors and signalling. OMV-mediated fusion of virulence factors via lipid raft domains is a common strategy of Gram-negative bacteria to interact with the human host (82). *Moraxella* OMVs can be found intracellularly in epithelial cells and the uptake mechanism is in this cell type also related to lipid rafts (88).

Similar to these findings, it has been shown in another study that OMVs derived from *Neisseria lactamica* mediate a B cell-dependent proliferative response that is associated with the production of polyclonal IgM. This mitogenic B cell response is dependent upon the BCR and occurs independently of T cell help. An unknown protein in the outer membrane of *N. lactamica* appears to be responsible for B cell activation. It is hypothesized that the mitogen is a B-cell superantigen with high affinity for IgM and IgD that allows *N. lactamica* to specifically target the activation of innate B cell subsets which include $CD5^+$ B1 cells and marginal zone cells, resulting in the production of natural Abs which might be maintaining immunological ignorance in the host (89). However, no contribution of TLR9 was observed during activation of B cells by *N. lactamica* OMVs. In another study on mouse B cells and *Nesisseria* OMVs, it was shown *in vitro* that the B cell mitogenic effect of OMVs is not critically dependent on the presence of LPS suggesting that it was not mediated by the TLR4 machinery (90).

5. IMPLICATIONS OF POLYCLONAL B CELL ACTIVATION BY *MORAXELLA*

Most of the existing data regarding the interaction of *Moraxella* with IgD^+ B cells have been

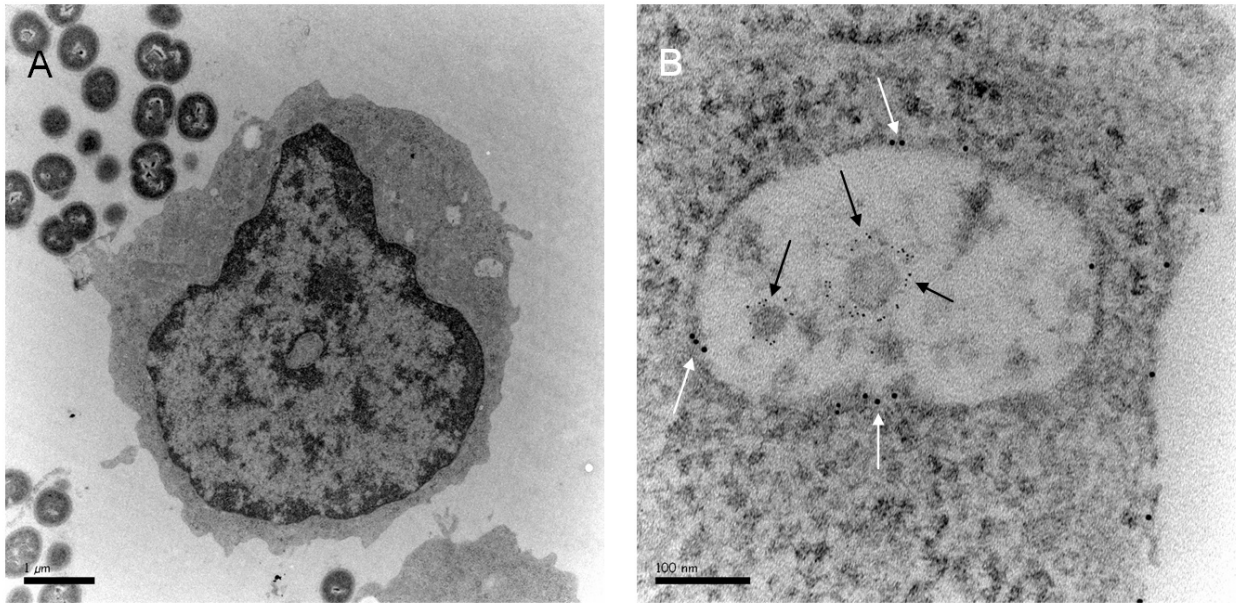

Figure 5. B cells and *M. catarrhalis* OMV interactions analyzed in TEM, showing internalisation of MID containing OMVs. The endosome with two OMVs is magnified in (B) and gold-labelled antibodies against IgD (large granules; white arrows) and MID (small granules; black arrows) are indicated. The scale in (A) and (B) represents 1 µm and 100 nm in length, respectively. Figure reproduced with permission from ref. 87.

accumulated via a series of *in vitro* experiments. When a pure human B cell population is analyzed *in vitro*, MID-dependent polyclonal activation of B cells leads to the production of non-*Moraxella* specific antibodies. This led to the hypothesis that *Moraxella* redirects the immune system by promoting the production of polyclonal low specificity Igs and thus results in avoidance of specific antibody response against the bacteria. Such polyclonal antibodies tend to recognise host components as well and thus can be detrimental to host as autoantibodies. However, the physiological relevance of the fate of B cells following MID mediated activation remains to be elucidated and it would shed further light on the role of polyclonal B cell activation in *Moraxella* pathogenesis. Learning from *in vitro* experiments with whole *Moraxella* and OMVs, it is evident that stimulation of BCR and TLR leads to initial IgM production but not to class switching (39, 85). On the other hand, if additional T_H2 cytokines are provided, BCR cross-linking by MID induces class switching (37). Thus, in a complex immune environment in the host, it might be possible that the outcome of polyclonal B cell activation by *Moraxella* would be highly modulated. This hypothesis is further supported by the fact that there are discrepancies in observations made during experiments done with *Staphylococcal* B-cell superantigen SpA to induce polyclonal B cell activation. SpA binds the Fc region of IgG and the Fab region of the Ig VH_3 gene family (91) allowing the binding to almost all classes of antibodies. SpA, purified or cell associated induces B cell proliferation in human B cells *in vitro*, whereas purified SpA administration to mice results in an initial activation followed by an apoptotic loss of B cells expressing the Ig VH_3 (46, 92, 93), though different B cell subsets are targeted with different kinetics. A permanent loss of circulating SpA-binding IgMs is also observed after a single exposure of purified SpA in mice. The deleterious effect of superantigen-mediated B cell activation is further confirmed by evidence that *Peptostreptococcus* PpL introduction in mice leads to Vk-targeted B cell deletion (94).

As in the case of SpA- and PpL-mediated activation, activation of IgD^+ B cells by *Moraxella* OMVs induce early signaling events such as increased Ca^{2+} influx, characteristic of conventional BCR engagement. Usually the presence of suitable rescuing signals such as IL-4 and CD40L favours the further pro-survival and proliferative response of B cells. However, for SpA- and PpL-induced B cell depletion, it is suggested that the rescuing signals are not sufficient owing to extensive signals provided by B-cell superantigen interaction (23). An analogous situation can be envisioned for *Moraxella*-induced B cell responses *in vivo*, albeit *Moraxella* does not induce apoptosis signals *in vitro* (46). The proof that extensive cross-linking of the IgD BCR may also result in B cell deletion is provided from a recent study where an IgD reactive "superantigen" was generated in mice. Membrane-tethered single-chain Abs reactive to the IgD constant region were expressed in mice and the effect of BCR cross-linking by these Abs was evaluated by analysis of the B cell populations. Administration of B-cell superantigen-carrying cells into mice led to deletion of follicular B cells and most mantle zone B cells followed by the induction of tolerance in mature B cells (95).

In vivo studies to estimate the MID-dependent B cell response are impeded by the fact that murine IgD does not bind MID (40, 46). Despite of the *in vitro* experiments, which show that *Moraxella* induces proliferation of IgD^+ B

cells, it is unclear whether MID induces the depletion of specific B cell subsets *in vivo*, analogous to other B-cell superantigens. Conversely, it is possible that interaction between whole bacteria and engagement of TLRs can provide much needed rescue signals and indeed the outcome may be clonal expansion of IgD^+ cells, without any cell death. A similar scenario has been suggested for *in vivo* B cell activation by whole *S. aureus* bacteria where B cell activation is achieved by synergistic stimulation of the BCR and TLR2 (79). Moreover, infection studies done with mice using *S. aureus* suggest that the outcome of B cell activation, i.e expansion or deletion is basically governed by the bacterial load and the extent of cross-linking of BCR (96).

It is intriguing that *Moraxella* binds IgD, given that the immunological role of IgD still remains to be elucidated. IgD exist in either cell associated or secreted form where cell associated IgD serves as the antigen receptor on human B cells prior to antigenic stimulation and class switch recombination (CSR). IgD deficient mice, as compared to the wild type mice do not show any abnormal B cell development or function. Thus, no independent function has been attributed to IgD except for detection of abnormal serum levels of soluble IgD in some autoinflammatory disorder, autoimmune diseases and chronic bacterial infections (97, 98). Interestingly, a large number of IgD-producing cells and higher serum IgD levels are reported in upper aerodigestive associated tissues including tonsils, adenoids and nasal mucosa as compared to other anatomical site such as peripheral lymph nodes, intestinal mucosa and liver (99-101). These observations suggest an important role of IgD in the immune response of the upper respiratory tract. The fact that *Moraxella* binds IgD is an indication that this phenomenon plays an important role in the pathogenesis of colonization/ infection by the species.

Moraxella is a human nasopharyngeal commensal and suggested to colonise tonsillar lymphoid tissue (32). Moreover, release of MID-containing OMVs can establish the virulence factors distant from the site of colonization. In such a case, constant exposure to MID-containing entities can downregulate surface IgD levels, induce tolerance or anergize the IgD^+ B cell subsets. One of the proposed consequences of binding of B-cell superantigens outside the conventional antigen binding on IgD is the generation of an atypical B cell subset, characterized by IgD^+IgM^- phenotype (102). This B cell subset is suggested to be the result of an IgD-binding B-cell superantigen-dependent activation followed by an unconventional IgM to IgD class switch recombination mechanism. An increased frequency of IgD only B cell subset is reported in recurrent tonsillitis and hyperplastic adenoids (97, 103). Taken together, targeting IgD^+ B cells as well as neutralizing soluble IgD may be a possible common mechanism employed by this intriguing pathogen.

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Abbreviations: BCR: B cell receptor, TLR: Toll like receptor, IL: interleukins, Ig: immunoglobulin, SpA: *Staphylococcus aureus*, protein A, PpL: *Peptostreptoccocus magnus* protein L, OMV: outer membrane vesicle

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