Multicomponent *Moraxella catarrhalis* outer membrane vesicles induce an inflammatory response and are internalized by human epithelial cells

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Summary

*Moraxella catarrhalis* is an emerging human respiratory pathogen in patients with chronic obstructive pulmonary disease (COPD) and in children with acute otitis media. The specific secretion machinery known as outer membrane vesicles (OMVs) is a mechanism by which Gram-negative pathogens interact with host cells during infection. We identified 57 proteins in *M. catarrhalis* OMVs using a proteomics approach combining two-dimensional SDS-PAGE and MALDI-TOF mass spectrometry analysis. The OMVs contained known surface proteins such as ubiquitous surface proteins (Usp) A1/A2, and *Moraxella* IgD-binding protein (MID). Most of the proteins are adhesins/virulence factors triggering the immune response, but also aid bacteria to evade the host defence. FITC-stained OMVs bound to lipid raft domains in alveolar epithelial cells and were internalized after interaction with Toll-like receptor 2 (TLR2), suggesting a delivery to the host tissue of a large and complex group of OMV-attributed proteins. Interestingly, OMVs modulated the pro-inflammatory response in epithelial cells, and UspA1-bearing OMVs were found to specifically downregulate the reaction. When mice were exposed to OMVs, a pulmonary inflammation was clearly seen. Our findings indicate that *Moraxella* OMVs are highly biologically active, transport main bacterial virulence factors and may modulate the epithelial pro-inflammatory response.

Introduction

*Moraxella catarrhalis* is a Gram-negative aerobic dipplococcus and an exclusive human respiratory pathogen. After *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*, *M. catarrhalis* is the third most common cause of bacterial respiratory tract infections, including acute otitis media (AOM), sinusitis, laryngitis and pneumonia. Increasing evidence underlines the importance of *M. catarrhalis* as an emerging pathogen responsible for recurrent and persistent respiratory tract infections in patients suffering of chronic obstructive pulmonary disease (COPD), which is one of the leading causes of morbidity and mortality worldwide (Murphy *et al*., 2000; Pauwels *et al*., 2001; Sethi *et al*., 2007; Perez Vidakovics *et al*., 2010). *M. catarrhalis* causes approximately 10% of all COPD exacerbations and significantly contributes to the chronic airway inflammation, which is a hallmark of the disease (Sethi *et al*., 2007). During exacerbations of COPD, the respiratory tract mucosa is exposed to bacterial components/antigens that induce a potent inflammatory response; this includes, for example, endotoxin, peptidoglycan fragments and lipoproteins. A number of *Moraxella* virulence factors have been identified and characterized (de Vries *et al*., 2009; Perez Vidakovics *et al*., 2010). Recently, the first complete genome sequence of *M. catarrhalis* has been reported (de Vries *et al*., 2010), which will greatly facilitate our ability to understand *Moraxella* pathogenesis and provide the basis...
for proteomics research. Ubiquitous surface proteins (UspA) and the Moraxella immunoglobulin (Ig) D-binding protein (MID), also designated haemagglutinin (Hag), are among the most extensively studied virulence factors of M. catarrhalis. UspA, which exists as three variant proteins, UspA1, UspA2 and the hybrid protein designated UspA2H, are oligomeric coiled-coil adhesins with binding sites for multiple host macromolecules such as fibronectin and vitronectin (Tan et al., 2005; Attia et al., 2006; Tan et al., 2006; Singh et al., 2010). Interestingly, UspA1 has been reported to include a critical binding site for carcionembryonic antigen-related cell adhesion molecule-1 (CEACAM-1), which is widely distributed on epithelial cells including those of the oropharynx and lower respiratory tract (Hill et al., 2003). A recent study demonstrated that the interaction between CEACAM-1 and M. catarrhalis UspA1 resulted in reduced Toll-like receptor-2 (TLR2)-initiated NFκB-dependent inflammatory responses of pulmonary epithelial cells (Slevogt et al., 2008). In parallel to the UspAs, MID is another well-studied and highly conserved OMP. MID has a unique ability to bind IgD in a non-immune manner, and functions as an adhesin by mediating attachment to lung epithelial cells (Forsgren et al., 2003; Riesbeck et al., 2006).

Outer membrane vesicles (OMVs) secreted by pathogenic bacteria are recognized as long-distance delivery vehicles which transport diverse virulence factors and allow pathogens to interact with the host, and hence have the possibility to alter the immune response without close contact (Ellis et al., 2010). These spherical bilayered OMVs are released from the outer membrane and range in size from 50–250 nm in diameter. OMVs produced by pathogenic bacteria contain adhesins, invasins and immunomodulatory compounds such as lipopolysaccharide (LPS) (Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006). Several studies have demonstrated that OMVs play a role as protective transport vesicles, delivering toxins, enzymes and DNA to eukaryotic cells as well as being key factors in natural competence (Deich and Hoyer, 1982; Dorward et al., 1989; Kadurugamuwa and Beveridge, 1998; Kesty et al., 2004; Renelli et al., 2004). OMVs can also improve bacterial survival in the host by directly mediating bacterial binding and invasion, causing cytotoxicity and modulating the host immune response (Shoberg and Thomas, 1993; Bomberger et al., 2009). We have previously shown that OMVs from M. catarrhalis contribute to an increased survival of Haemophilus influenzae in human serum by binding and neutralizing C3 in vitro (Tan et al., 2007). Interestingly, OMVs secreted by Moraxella also bind to tonsillar B cells through the superantigen MID (Perez Vidakovics et al., 2010). The interaction between MID and the B cell receptor induces Ca²⁺ mobilization and receptor clustering in lipid raft motifs followed by internalization of vesicles. Mainly TLR9, a DNA recognition receptor of the innate immune system, participates in the signalling induced by OMVs through sensing of DNA associated with the vesicles. The OMV-dependent B cell activation induces upregulation of surface activation markers in addition to IL-6 and IgM secretion.

In this study, we report the global proteome of highly purified M. catarrhalis OMVs using 2D gel electrophoresis and MALDI-TOF analysis. Major outer membrane proteins, i.e. MID, UspA, OMPs J, G1b, CD, E, M35 and TbpB, were carried by OMVs of which several are reported to contribute to the bacterial pathogenesis. Periplasmic, peptidoglycan-associated as well as hypothetical proteins with unknown functions were also present. Using a functional approach, we analysed binding and internalization of OMVs to human alveolar epithelial cells and alteration of cytokine profiles, which are critical activities during the Moraxella–host interaction. Finally, when OMVs were administered to a series of mice, pulmonary inflammation was observed suggesting an important role for OMVs also in vivo.

Results

M. catarrhalis OMVs contain LOS and DNA

We have previously shown that M. catarrhalis has the capacity to produce OMVs in vitro as well as in vivo during infection (Tan et al., 2007; Perez Vidakovics et al., 2010). The OMVs secreted in vitro by M. catarrhalis BBH18 were spherical, bilayered proteolipids with an average diameter of 50–150 nm, which is consistent with data on OMVs released by other Gram-negative bacteria (Bumann et al., 2002; Lee et al., 2007; Galka et al., 2008; Roberts et al., 2008) (Fig. 1A and B). The bacteria produced OMVs in both logarithmic and stationary phases, and OMVs were released from intact bacterial membranes indicating that the OMVs were not merely the result of bacterial cell lysis. The presence of LOS, a general component of the outer membrane of Gram-negative bacteria (Fig. 1C), and the well-known surface proteins MID and UspA1 and UspA2 (Fig. 1D) indicated that OMVs released by Moraxella were indeed derived from the outer membrane as previously defined in EDTA-induced OMVs by Nordstrom et al. (2004). This was also done in order to confirm the presence of UspA1 and A2 in naturally produced OMVs, as these proteins could not be seen in the proteomic analysis later performed due to a size cut-off. M. catarrhalis is among a few Gram-negative bacteria with LPS/LOS containing 3-hydroxydodecanoic acid as the sole 3-hydroxy fatty acid (Wilkinson, 1988), which was confirmed by GC/MSMS analysis of the OMVs.

We have recently shown that OMVs carry DNA that interacts with TLR9 in B lymphocytes (Perez Vidakovics...
To quantify the DNA content in OMVs, *Moraxella* OMVs were lysed by incubation in lysis solution containing Sarkosyl to release DNA. The wild-type OMVs and MID-deficient OMVs contained 23.5 pg DNA per mg OMV protein and 23.3 pg DNA per mg OMV protein respectively. Similar values were detected in UspA1-deficient OMVs (23.6 pg DNA per mg OMV protein), indicating that the absence of this particular virulence factor did not alter DNA content of the OMVs.

### Proteomic analysis of OMVs

Two-dimensional electrophoresis (2D-SDS PAGE) followed by MALDI-TOF mass spectrometry analysis was used to characterize the *M. catarrhalis* OMV proteome. In Fig. 2, the *M. catarrhalis* OMV proteome 2D-reference map is shown. On the average 115 protein spots per gel were detected by silver staining. The resulting peptide mass fingerprints were analysed using a *M. catarrhalis* RH4 protein data set (de Vries et al., 2010). We were able to successfully identify 85 distinct spots corresponding to 57 different proteins (Table 1), displaying molecular masses between 14 and 115 kDa. Several proteins were found to be present in more than one spot due to pI or mass variations and in some cases a protein was dispersed among multiple spots. As expected, the largest group of OMV-associated proteins identified (37%) were outer membrane and periplasmic proteins. Most entries were found for proteins belonging to the functional protein categories of energy metabolism, protein synthesis, and cell envelop, the latter predominately containing outer membrane proteins (Fig. 3). Some of these proteins have a role in *M. catarrhalis* pathogenesis as for example CopB and OMP E (Aebi et al., 1996; Murphy et al., 2000). Additionally, several proteins with unknown or putative function (conserved hypothetical proteins) were also identified. Taken together, proteome mapping demonstrated that a large proportion of *Moraxella* virulence factors thus are delivered by OMVs.

**OMVs bind to and enter respiratory epithelial cells via a lipid raft-mediated pathway**

Outer membrane vesicles are vehicles by which virulence factors including adhesins can be efficiently transported to host cells or tissues during bacterial colonization. To determine whether OMVs released by *M. catarrhalis* interact with human respiratory epithelial cells, the type two
alveolar epithelial cell line A549 was incubated with purified OMVs. Analysis by fluorescence microscopy revealed several OMVs attached to epithelial cells as shown with green fluorescence (Fig. 4). The acquisition of fluorescence was dependent upon the presence of OMVs since cells that were not incubated with OMVs did not exhibit any detectable green fluorescence. Binding of OMVs was independent of the presence of UspA1 since vesicles isolated from UspA1-deficient mutants bound equally well to A549 cells as compared with OMVs isolated from wild-type bacteria (Fig. 4).

We have recently demonstrated that *M. catarrhalis* OMV-dependent interactions with human B cells is initiated by clustering of IgD B cell receptors into lipid rafts motifs and is depending on protein MID (Pérez Vidakovics *et al.*, 2010). To examine whether these cholesterol
| Sample ID | Locus | Gene | Score | Pept.* | Mass | % Vol | Product General role description | Predicted cellular destination |
|-----------|-------|------|-------|--------|------|-------|---------------------------------|-------------------------------|
| 1 pL Mct1 | MCR_1393 |      | 84    | 6      | 16485 | 3.64 ± 0.23 | Putative lipoprotein | Cell envelope Outer membrane |
| 2 LpxD    | MCR_0547 | lpxD | 23    | 3      | 35416 | 0.24 ± 0.01 | UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase LpxD | Cell envelope Cytoplasm |
| 3 OMP J   | MCR_0366 | ompJ | 124   | 8      | 18838 | 8.01 ± 0.14 | Outer membrane protein J | Cell envelope Outer membrane |
| 4 pL Mct2 | MCR_0117 |      | 180   | 11     | 20319 | 2.13 ± 0.11 | Putative lipoprotein | Cell envelope Outer membrane |
| 5 LysM    | MCR_1551 |      | 183   | 12     | 18036 | 0.89 ± 0.20 | LysM domain protein | Cell envelope Cytoplasm |
| 6 OMP G1b | MCR_1380 | onpgG1b | 108  | 9      | 26975 | 3.97 ± 0.34 | Outer membrane protein G1b | Cell envelope Outer membrane |
| 7 Lgt1    | MCR_1095 | lgt1 | 23    | 3      | 35968 | 0.25 ± 0.02 | Glucosyltransferase Lgt1 | Cell envelope Cytoplasm |
| 8 CopB    | MCR_0492 |      | 302   | 26     | 83023 | 6.67 ± 0.18 | Outer membrane protein CopB | Cellular processes Outer membrane |
| 9 OMP CD  | MCR_1698 | ompCD | 295   | 24     | 48248 | 9.03 ± 0.41 | Outer membrane protein CD | Cellular processes Extracellular |
| 10 Cysl   | MCR_1130 | cysl | 25    | 3      | 63043 | 0.37 ± 0.023 | Sulphite reductase | Central intermediary metabolism |
| 11 CcmE   | MCR_0175 | ccmE | 24    | 2      | 18848 | 0.52 ± 0.09 | Cytochrome c-type biogenesis protein CcmE | Energy metabolism Inner membrane (Periplasm) |
| 12 CcoP   | MCR_1320 | ccoP | 35    | 3      | 39238 | 0.02 ± 0.002 | Phospho mannose mutase | Energy metabolism Cytoplasm (Possibly secreted via non-classical pathways) |
| 13 PmmA   | MCR_1842 |      | 31    | 5      | 52153 | 0.03 ± 0.002 | Putative polyhydroxyalkanoic acid system protein | Energy metabolism Cytoplasm |
| 14 pPhaP  | MCR_1252 |      | 30    | 2      | 10728 | 0.12 ± 0.003 | Phospho mannose mutase | Energy metabolism Cytoplasm |
| 15 Pgi    | MCR_1808 | pgi  | 24    | 4      | 60198 | 0.03 ± 0.005 | Glucose 6-phosphate isomerase | Energy metabolism Cytoplasm |
| 16 AceA   | MCR_1622 | aceA | 47    | 8      | 59480 | 0.03 ± 0.006 | Isocitrate lyase | Energy metabolism Cytoplasm |
| 17 OMP E  | MCR_0858 | onpe | 343   | 24     | 49372 | 2.36 ± 0.20 | Outer membrane protein E | Fatty acid and phospholipid metabolism |
| 18 HP Mct5| MCR_0189 |      | 28    | 2      | 4791  | 0.17 ± 0.015 | Hypothetical protein | Hypothetical proteins Cytoplasm |
| 19 HP Mct3| MCR_1456 |      | 39    | 3      | 5276  | 0.01 ± 0.001 | Hypothetical protein | Hypothetical proteins Cytoplasm |
| 20 chp Mc1| MCR_1042 |      | 190   | 15     | 15153 | 3.33 ± 0.23 | Conserved hypothetical protein | Hypothetical proteins Outer membrane |
| 21 HP Mc2 | MCR_0996 |      | 63    | 4      | 16202 | 0.95 ± 0.07 | Hypothetical protein | Hypothetical proteins Periplasm |
| 22 HP Mc9 | MCR_1288 |      | 20    | 2      | 17273 | 0.03 ± 0.002 | Hypothetical protein | Hypothetical proteins Inner membrane |
| 23 HP Mc4 | MCR_1230 |      | 243   | 17     | 30068 | 2.51 ± 0.19 | Hypothetical protein | Hypothetical proteins Extracellular |
| 24 chp Mc10 | MCR_0298 |      | 26    | 2      | 24154 | 0.37 ± 0.02 | Conserved hypothetical protein | Hypothetical proteins Cytoplasm |
| 25 HP Mc7 | MCR_0038 |      | 22    | 2      | 10640 | 0.40 ± 0.03 | Hypothetical protein | Hypothetical proteins Inner membrane (Periplasm) |
| 26 chp Mc8 | MCR_0285 |      | 38    | 4      | 24535 | 0.06 ± 0.001 | Hypothetical protein | Hypothetical proteins Cytoplasm |
| 27 HP Mc6 | MCR_1643 |      | 35    | 2      | 4959  | 0.06 ± 0.002 | Hypothetical protein | Hypothetical proteins Cytoplasm |
| 28 HP Mc11 | MCR_1167 |      | 46    | 3      | 4614  | 0.007 ± 0.004 | Hypothetical protein | Hypothetical proteins Cytoplasm |
| 29 HP Mc12 | MCR_0740 |      | 32    | 2      | 8131  | 0.38 ± 0.03 | Hypothetical protein | Hypothetical proteins Cytoplasm |
| 30 PmlL   | MCR_0964 |      | 48    | 6      | 24742 | 0.14 ± 0.015 | Phage minor tail protein L | Mobile and extrachromosomal element functions |
| 31 LolB   | MCR_0186 | loliB | 42    | 3      | 20295 | 0.19 ± 0.02 | Membrane lipoprotein LolB | Protein fate Inner membrane (Periplasm) |
| 32 TatB   | MCR_0126 | tatB | 27    | 2      | 20608 | 0.014 ± 0.003 | Twin-arginine translocation protein subunit TatB | Protein fate Inner membrane (Periplasm) |
| 33 PpiC   | MCR_1115 |      | 335   | 27     | 47783 | 0.36 ± 0.03 | PpiC-type peptidyl-prolyl cis-trans isomerase | Protein fate Outer membrane |
| 34 MsrAB  | MCR_0686 | msrAB | 327   | 28     | 62017 | 0.32 ± 0.02 | Peptide methionine sulfoxide reductase MsrA/MsrB | Protein fate Outer membrane |
| 35 CtpP   | MCR_0264 |      | 92    | 9      | 81340 | 0.15 ± 0.01 | C-terminal processing peptidase-1 | Protein fate Extra cellular |
| 36 PepN   | MCR_1376 | pepN | 36    | 4      | 97773 | 1.01 ± 0.23 | Aminopeptidase N | Protein fate Cytoplasm |
| # | Protein | Accession | Gene | MW | p-value | Ubiquity | Subcellular Location | Description |
|---|---------|-----------|------|-----|---------|----------|---------------------|-------------|
| 37 | RplN | MCR_1575 | rplN | 36 | 4 | 13.432 | 0.41 ± 0.02 | 50S ribosomal protein L14 | Protein synthesis Cytoplasm |
| 38 | RsuA | MCR_0053 | rsuA | 29 | 2 | 26.108 | 0.01 ± 0.004 | Ribosomal small subunit pseudouridine synthase A | Protein synthesis Cytoplasm |
| 39 | RliB | MCR_0847 | rliB | 35 | 4 | 37.338 | 0.18 ± 0.04 | Ribosomal large subunit pseudouridine synthase B | Protein synthesis Cytoplasm |
| 40 | RpsD | MCR_1562 | rpsD | 34 | 3 | 24.206 | 0.02 ± 0.006 | 30S ribosomal protein S4 | Protein synthesis Cytoplasm |
| 41 | RpsH | MCR_1571 | rpsH | 46 | 4 | 14.381 | 0.06 ± 0.01 | 30S ribosomal protein S8 | Protein synthesis Cytoplasm |
| 42 | GluL | MCR_0967 | glul | 30 | 4 | 34.928 | 0.02 ± 0.04 | Glutamate-riRNA ligase | Protein synthesis Cytoplasm |
| 43 | IcIR | MCR_0785 | icIR | 28 | 2 | 9.769 | 0.09 ± 0.006 | IclR family transcriptional regulator | Regulatory functions Cytoplasm |
| 44 | RpoC | MCR_0258 | rpoC | 34 | 10 | 15.532 | 0.30 ± 0.05 | DNA-directed RNA polymerase subunit beta' | Transcription Cytoplasm |
| 45 | NusG | MCR_0252 | nusG | 38 | 3 | 20.284 | 0.24 ± 0.01 | Transcription termination/antitermination protein NusG | Transcription Cytoplasm |
| 46 | OppB | MCR_1307 | oppB | 23 | 2 | 53.631 | 0.01 ± 0.002 | Oligopeptide ABC transport system permease protein OppB | Transport and binding proteins Inner membrane |
| 47 | OMP M35 | MCR_1247 | mmp | 282 | 21 | 38.028 | 4.87 ± 0.21 | Outer membrane porin M35 | Transport and binding proteins Outer membrane |
| 48 | TbpB | MCR_0694 | tbpB | 73 | 7 | 75.757 | 1.47 ± 0.18 | Transterrin binding protein B TbpB | Transport and binding proteins Outer membrane |
| 49 | HcbP | MCR_1347 | hcbP | 42 | 4 | 18.175 | 0.19 ± 0.03 | Haemerythrin HHE cation binding protein | Unknown function Cytoplasm |
| 50 | OtpA | MCR_1761 | otpA | 175 | 12 | 25.622 | 3.55 ± 0.38 | Opa-like protein A | Unknown function Outer membrane |
| 51 | DSBA | MCR_1827 | dsba | 128 | 10 | 22.953 | 0.01 ± 0.01 | DSBA oxidoreductase | Energy metabolism Periplasm |
| 52 | TprA | MCR_0405 | tprA | 48 | 4 | 33.717 | 0.05 ± 0.004 | Tetraicopeptide repeat family protein | Unknown function Outer membrane |
| 53 | KGW LIP | MCR_1521 | kgw | 120 | 10 | 31.766 | 0.19 ± 0.03 | KGW Leptospira family protein | Unknown function Periplasm |
| 54 | PP-1IP | MCR_1101 | pp-1ip | 20 | 2 | 42.792 | 0.008 ± 0.001 | PP-loop family protein | Unknown function Cytoplasm |
| 55 | pFibP | MCR_1750 | pfibp | 58 | 4 | 27.928 | 0.007 ± 0.002 | Putative phospholipid binding protein | Unknown function Outer membrane |
| 56 | FltP | MCR_0884 | fltP | 22 | 2 | 23.130 | 0.01 ± 0.001 | Fumarylcoacetate hydrolase family protein | Unknown function Cytoplasm |
| 57 | YilO | MCR_1053 | yilO | 64 | 1 | 39.655 | 0.03 ± 0.007 | DNA uptake lipoprotein-like protein | Unknown function Outer membrane |

*Corresponds to the number of peptides identified per protein.
enriched domains are involved in the interaction of OMVs with respiratory epithelial cells, A549 cells were exposed to untreated OMVs, filipin-treated OMVs or formaldehyde-fixed whole bacteria (positive stimulus). After stimulation, A549 cell membranes were isolated by ultracentrifugation, and aliquots of the Triton-insoluble fraction were separated on discontinuous sucrose gradients, and screened by immunoblots using specific antibodies (Fig. 5A). Flotillin and caveolin were used as markers for raft fractions (Dermine et al., 2001). Importantly, TLR2 was found in the raft fractions of cells stimulated with either OMVs or whole bacteria. To confirm the compartmentalization of TLR2 into lipid rafts, we treated epithelial cells with filipin, which intercalates into lipid motifs and thereby disrupts lipid raft structures (Orlandi and Fishman, 1998). The partitioning of TLR2 induced by OMVs was prevented in A549 cells treated with filipin (Fig. 5A). OMVs colocalized with flotillin as soon as 30 min after interaction with alveolar epithelial cells (Fig. 5B). Colocalization of TLR2 and OMVs in A549 cells was also observed (Fig. 5B). The interaction between OMVs and the lipid raft marker (flotillin) or TLR2 was independent of the presence of UspA1 in OMVs as similar patterns were detected using OMVs obtained from the wild-type M. catarrhalis as compared with the Moraxella UspA1-deficient mutant (Fig. 5B). To confirm the internalization of OMVs, A549 cells were incubated with rhodamine-stained OMVs for 16 h. After fixation, permeabilized cells were incubated with rabbit anti-MID antibodies followed by incubation with an Alexa-conjugated secondary pAb. As shown in Fig. 5C, OMVs were localized intracellularly. Thus, Moraxella OMVs can be found intracellularly in epithelial cells and the uptake mechanism is related to lipid rafts.

A pro-inflammatory response is induced by OMVs, and UspA1-bearing OMVs inhibit the activation

To analyse whether the interaction of M. catarrhalis OMVs trigger a pro-inflammatory response, we analysed IL-8 and ICAM-1 expression in A549 epithelial cells. Formaldehyde-fixed M. catarrhalis and a mutant deficient in the UspA1 were included as positive controls, as UspA1 contains a CEACAM1-binding motif that recently has been shown to abrogate the TLR2 driven NFκB response (Slevogt et al., 2008). The OMV exposure induced a significant increase in IL-8 synthesis as compared with non-stimulated control cells (Fig. 6A). The IL-8 concentration reached a maximum after 24 h and did not

Fig. 3. Distribution of the M. catarrhalis OMV proteins identified by MALDI-TOF based on their functional classes. Proteins found were categorized according to their presumed function as indicated in the pie chart (see Table 1 for a complete list of all proteins identified).

Fig. 4. M. catarrhalis OMVs bind to human epithelial cells. The type II alveolar epithelial cell line A549 was incubated for 1 h with OMVs (50 µg ml⁻¹) isolated from M. catarrhalis wild-type (OMV wt) or an UspA1-deficient mutant (OMV ΔuspA1). OMVs were detected using rabbit anti-MID and anti-rabbit Alexa Fluor 488 antibodies (green). Cell nuclei were labelled with DAPI.
change at later time points (data not shown). Furthermore, UspA1-deficient OMVs induced a higher IL-8 secretion than wild-type OMVs. The mitogenic effect of OMVs was concentration-dependent and saturated above 10 μg ml⁻¹ (Fig. 6B). We also incubated Detroit 562 pharyngeal epithelial cells with *Moraxella* OMVs. In parallel with A549, a pro-inflammatory was observed with Detroit 562 (data not shown). To analyse whether the IL-8 protein levels correlated with IL-8 mRNA concentrations, the IL-8 mRNA levels in OMV-stimulated A549 cells were assessed by a semi-quantitative reverse transcription (RT)-PCR. In accordance with the IL-8 measurements, the IL-8 mRNA levels in OMV-stimulated A549 cells were higher compared with unstimulated cells and reached a maximum after 12 h in the epithelial cells stimulated with UspA1-deficient OMVs (Fig. 6C).

We also examined the capacity of *Moraxella* OMVs to stimulate ICAM-1 expression in respiratory epithelial cells. The number of ICAM-1 molecules was significantly higher on A549 cells incubated with OMVs than on unstimulated cells after 6 h of incubation (Fig. 6D). Furthermore, the kinetics of ICAM-1 expression correlated with the levels of IL-8 produced after OMV stimulation. In conclusion, OMVs are strong activators of both IL-8 synthesis and ICAM-1 expression in respiratory epithelial cells.

**OMVs induce a pulmonary inflammation in mice**

To investigate whether OMVs can trigger an inflammatory response *in vivo*, we harvested OMVs from overnight cultures and administered the OMVs into the lungs of mice. After 3 and 6 h lungs were stained with eosin and haematoxylin followed by examination by light microscopy. Interestingly, an increase in exudate was observed, and the lung epithelial surface developed a more ruffled appearance compared with controls receiving PBS only (Fig. 7A–C). To in detail examine the inflammatory response, tissue specimens were also stained with the anti-neutrophil mAb NIMP-R14, which recognizes a to-date-undetermined structure on the murine neutrophil cell membrane. In the lung parenchyma from mice administered with *Moraxella* wild-type OMVs, a clear neutrophil infiltration was observed after 6 h (Fig. 7F), quantified as average neutrophils per field (Fig. 7G). Taken together, in addition to the OMV-dependent pro-inflammatory response seen with epithelial cells *in vitro*, OMVs were highly biologically active in the mouse lung.

**Discussion**

In the present study, we have determined the proteome of *M. catarrhalis* OMVs and have gained new insights in the biological function of these protein-carrying lipid structures. Extracellular OMV proteomes of various Gram-positive and Gram-negative bacterial pathogens have been characterized (Bumann et al., 2002; Nally et al., 2005; Lee et al., 2007; Williams et al., 2007; Galka et al., 2008; Roberts et al., 2008). Both lipid and protein analyses of *M. catarrhalis* OMVs suggest that, as expected, the OMVs are derived from outer membranes. Putative periplasmic and/or peptidoglycan-associated proteins (pFlbp, OppB, Lgt1, pLMc1 and pLMc2) and 12 hypothetical proteins (HP Mc) with unknown functions were also present. These properties are consistent with the characteristics of OMVs recovered from other Gram-negative bacterial species (Beveridge, 1999; Lee et al., 2007; Galka et al., 2008; Roberts et al., 2008).

Several known major OMPs of *M. catarrhalis*, i.e. MID, UspA1, UspA2, TbpB and OlpA were identified in OMVs, of which the autotransporter proteins UspA1 and MID play an important role in adhesion to host cells. Further, the OMPs: J, E, CD, and M35 were detected in OMVs as well as OMP G1b, which shares homology with *Acinetobacter* species proteins associated with resistance against beta-lactam antibiotics (imipenem and carbapenem).

We recently demonstrated that OMVs released by *Moraxella* activate human tonsillar B cells (Perez Vidakovics et al., 2010). Two components of the OMVs, the MID protein and unmethylated CpG-DNA motifs, were found to be critical for B cell activation. Similar to the results obtained with epithelial cells in the present paper, lipid raft motifs were seen in B-cells and seemed to be crucial for the recruitment of receptors and signalling.

*Moraxella catarrhalis* expresses surface receptor proteins involved in iron acquisition pathways that specifically bind host transferrin and lactoferrin. Two of these proteins, TbpB and CopB, were found in OMVs. TbpB is a peripheral outer membrane lipoprotein possessing transferrin-binding properties (Campagnari et al., 1996; Myers et al., 1998). CopB is upregulated under iron limiting conditions and is involved in the binding of iron from transferrin and lactoferrin sources (Aebi et al., 1996). A haem chaperone protein, CcmE, could also be identified in OMVs that is involved in the biogenesis of c-type cytochromes (Ren, Q. et al., 2002). The OMP E protein was predicted to be the FadL homologue in *M. catarrhalis* (de Vries et al., 2010) and could therefore be potentially involved in binding and transport of fatty acids and OMP G1b, which shares homology with several known and hypothetical copper binding lipoproteins (Adlowitz et al., 2004). Interestingly, one protein potentially involved in DNA transport was identified: a DNA uptake lipoprotein-like protein designated YfIO, named after the *Escherichia coli* homologue, which plays an essential role in assembly and targeting of OMPs to the outer membrane (Malinverni et al., 2006). The YfIO homologue in *Neisserial* species, named BarnD/ ComL, is suggested to have a role in periplasmic DNA transport (Volokhina et al., 2009).
The lack of some predicted or known outer membrane proteins in the obtained 2D pattern might be the result of the problematic MS identification of low-molecular-mass proteins, low abundance, or large molecular mass proteins (> 150 kDa), which are commonly missing from 2D maps. High-molecular-weight proteins might have been lost during the separation procedure, either at the point of IPG re-swelling or during IEF by precipitation at the isoelectric point. Additionally, 2D analysis of membrane extracts is a hard task to be accomplished. The hydrophobicity of the proteins turns them refractory to common solubilization protocols and once in the IPG they often precipitate at their pI, reducing their transfer to the second-dimension gel. In our experiments, we included trifluoroethanol (TFE) in the in-gel sample rehydration buffer to improve membrane protein IEF separation. This procedure was previously used in the proteomic analysis of Bordetella pertussis outer membrane fractions (Vidakovics et al., 2007), and in agreement with their results we found a noticeable improvement in the number and resolution of the protein spots.

Gram-negative pathogens have developed type I to VII secretion systems to transport active virulence factors. Secretion of OMVs is thought to be another means by which bacterial pathogens can secrete virulence factors to establish colonization and subsequent infection, eliminating the need for bacterial contact with the host cell. Thus, OMVs have been proposed to be specifically targeted transport vehicles that mediate entry of virulence factors into host cells. The present experiments showed that Moraxella OMVs adhere to human alveolar epithelial cells and then enter via a lipid raft-dependent endocytic pathway. It was previously reported that OMVs released by E. coli undergo pinocytosis via cholesterol-rich lipid rafts (Kesty et al., 2004). Porphyromonas gingivalis, a Gram-negative bacterium that causes periodontitis in humans, also release OMVs that enter human epithelial cells via an endocytic pathway (Furuta et al., 2009). In a recent report, Bomberger et al. demonstrated that Pseudomonas aeruginosa OMVs deliver multiple virulence factors into host airway epithelial cells (Bomberger et al., 2009). In accordance with our results, this occurred via OMV fusion with the host cell lipid raft machinery. Based on our work and previous reports on OMV interaction with host cells, we propose that lipid raft-mediated OMV delivery into host cells likely represent a general strategy utilized by pathogenic Gram-negative bacteria to interact with the host (Kesty et al., 2004; Bomberger et al., 2009; Furuta et al., 2009; Kaparakis et al., 2010).

Outer membrane vesicles can also deliver bacterial factors across host barriers such as mucus layers and provide a mechanism for delivering a concentrated container of virulence factors at the same time. We demonstrate that Moraxella OMVs induce a pro-inflammatory response in epithelial cells with increased IL-8 secretion and ICAM-1 expression, which is expected to be the result of the recruitment of TLR2 to lipid raft domains after OMV binding. A recent study demonstrated the NFkB-dependent pro-inflammatory activities of Helicobacter pylori OMVs in human gastric adenocarcinoma (AGS) and epithelial (HEK239) cell lines. NOD1 dependency but not TLR signalling was observed for these cell responses to OMVs (Kaparakis et al., 2010). In contrast to A549 type II alveolar epithelial cells, neither HEK293 nor AGS possess a functional form of TLR2 (Girardin et al., 2003; Kurt-Jones et al., 2004). NOD1 mRNA as well as TLR2 mRNA and protein were, however, detected in different lung epithelial cells (Slevogt et al., 2007), whereas at least A549 cells expressed little or no TLR4 and responded poorly to LPS (Cowland et al., 2003; Tsutsumi-Ishii and Nagaoka, 2003). Whole M. catarrhalis bacterial cells have the capacity to invade bronchial (BEAS-2B), alveolar (A549) and primary small airway epithelial cells (SAEC) (Slevogt et al., 2007). In this study, Slevogt et al. demonstrated an involvement of both TLR2 and NOD1 in the IL-8 response to M. catarrhalis by gene silencing experiments; knock-down of NOD1 reduced the IL-8 production by approximately 30–35%, whereas a 60–70% reduction was observed by TLR2 silencing. Thus, in parallel to those results, we observed that a TLR2-dependent pathway most likely is induced by Moraxella OMVs. We cannot,
Fig. 6. OMVs binding to human alveolar epithelial cells results in a pro-inflammatory response and a decreased reaction is seen in the presence of UspA1.

A and B. IL-8 production by epithelial cells exposed to OMVs. Cell free supernatants from A549 cells either left unstimulated (control) or stimulated with fixed *M. catarrhalis* (*M. cat.-fixed*), wild-type OMVs (OMV *wt*) or UspA1-deficient OMVs (OMV *ΔuspA1*) were analysed for IL-8 production by ELISA. The data are means (± standard deviations) of at least three independent experiments.

C. IL-8 and GAPDH mRNA analysis of total RNA derived from A549 cells stimulated with *M. catarrhalis* fixed bacteria or OMVs as indicated. The kinetics of activation by the different OMVs was studied at four different time points. The graph represents the ratio of the density of IL-8 relative to GAPDH.

D. Analysis of ICAM-1 expression in A549 cells stimulated with *M. catarrhalis* or the different OMV preparations. Epithelial cells were labelled with FITC-conjugated rabbit anti-human ICAM-1. Thereafter, ICAM-1 density was analysed by flow cytometry. Mean fluorescence intensity (mfi) values are shown.

E. Raw data of one representative experiment of the ones shown in (C). In (C) and (E), total RNA was isolated after 12 h of stimulation and reversely transcribed. Various amounts of resulting cDNA were used in a semi-quantitative IL-8 PCR. GAPDH was included as a control reference gene. For all experiments, data shown are means and standard deviations of at least three independent experiments. *P ≤ 0.05, **P ≤ 0.01, where UspA1-deficient OMVs were compared with the unstimulated control in an unpaired Student's t-test.
however, exclude a potential role also of NOD1 in the activation of epithelial cells.

The potential role of TLR2 in the IL-8 response induced by OMVs was shown in experiments performed with OMVs isolated from a Moraxella UspA1-deficient mutant. A previous study demonstrated that the interaction of CEACAM1 with UspA1 resulted in reduced TLR2-initiated NFκB-dependent inflammatory responses in primary pulmonary epithelial cells (Slevogt et al., 2008). Therefore, after demonstrating the presence of both UspA1 and UspA2 in our wild-type OMVs, we used OMVs from a single UspA1-mutant in subsequent experiments. We found that UspA1-deficient OMVs induced more IL-8 secretion than wild-type OMVs, which corroborates the importance of UspA1 in the inhibition of TLR2-dependent cytokine production.

In this study, we also demonstrated that wild-type OMVs from M. catarrhalis cause inflammation in mouse lung parenchyma. In contrast to experiments with epithelial cells in vitro, no inflammation as quantified by neutrophil influx was observed in mice exposed to UspA1-deficient OMVs (data not shown). Since Moraxella is a human-specific pathogen limitations of the mouse model have to be taken into account. Consequently, the rodents could not accurately be used to compare the inflammatory response with the corresponding reaction in human epithelial cells. However, with this experiment we could clearly confirm that Moraxella OMVs were also stimulatory in vivo, proving the relevance of OMVs from a clinical point of view. Using TEM we have observed released Moraxella OMVs in the nasopharyngeal site of a patient (Tan et al., 2007; Perez Vidakovics et al., 2010), but the ‘physiological’ concentration of OMVs in vivo is presently unknown except for lethal cases of meningococcal disease (Namork and Brandtzaeg, 2002). Most likely Moraxella would release a considerably lower number of OMVs upon infection, in contrast to our mouse experiments with highly purified OMVs.

Collectively, our results show a novel molecular basis for the role of OMVs in M. catarrhalis pathogenesis. The protein delivery of virulence factors by OMVs, the small size of OMVs, which potentially allows interaction with tissue structures not readily accessible to whole bacteria, and the possibility to work as ‘bacterial missiles’ altering both innate and adaptive host responses, support the view that OMVs are an important part of Moraxella pathogenesis.

**Experimental procedures**

*Antibodies and reagents*  
Rabbit anti-MIL902–1200 antiserum was prepared as described earlier (Nordstrom et al., 2002). FITC-conjugated mouse anti-human ICAM-1 mAbs and FITC-conjugated swine anti-rabbit pAbs were purchased from DAKO (Glostrup, Denmark). Anti-human TLR2 mAbs were obtained from Imgenex (San Diego, CA, USA). Mouse anti-human flotillin-2 and mouse anti-human caveolin-1 were purchased from BD Bioscience (San Diego, CA, USA). Filipin III was purchased from Sigma-Aldrich (St Louis, MO, USA). Alexa Fluor 594 goat anti-mouse IgG and ProLong Gold antifade reagent with DAPI (4′, 6-diamidino-2-phenylindole) were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA).
**Bacterial strains and growth conditions**

*Moraxella catarrhalis* wild-type BBH18 and directed BBH18 mutants (previously described in (Mollenkvist et al., 2003; Nordstrom et al., 2004; 2006) were routinely cultured in brain heart infusion (BHI) liquid broth or on BHI agar plates at 37°C. The MID-deficient mutant was cultured in BHI supplemented with 50 μg ml⁻¹ kanamycin. The UspA1-deficient mutant was cultured in BHI supplemented with 1.5 mg ml⁻¹ chloramphenicol (Sigma-Aldrich). Both chloramphenicol (1.5 mg ml⁻¹) and zeocin (7 μg ml⁻¹, Invitrogen) were added to BHI medium for growth of the UspA1/A2 double mutant.

**Isolation of outer membrane vesicles**

Outer membrane vesicles were prepared from overnight cultures according to the Rosen method (Rosen et al., 1995). Briefly, cell free supernatants were filtered through a 0.2 μm-pore size filter (Sartorius, Epson, UK) and concentrated using 100 kDa Vivaspin centrifugal concentrators (Vivascience, Hannover, Germany). The concentrated supernatants were thereafter centrifuged at 100 000 g for 60 min. The pellet containing OMVs were washed three times in phosphate-buffered saline (PBS) followed by a final sterile filtration to definitely exclude cellular contamination from the parent OMV-producing *M. catarrhalis*. Protein content was determined by spectrophotometry using NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). The purity of OMV samples was examined by transmission electron microscopy (TEM) and by excluding bacterial growth of any remaining parent cells on BHI agar.

**DNA analysis in OMVs**

DNA associated with OMVs was quantified by spectrophotometry using NanoDrop. Briefly, *Moraxella* OMV samples (20 μg protein) were lysed by incubation in 0.5% (v/v) Sarkosyl, 100 mM EDTA lysis solution to release DNA from OMVs. DNA in OMV samples was also visualized by electrophoresis in agarose gel. Whole cell genomic DNA was included as a positive control.

**Lipo-oligosaccharide analysis in OMVs**

Lipo-oligosaccharide (LOS) from OMVs were solubilized in Laemmli sample buffer (Laemmli, 1970) and heated at 100°C for 10 min. Proteinase K (50 μg ml⁻¹) were added per 50 μg of OMV proteins and incubated in a water bath at 60°C for 1 h with occasional vortexing. The presence of LOS in Proteinase K-treated OMV samples was analysed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by visualization with a sensitive silver stain method as described (Tsai and Frasch, 1982).

The composition of LOS in OMVs was analysed by a gas chromatography/ion trap mass spectrometry (GC/MS) method. Briefly, OMV samples, in Teflon-lined glass test tubes, were dried, heated in 1 ml of 2 M methanolic HCl at 85°C overnight, and extracted with 1.5 ml of water: n-heptane (1:2 v/v). The heptane (upper) layer was evaporated under a stream of nitrogen at room temperature, redissolved in 1 ml of heptane : dichloromethane (1:1 v/v), and purified using a disposable silica gel column (100 mg). Prior to use the silica gel column was washed twice with 1 ml of diethyl ether and twice with 1 ml of heptane : dichloromethane, thereafter the methyl ester-containing mixture was added. Heptane : dichloromethane (2 ml) was added to the column to elute the non-hydroxylated fatty acid esters, the eluate was discarded. Diethyl ether (2 ml) was then added to the column to elute the hydroxy fatty acid esters; the eluate was evaporated at room temperature. Trimethylsilyl (TMS) derivatives of the hydroxy fatty acid esters were prepared by adding N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) (50 μl) and pyridine (5 μl) followed by heating for 20 min at 80°C. Heptane (50 μl) was then added. The preparations were analysed following storage at 4°C overnight (Sebastian and Larsson, 2003).

A Saturn 2000 ion-trap gas chromatography-mass spectrometry (GC/MS) instrument (Varian, Palo Alto, CA, USA) equipped with a fused-silica capillary column (CP-Sil 8 CB low bleed, 0.25 μm film thickness, 30 m × 0.25 mm i.d.) (Chrompack, Middelburg, the Netherlands) was used. Volumes of 2 μl were injected in the splitless mode with helium head column pressure of 69 kPa using a Combi Pal autosampler (CTC Analytics, Zwingen, Switzerland). The temperature of the column was programmed from 90–280°C at 20°C min⁻¹; the temperature of injector was 280°C and that of transfer line (between GC and MS system) was 290°C. The ion trap temperature was 180°C. All analyses were made in the electron impact (EI) mode (Sebastian and Larsson, 2003). Mass spectra of the methyl ester/TMS 3-OH FA derivatives show abundant ions of m/z (M-15), due to loss of a CH₃ group, and m/z 175, due to cleavage of C3-C4 linkage. The derivative acids were measured by monitoring m/z 131 (a product ion of m/z 175) in GC-tandem MA (GC/MS/MS) (Sebastian and Larsson, 2003).

**SDS-PAGE and Western blots**

The protein content of OMVs was analysed on a 6% and 10% SDS-PAGE stained with Bio-Rad Silver Stain Plus kit (Munich, Germany). Proteins were transferred at 20 V overnight to an Immobilon-P membrane (Millipore, Bedford, MA, USA). After transfer, the membranes were blocked for 2 h using PBS with 0.1% Tween (PBS-Tween) and 5% skim milk powder. After several washes with PBS-Tween, the membrane was incubated with a rabbit anti-MID961-1020 antisera (described or described as a rabbit anti-UspA antisera (Nordstrom et al., 2002; 2006). Repeated washes with PBS-Tween were followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit pAbs (Dakopatts, Copenhagen, Denmark) for 1 h. The transferred proteins were detected using ECL Western blot detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden) and performed with the Quantity One densitometry software from Bio-Rad.

**2D gel electrophoresis**

For isoelectric focusing (IEF), precast 18 cm pH 4–7 IPG gels (Immobiline DryStrips, Amersham Biosciences, Uppsala, Sweden) were used. OMV samples were solubilized during 1 h in 1 ml of TFE-rehydration buffer (Deshusses et al., 2003) [5 M urea, 2 M thiourea, 50% (v/v) 2,2,2-Trifluoroethanol (TFE, 99.0%; Fluka, Buchs, Switzerland), 2% (w/v) Triton X-100, 65 mM DTT, 0.5% (v/v) Pharmalyte pH 4–7 or pH 6–11 (Amersham...
et al by in-gel sample rehydration as previously described (Sanchez et al., 1999). IEF was performed in an IPGphor II electrophoresis unit (Amersham Biosciences) using the following program: 500 V for 0.01 h (1 Vh), 3500 V for 1.30 h (gradient, 3000 Vh), 3500 V for 5.40 h (20 kVh), resulting in a total voltage of 23 kVh. Focused strips were either used immediately for the second dimension or were stored at −80°C until use. After IEF, IPG strips were soaked (15 min) in 10 ml of equilibration buffer [50 mM Tris-Cl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 20 mM DTT, and 0.002% (w/v) bromophenol blue] followed by incubation (15 min) in the same solution but replacing DTT for 20 mM iodoacetamide. After the reduction/alquilation step, the incubation (15 min) in the same solution but replacing DTT for 20 mM DTT, and 0.002% (w/v) bromophenol blue] followed by Tris-Cl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 20 mM iodoacetamide. After the reduction/alquilation step, the IPG strips were placed on the top of a 10% SDS-PAGE and sealed with 0.5% agarose in electrophoresis buffer. Electrophoresis was performed at a constant current (25 mAmp per gel) at 20°C until the dye front reached the lower end of the gel, using a PROTEAN II 2D cell electrophoresis unit (Bio-Rad, Hercules, CA, USA) connected to a Multitemp II cooling bath (GE Healthcare). Proteins were routinely visualized by silver staining (Blum et al., 1987) for image analysis and alternatively by Coomassie blue staining (Gorg et al., 1988) for spots to be submitted to tryptic digestions and MALDI-TOF-MS.

Identification of protein spots by MALDI-TOF MS

Protein identification was performed at the Proteomic Service Facility from Universidad Complutense de Madrid-Parque Cientifico de Madrid, Spain (UCM-PCM). Briefly, Coomassie stained spots were manually excised and washed twice with double-distilled water. Samples were then reduced with 10 mM DTT in 25 mM ammonium bicarbonate for 30 min at 56°C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Finally, samples were digested with 12.5 ng ml⁻¹ sequencing grade trypsin (Roche Molecular Biochemicals, Mannheim, Germany) in 25 mM ammonium bicarbonate (pH 8.5) overnight at 37°C. After digestion, the supernatants were collected and 1 μl was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.5 μl of a 3 mg ml⁻¹ of CHCA matrix (Sigma) in 50% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) was added to the dried peptide digested spots and allowed to air-dry again.

The MS analyses were performed in a MALDI-TOF/TOF spectrometer 4700 Proteomics Analyzer (PerSeptives Biosystems, Framingham, MA, USA). The instrument was operated in reflector positive ion mode, with an accelerating voltage of 20 000 V. All mass spectra were internally calibrated using auto-digested trypsin peptides. Proteins that could not be identified with certainty by peptide mass fingerprinting were subjected to MS/MS sequencing analyses. Fragmentation was carried out using the acquisition method 1 kV ion reflector mode CID on and precursor mass window ± 10 Da. The resulting peptide mass fingerprints and MALDI TOF/TOF fragmentation spectra were analysed using a M. catarrhalis RH4 protein database (de Vries et al., 2010) with the MASCOT search engine (Matrix Science) and the genome of M. catarrhalis (de Vries et al., 2010).

Epithelial cells

Human alveolar (A549) and pharyngeal (Detroit 562) epithelial cells were grown in DMEM supplemented with 10% FCS and 50 μg ml⁻¹ gentamicin at 37°C and incubated at a 90% humidity and 5% CO₂. The cells were allowed to grow until confluency and were trypsinized and seeded in multi-well cell culture plates or eight-well chamber slide system (Lab-Tek II; Thermo Fisher Scientific, Roskilde, Denmark) for microscopy experiment.

Isolation of lipid rafts

A modification of the method of Brown and Rose (1992) was used to isolate lipid rafts. A549 cells were exposed to M. catarrhalis (1 × 10⁷ cfu ml⁻¹) or OMVs (10 μg ml⁻¹) for 30 min and/or filipin 20 μg ml⁻¹ for 30 min prior to stimulation. The cells were washed with ice cold PBS and lysed with 0.5% Triton X-100 in TNE buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl and 5 mM EDTA) plus protease inhibitors for 20 min on ice. Lysed cells were harvested and homogenized with a loose-fitting followed by a tight-fitting Dounce homogenizer. Whole cells and nuclei were removed by centrifugation at 1000 g for 10 min. An equal volume of 90% sucrose in TNE buffer was added to the supernatant. This 45% layer was overlaid with 30% and 5% sucrose in TNE buffer to form a discontinuous gradient. Samples were centrifuged at 200 000 g for 18 h at 4°C followed by collection of 1 ml fractions. Protein concentrations were determined using a NanoDrop and 5 μg was analysed on a 12% SDS-PAGE. After transfer to PVDF Immobilon-P (Millipore), blots were incubated in 5% skim milk blocking solution for 1 h at room temperature. Membranes were incubated overnight at 4°C and in the presence of mouse mAbs against human flotillin-1, caveolin or TLR2. After several washes in PBS-Tween, HRP-conjugated anti-mouse IgG (DAKO) and Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) were used for visualization.

Flow cytometry analysis

Surface expression of ICAM-1 after addition of OMVs was monitored using flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). A549 cells were incubated in complete medium with different concentrations of OMVs ranging from 0.625–10 μg ml⁻¹ and analysed for ICAM-1 expression at various time points (0–24 h). Harvested cells were washed twice and incubated in PBS containing 2% BSA with FITC-conjugated rabbit anti-human ICAM-1 mAb for 1 h on ice. After several washes in PBS, A549 cells were screened for ICAM-1 density using flow cytometry.

IL-8 enzyme-linked immunosorbent assay (ELISA)

To analyse interleukin-8 (IL-8) production, 1 × 10⁵ A549 cells were cultured in 12-well flat-bottom plates (Nunc-Immuno Module) with or without 10 μg ml⁻¹ OMVs. The cell-free supernatant was harvested after 24 h. IL-8 production was determined using a commercial ELISA from R&D systems (Minneapolis, MN, USA).

Fluorescence, confocal and transmission electron microscopy

For fluorescence and confocal microscopy, A549 cells were grown in eight-well chamber slide system (Lab-Tek II). Slides...
were thereafter incubated with OMVs (50 μg ml⁻¹) in DMEM complete medium for 1 h at 37°C or 18 h at 37°C for internalization experiments (Kaparakis et al., 2010). After several washes with ice cold PBS, cells were fixed with 4% paraformaldehyde solution for 10 min at room temperature, washed and stained with 40,6-diamidino-2-phenylindole (DAPI). Following incubation with 5% normal serum blocking solution (20 min at room temperature), rabbit anti-MID pAbs were added for 1 h at room temperature. After several washes, Alexa Fluor-conjugated secondary antibodies were added in the dark for 1 h at room temperature. For intracellular OMV staining, cells pre-incubated with rhodamine-labelled OMVs were fixed and incubated in permeabilization buffer (0.03% Triton X-100 and 5% normal serum blocking solution in PBS) before adding rabbit anti-MID pAbs. Control cells were fixed and incubated with 5% normal serum blocking solution without Triton X-100. Alexa Fluor-conjugated secondary antibodies were added in the dark for 1 h at room temperature. After washing twice with PBS, cells were fixed to the glass surface with DAPI containing Prolong Gold antifade reagent overnight and examined by immunofluorescence microscopy or by confocal microscopy using a Bio-Rad Radiance 2000 confocal system fitted on a Nikon microscope with a ×60/NA 1.40 oil lens. A549 cells showing polarization of lipid rafts were counted by microscopic examination of 25 randomly selected fields, showing a minimum of three cells per field.

For electron microscopy, OMV samples were fixed in 0.15 M sodium cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde for 1 day at room temperature. They were rinsed in isotonic cacodylate buffer, dehydrated in grades of ethanol, embedded in Epon 812. Thin sections were cut then rinsed in isotonic cacodylate buffer, dehydrated in graded series of ethanol, embedded in Epon 812. Thin sections were cut on a Leica Ultracut S (Deerfield, IL, USA). Sections were poststained with 5% uranyl acetate for 2 h and then in a saturated lead citrate solution for 7 min. Specimens were observed in a Jeol JEM 1230 electron microscope (JEOL, Tokyo, Japan) operated at 60 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera (Gatan, Pleasanton, CA, USA).

RNA extraction and semi-quantitative RT-PCR analysis

Extraction of total RNA was performed by use of Trizol reagent (Invitrogen, CA, USA) according to manufacturer’s instructions. The samples were thereafter treated with the amplification grade DNasel (Sigma) solution. To determine residual DNA contamination of the RNA extracts, 1 μl samples of the DNase-treated RNA samples were used as templates for PCR reactions without previous reverse transcription. All devices and solutions were treated or prepared with diethyl pyrocarbonate (DEPC) to inactive RNases. The quantity and purity of the total RNA were determined by spectrophotometry (NanoDrop, NanoDrop Technologies) using the A260/A280 ratio. One microgram of RNA was reverse transcribed using AMV reverse transcriptase (Roche) according to the manufacturer’s instructions. The cDNA yield of each sample of the DNase-treated RNA was determined by spectrophotometry (NanoDrop, NanoDrop Technologies). The cDNA was amplified using specific amplification primers (5′-CACCACCATGGAGAAGGGCTGG-3′ and 5′-GACCTGGTGTAGAAGTTT-3′) and IL-8 specific primers (5′-ATGACCTCCAAGCTGCGTAT-3′ and 5′-GGAGATTGCTTTATGACCTGAT-3′), according to the following thermal cycling protocol: 95°C for 3 min, 95°C 45 s, 53°C 1 min, 72°C 2 min (30 cycles), 72°C 8 min. The PCR products were analysed on 1.5% agarose gels, stained with ethidium bromide and subsequently visualized. The intensities of the final IL-8 RT-PCR products were quantified by using density scanning analysis on each band relative to the corresponding GAPDH band.

Mouse pulmonary inflammation model

Female BALB/c mice (8–10 weeks old) were used and experiments were performed in accordance with guidelines of the Swedish National Board for Laboratory Animals at Lund University, Lund, Sweden. Anesthetized mice were challenged with 50 μg OMV proteins in 50 μl PBS. The suspension was administered into both nostrils. Control mice were correspondingly administered 50 μl PBS only. Mice were euthanized, lungs were removed and fixed with 10% formalin in PBS at 3 h (3 mice in each group) and 6 h (2 mice in each group). Several lung specimens from each mouse were embedded in paraffin, sectioned and stained with eosin and haematoxylin or with a rat anti-mouse neutrophil mAb (NIMP-R14; Abcam, Cambridge, MA, USA) and examined for signs of inflammation. Neutrophils were counted in three random microscopy fields, and the average count per field is reported. Significant values were defined as *P ≤ 0.05; **P ≤ 0.01. All data are expressed as mean ± SEM, and n corresponds to the number of experiments performed.

Statistical analysis

The Student’s t-test was used to determine statistical differences for unpaired comparisons with Welch correction if variances were non-homogenous. Significant values were defined as *P ≤ 0.05; **P ≤ 0.01. All data are expressed as mean ± SEM, and n corresponds to the number of experiments performed.

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