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OTITIS (DP SKONER, SECTION EDITOR)

Molecular Mechanisms of *Moraxella catarrhalis*-Induced Otitis Media

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Abstract *Moraxella catarrhalis* is a Gram-negative bacterium, exclusively present in humans and a leading causative agent of otitis media (OM) in children. Most children (80 %) experience at least one episode of OM by their third birthday and half suffer multiple episodes of infection. Over the last 10 years, increased evidence suggests that *M. cat* possesses multiple virulence factors which can be carried through biologically active outer membrane vesicles (OMVs) that are themselves able to activate host-immune responses. It has also been noted that multiple toll-like receptors are responsible for *M. cat* recognition. This review is intended to summarize the key findings and progress in recent years of the molecular mechanisms of *M. cat*-induced otitis media with particular emphasis on adhesion, invasion, and activation of the host immune system, biofilm formation, and vaccine development.

Keywords *Moraxella catarrhalis* · Virulence · Adhesion · Invasion · Host activation · Toll-like receptors · Vaccine · Biofilm · Otitis media

Introduction

Otitis media (OM) is the most common infectious disease in pediatric population and caused by virus, bacteria, or concurrent infection by both. The total health care costs for OM are projected to be US$3.8 billion annually due to medical costs and lost wages [1]. If untreated, OM can lead to conductive hearing loss, followed by delays in language and cognitive development. Until a few years back, *Streptococcus pneumonia* was the leading causative pathogen of OM; however, as a consequence of the introduction of the newly developed heptavalent pneumococcal vaccine, the microbiology of OM has changed and nontypeable *Haemophilus influenzae* (NTHi) has become the leading pathogen and *Moraxella catarrhalis (M. cat)* ranks third and accounts for 15–20 % of total bacterial infections [2, 3]. In addition to OM, *M. cat* also causes lower tract infection in adults leading to exacerbations of chronic obstructive pulmonary disease (COPD) [4].

It is generally believed that, in order to establish any successful infection, the pathogen has to adhere on the host cell, invade, survive host defense mechanisms, and activate the innate immune system. It has also been recently reported that they can form biofilm, thus making it difficult to treat by antibiotics and often the reason for chronic and recurrent otitis media [5]. This review is intended to summarize the key findings and progress in recent years of the molecular mechanism of *M. cat*-induced otitis media. Due to space restriction, discussion will be limited to adhesion, invasion, and activation of host immune system, biofilm formation, and vaccine development.

Bacterial Adherence to Host Cells

Bacterial adherence to mucosal surface is considered to be a major step to establish bacterial colonization on epithelium. It is not only allowing the pathogen to remain firmly attached with the host cells but also additionally acts as a defense mechanism to escape and survive the complement system. *M. cat* expresses several adhesions molecules such as ubiquitious surface protein A family (UspAs), the human erythrocyte agglutinin/M.cat immunoglobulin D binding protein or Hag/MID, adherence protein (McaP), the outer membrane vesicles (OMVs), and lipooligosaccharide (LOS) [6]. UspAs consists of three major proteins including UspA1, UspA2, and a closely related protein known as hybrid UspA2H, since it has the

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property of both UspA1 and UspA2 and contributes both in adhesion and serum resistance [7–9]. Recently, the complete genomic sequence of *M. cat* strain RH4 (16s rRNA subtype 1 strain) has been reported [10••]. The authors found that two of these genes (*uspA1* and *usp2H*) are highly expressed during the log, stationary, and exponential growth phage of *M. cat* indicating that they are capable of establishing infection at early stage and remain firmly attached to the host cell throughout their life cycle. However, in some *M. cat* strains, like 16s rRNA subtypes 2 and 3, *uspA1* gene expression could be significantly induced by cold-shock treatment [11]. Cold-shock-induced up-regulation of *uspA1* has the potential to benefit the pathogen to survive in the nasopharyngeal region which often experiences lower temperatures than the rest of the body. UspA1 binds to host cells through carcino-embryotic antigen-related cell adhesions-molecule 1 (CECAM1) which is expressed in a wide variety of human tissues including respiratory epithelial cells and leukocytes [12–14]. Using X-ray crystallography Conners et al. showed that the CEACAM1 receptor-binding region of UspA1 unusually consists of an extended, rod-like, left-handed trimeric coiled-coil, thus allowing the pathogen to establish closer contact with the surface of the epithelial cells [15]. The binding of UspA1 to CEACAM1 also induces apoptosis in host cells. Interestingly, it has been found that interaction of CEACAM1 and UspA1 inhibits toll-like receptor 2 (TLR2)-mediated inflammation through the NF-κB signaling pathway in primary pulmonary epithelial cells [16]. On the other hand, UspA2 interacts with extracellular matrix proteins, vitronectin, fibronectin, and laminin, and unlike UspA1, does not bind with CEACAM1 [17, 18].

Haemagglutinin (Hag), also known as MID (Superantigen *Moraxella* immunoglobin D binding protein), is an important surface protein that mediates the adherence of *M. cat* to various host cells, most notably human middle ear epithelial cells (HMEEC) [19, 20]. Genetic sequencing of *M. cat* RH4 strain revealed that expression of Hag/MID gene is high during lag phase and stationary phase but intermediate at exponential phase [10••]. This result indicates that the Hag/MID protein plays an important role in the early phase of infection and enables the pathogen to adhere to host cells such as HMEEC, Chang cells, and NCIH292 lung epithelial cells [19, 21]. MID has also been shown to activate tonsilar B cells through TLR9 and is independent of T cell involvement [22•].

Outer membrane vesicles (OMVs) secreted by pathogens are recognized as long-distance delivery vehicles that carry various types of virulence factors and allow pathogens to interact with host cells and influence the immune response. Using 2D gel electrophoresis and MALDI-TOF spectrometry analysis, Schaar et al. identified 57 proteins that are carried by OMVs including UspA1, UspA2, MID, LOS, and DNA [23••]. OMVs bind and enter into respiratory epithelial cells A549 through TLR-2 leading to ICAM-1 expression and induce secretion of pro-inflammatory cytokines IL-8. When harvested OMVs were administered into mice lung in vivo, an increase exudate was observed, and the lung epithelial surface developed a more ruffled appearance compared with controls receiving PBS only, confirming that OMVs are highly biologically active in the mouse lung [23••]. Very recently, it was documented that *M. cat* OMVs could shield active β-lactamase from the anti-β-lactamase IgG and could potentially contribute to the spread of antibiotic resistance [24].

Another important protein, the *M. cat* adherence protein (McaP), was first identified by Lafontaine et al. and later described by the same group as one of the essential outer membrane protein responsible for adhesion [25, 26]. Sequence analysis showed that McaP is highly conserved with 98–100 % identity among nine isolates that were tested. *E.coli* expressing recombinant Mcap showed increased adherence to Chang cells, A549 cells, and human bronchial cells by 50- to 100-fold [25]. Surprisingly, no significance reduction of adherence was found in mutant *M. cat* that lacked only the McaP gene compared to wild-type *M. cat*. The authors concluded that other adhesion molecules, such as UspA1, UspA2, and Hag, could compensate the function of McaP. In fact, a mutant *M. cat* strain lacking all four molecules UspA1, UspA2, Hag, and Mcap showed reduced adherence in Chang cells compared to a mutant strain that lacked only UspA1, UspA1, and Hag but not McaP.

Similar to other Gram-negative bacteria, *M. cat* possesses lipooligosaccharide (LOS), which is found in the outer membrane of the bacteria and considered one of the major virulence factors [27, 28]. LOS is responsible for serum resistance, adherence to epithelia cells, and initiating host activation, details of which will be described later. The LOS is structurally distinct from typical lipopolysaccharide (LPS). LOS consists of an oligosaccharide (OS) core and associated lipid A without the presence of repeating O-antigen polysaccharide side-chains that are commonly found in LPS [29]. Two late acyltransferase gene, lpxX and lpxL, are responsible for the incorporation of acyloxyacyl-linked secondary acyl chains into lipid A during LOS biosynthesis [30•]. In comparison with the O35E parental strain and the single mutants O35ElpxX and O35ElpxL, the double mutant O35ElpxXL displayed prominently decreased endotoxin content, reduced resistance to normal human serum, and accelerated bacterial clearance at 0, 3, and 6 h after an aerosol challenge in a mouse model of bacterial pulmonary clearance. These results indicate that these two genes encoding late acyltransferases responsible for lipid A biosynthesis jointly contribute to the biological activities of LOS and pathogenicity of *M. cat*.

Bacterial Invasion into Host Cells

Like other respiratory tract pathogen such as *Haemophilus influenzae* and *Streptococcus pneumonia, M. cat* is also able to invade the epithelial cells. The ability to invade epithelial

cells has been discussed as a useful bacterial strategy to colonize the respiratory tract and to avoid extracellular host-immune recognition. An isogenic mutant of strain O35E, which lacked expression of the UspA1 adhesin, demonstrated not only severely impaired adherence (86 %) to but also reduced invasion (77 %) into Chang conjunctival cells in comparison with the wild-type strain [31]. The isogenic, LOS-deficient mutant strain O35E.lpxA was attenuated in adherence (93 %) and its capacity to invade was severely reduced (95 %). Invasion of *M. cat* into host cells was further confirmed in vivo by a separate research team. Using confocal laser microscopy, Heiniger et al. identified intracellular presence of *M. cat* in macrophages and B cells in lymphoid follicles obtained from patients [32]. Invasion of A-549 cells and primary small airway epithelial cells (SAEC) by *M. cat* was found to be associated with formation of lamellipodia and internalized bacteria were located within vacuoles. Using scanning and transmission microscope, it was documented that *M. cat* was surrounded by both lamellipodia and filopodia with an involvement of active cytoskeleton [33]. This was further confirmed by pretreatment of cytochalasin D, a potent inhibitor of cytoskeleton polymerization. Cells treated with cytochalasin D followed by *M. cat* infection had profound effect on invasion with almost 80–90 % of total reduction compared with untreated cells. This phenomenon is highly influenced by TLR2 and NOD-1. When TLR2 and NOD-1 was silenced by siRNA, it strongly inhibited *M. cat*-induced activation of NF-κB and abolished the secretion of IL-8 in A549 cells.

Activation of Host-Immune Response

It is generally recognized that inflammation mediated by microbes usually begins when immune cells recognize microbial products through one or more innate immune receptors termed Toll-like receptors (TLRs). As discussed earlier, *M. cat* LOS is not only a major molecule responsible for bacterial adherence but also one of the most important virulence factors that immediately activate the host immune system. Bacterial infection is considered to be a dominant etiology in acute otitis media, causing increased infiltration of leukocytes and macrophages in the middle ear which, in turn, secrete inflammatory cytokines such as TNF-α, IL-6, IL-1β, and IFN-γ. *M. cat* LOS can selectively up-regulate intracellular-adhesion molecule1 (ICAM-1) in THP1 cells and human primary monocytes through the CD14-TLR4-dependent signaling pathway [34]. It was further noted that up-regulation of ICAM-1 is mediated through the TNF-α–dependent autocrine mechanism and requires JNK1/2 and NF-κB p65 activities. Interestingly, *M. cat* LOS-activated monocytes were also able to stimulate adjacent naïve monocytes to produce TNF-α partially by surface ICAM-1 expression and IL-8 secretion. In addition to cytokine secretion, *M. cat* LOS is capable of inducing secretion of matrix metalloproteinases-9 (MMP-9) from murine macrophage RAW 264.7 cells but does not affect MMP-2 production [35•]. MMP-9 belongs to a family of zinc-dependent endopeptidase that functions to promote degradation of the extracellular matrix. Recently, MMP-2 and MMP-9 have been detected in patients with OM with effusion, as well as in patients with chronic OM with effusion [36, 37]. It was further documented that secretion of MMP-9 in response to LOS stimulation was regulated by p38 and ERK1/2, two members of the mitogen-activated protein kinase (MAPK) family. In contrast, JNK1/2, a third member of the MAPK family, negatively regulates secretion of MMP-9, and inhibition of JNK1/2 activation increases secretion of active MMP-9 in murine macrophages. Enhanced secretion of MMP-9 was also involved in increased cellular invasion and migration [35•]. The results of these studies contributed to an increased understanding of the underlying pathophysiology of OM caused by *M. cat*.

Several clinical studies have shown the association between TLRs and otitis media (OM). Lee et al. recently reported that TLR2 and TLR4 are expressed in all the middle ear fluid samples of OM with effusion [38]. Gene expressions of TLR3, TLR4, TLR5, and TLR7 were significantly lower in patients with chronic middle ear disease compared to control patients [39]. On the other hand, increased expressions of TLRs have also been reported. Granath et al. found increased TLR7 expression in the adenoids of children with OM with effusion [40]. Recently, we found that LOS and whole bacteria initiate distinct signaling pathways through one or more TLRs and were able to activate both MyD88- and TRIF-dependent signaling pathways in vitro [41••]. The details of the signaling pathways are shown in Fig. 1. While LOS required only the CD14–TLR4 complex and was able to initiate both MyD88- and TRIF-dependent host responses, both live and heat-killed *M. cat* required multiple TLRs (TLR2, TLR4, and TLR9), and recognition of whole bacteria did not necessarily require CD14. It was further documented that TLR4 mutant mice produced significantly reduced levels of pro-inflammatory cytokines such as TNF-α and IL-6 in response to live *M. cat* and had a significantly higher level of bacterial loads in the lungs compared to wild-type control mice. Association between TLR4 and *M. cat* was also found in pediatric population in Europe. In a prospective study from 2008 to 2010, 23 % of children aged 3 months was culture-positive for *M. cat*. The colonization rate of *M. cat* was significantly higher in subjects with variant types of TLR4 (Asp299Gly) than those with wild type [42].

Formation of Biofilm

Once the pathogen is adhered and has activated the host immune response, it is always beneficial for the pathogen to colonize and remain persistent in the host. Formation of

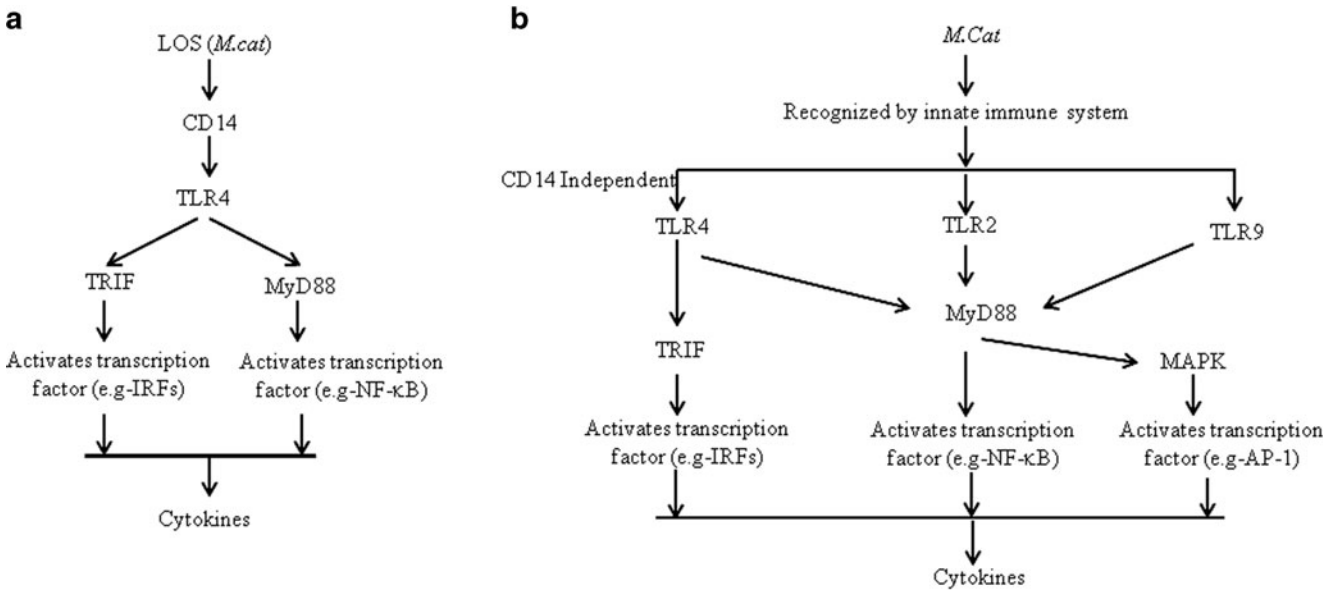

Fig. 1 Schematic diagram of *Moraxella catarrhalis*-induced activation of host signaling pathway. **a** *M. cat* LOS is recognized by TLR4 with the help of membrane bound CD14. Once recognized, LOS initiates both MyD88-dependent and TRIF-dependent signaling pathways which activate their respective transcription factors and induce secretion of pro-inflammatory cytokines. **b** Either heat-killed or live *M. cat* is recognized by multiple TLRs such as TLR2, TLR4, and TLR9 without the involvement of membrane bound CD14 and activates both MyD88- and TRIF-dependent signaling pathways. In addition, *M. cat* also induces secretion of TNF-α through activation of the TLR4-p38 MAPK-JNK1/2 pathway. All the activation leads to the translocation of transcription factors IRFs, NF-κB, AP-1, etc. to the nucleus and induces cytokine secretion

biofilm is one of the key factors that enable the pathogen to avoid adverse surrounding environmental condition including effect of antibiotics. Presence of biofilm is well documented in OM, a disease that is difficult to treat with antibiotics and often chronic and recurrent in nature [5]. Most of the information to date that are available is related to NTHi. The mechanism of biofilm formation by *M. cat* is less well understood. Pearson et al. first showed that *M. cat* was capable of forming biofilm in vitro and regulated by UspA1 and Hag [5, 43]. At the same time, biofilm has been directly detected on the middle-ear mucosa of children with chronic otitis media [44]. Recent report found that biofilm producing bacteria such as *Streptococcus pneumoniae* and *Moraxella catarrhalis* were found to be more frequently located near the ostium of the eustachian tube (ET) suggests that the adenoids are a reservoir for bacteria and indicates that hypertrophic adenoids (particularly hypertrophy near the ostium of the ET) play a role in recurrent acute otitis media and/or otitis media with effusion [45].

Development of Vaccine

The present treatment of *M. cat* infection has relied largely on antimicrobial agents. However, frequent use of antibiotics resulted in antibiotic resistance since greater than 90 % of the clinical isolates express a drug-resistance enzyme, beta-lactamase. Prevention of *M. cat* infections by effective vaccination would thus potentially have a significant impact on both public health and the economy. As expected, most of the vaccines are designed to target outer membrane protein of *M. cat* such as UspA1, Hag/MID, catarrhalis outer membrane protein B (copB), and CD [6]. Recently, *M. cat* LOS has become an attractive and promising target for vaccine development. Gu et al. have developed series of Vaccines against each of the serotypes of *M. cat* such as serotypes A, B, and C [46–49]. However, each of these conjugates has been found to cover only a portion of the pathogenic strains of *M. cats*. In order to overcome this limitation, LOS-based conjugate vaccines have been developed with wide coverage. Subcutaneous immunization elicited significant increases of serum immunoglobulin (Ig)G against O35E LOS in rabbits with or without an adjuvant [50]. More recently, mice immunized intranasally with LOS-conjugate vaccine showed enhanced pulmonary bacterial clearance of all three serotypes of *M. cat* strains in vaccinated mice [51•]. Mice vaccinated with the combined LOS conjugates also showed increased interferon (IFN)-γ, interleukin (IL)-12, and IL-4 in the lungs after challenges. Compared to the control group, mice immunized with the combined LOS conjugates also showed reduced lung inflammation after *M. cat* infections. The hyperimmune sera induced by the combined conjugates exhibited a broad cross-reactivity toward all three serotypes of *M. cat* under transmission electron microscopy [51•]. It was concluded that the combined vaccine of serotype A and B LOS conjugates provides protection against most *M. cat* strains by eliciting humoral and cellular immune responses and could be a potential vaccine candidate for clinical trial.

Conclusions

Once known as a commensal pathogen, *Moraxella catarrhalis* is now being recognized as an exclusive human pathogen and capable of causing upper respiratory tract infection including otitis media in children. In addition, *M. cat* is also responsible for an estimated 2–4 million exacerbations annually of chronic obstructive pulmonary disease (COPD) in the elderly. In the last few years, our knowledge of molecular mechanism of otitis media caused by *M. cat* has increased significantly. The complete genome sequence of *M. cat* strain RH4 is now available and has revealed that UspAs, Hag/MID, and LOS are important surface molecules that are almost equally expressed during the growth cycle of *M. cat*. Outer membrane vesicles carry most of the surface proteins and play pivotal roles in pathogenicity. Host cell receptors such as TLRs are important for any pathogen recognition. In fact, multiple TLRs (TLR2/4/9) are responsible for *M. cat*-induced host activation. *M. cat* also has the ability to invade bronchial epithelial cells and primary airway epithelial cells and initiate a TLR2 and partly NOD1-dependent inflammatory response. Signaling pathways that are activated by *M. cat* could be used as a potential target for therapeutic drug development. Understanding the molecular mechanism of biofilm formation by *M. cat* could change the course of present antibiotic treatment to be more effective. There are no vaccines available in market to prevent *M. cat* infection. However, there are several vaccines that showed promising results in animal models. Further studies are needed to validate these results in order to have full clinical trials and discover the efficacy in human.

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Compliance with Ethics Guidelines

Conflict of Interest Ferdaus Hassan declares that he has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by the author.

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