International Journal of Medical Microbiology 308 (2018) 882–889

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Enterohemorrhagic *Escherichia coli* O157 outer membrane vesicles induce interleukin 8 production in human intestinal epithelial cells by signaling via Toll-like receptors TLR4 and TLR5 and activation of the nuclear factor NF-κB

Martina Bielaszewska[a,b,*,1], Monika Marejková[b,1], Andreas Bauwens[a], Lisa Kunsmann-Prokscha[a], Alexander Mellmann[a,c], Helge Karch[a,c]

[a] Institute for Hygiene and the National Consulting Laboratory for Hemolytic Uremic Syndrome, University of Münster, Münster, 48149, Germany
[b] National Institute of Public Health, National Reference Laboratory for E. coli and Shigella, Prague, 100 42, Czech Republic
[c] Interdisciplinary Center for Clinical Research (IZKF), University of Münster, Münster, 48149, Germany

ARTICLE INFO

Keywords:
Enterohemorrhagic *Escherichia coli* O157
Outer membrane vesicles
Immunomodulation
Proinflammatory cytokines
Flagellin
Lipopolysaccharide

ABSTRACT

Proinflammatory cytokines play important roles in the pathogenesis of diseases caused by enterohemorrhagic *Escherichia coli* (EHEC) O157, but the spectrum of bacterial components involved in the proinflammatory responses is not fully understood. Here, we investigated the abilities of outer membrane vesicles (OMVs), nanoparticles released by EHEC O157 during growth, to induce production of proinflammatory cytokines in human intestinal epithelial cells. OMVs from both EHEC O157:H7 and sorbitol-fermenting (SF) EHEC O157:H$^-$ induced production of interleukin-8 (IL-8) in Caco-2, HCT-8, and HT-29 intestinal epithelial cell lines. H7 flagellin was the key IL-8-inducing component of EHEC O157:H7 OMVs, whereas cytolethal distending toxin V and O157 lipopolysaccharide (LPS) largely contributed to IL-8 production elicited by flagellin-lacking OMVs from SF EHEC O157:H$^-$. The H7 flagellin-mediated signaling via Toll-like receptor (TLR) 5, and O157 LPS-mediated signaling via TLR4/MD-2 complex, which were followed by activation of the nuclear factor NF-κB were major pathways underlying IL-8 production induced by EHEC O157 OMVs. The proinflammatory and immunomodulatory capacities of EHEC O157 OMVs have pathogenetic implications and support the OMVs as suitable vaccine candidates.

1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) strains of serotypes O157:H7/H$^-$ are major causes of diarrhea-associated hemolytic uremic syndrome (D+ HUS), which affects mostly children under 5 years and is a leading cause of acute renal failure in childhood (Siegler, 2003; Tarr et al., 2005). Classical non-sorbitol-fermenting (NSF) EHEC O157:H7 strains cause D+ HUS worldwide (Karch et al., 2005; Tarr et al., 2005), whereas sorbitol-fermenting (SF) EHEC O157:H$^-$ (non-motile) strains have emerged as causes of D + HUS in Europe (Buvens et al., 2009; King et al., 2014; Marejková et al., 2013; Mellmann et al., 2008; Orth et al., 2009; Pollock et al., 2010; Vygen-Bonnet et al., 2017). Like other pathogens, EHEC O157 secrete outer membrane vesicles (OMVs), nanoparticles composed of outer membrane, periplasmic, and cytoplasmic components (Bielaszewska et al., 2017). EHEC O157 OMVs carry a cocktail of EHEC O157 virulence factors including Shiga toxin 2a (Stx2a), cytolethal distending toxin V (CdtV), EHEC hemolysin (EHEC-Hly), and flagellin (Bielaszewska et al., 2017; Kolling and Matthews, 1999). The OMVs are internalized by human intestinal epithelial and microvascular endothelial cells, which are the major targets during D + HUS, and deliver the virulence factors intracellularly, hereby causing cell death (Bielaszewska et al., 2017).

OMVs produced by various pathogens exhibit immunomodulatory effects which result from the presence of pathogen-associated microbial patterns (PAMPs), such as lipopolysaccharide (LPS), peptidoglycan, flagellin, and outer membrane porins, within OMVs (reviewed in Ellis and Kuehn, 2010; Kaparakis-Liaskos and Ferrero, 2015). Recognition of the OMV-associated PAMPs by the host pattern recognition receptors such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-containing proteins (NOD1 and NOD2) triggers signaling cascades that ultimately lead to the production of proinflammatory cytokines and chemokines, the key players in innate immunity and modulators of adaptive immune responses (Kaparakis-Liaskos and Ferrero, 2015).

* Corresponding author at: National Institute of Public Health, Šrobárova 48, 100 42, Prague, Czech Republic.
E-mail address: martina.bielaszewska@szu.cz (M. Bielaszewska).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.ijmm.2018.06.004
Received 30 April 2018; Received in revised form 26 May 2018; Accepted 16 June 2018

Proinflammatory cytokines play multiple roles in the pathogenesis of D+ HUS (Proulx et al., 2001; Zoja et al., 2010). Patients with D + HUS have increased circulating levels of proinflammatory cytokines compared to patients with uncomplicated EHEC diarrhea (Proulx et al., 2001; Westerholt et al., 2000). High levels of interleukin 8 (IL-8) have been identified as a risk factor for developing D+ HUS (Fitzpatrick et al., 1992; Westerholt et al., 2000) and have been associated with high counts of polymorphonuclear leukocytes (Fitzpatrick et al., 1992), a predictor of a poor disease outcome (Walters et al., 1989). Hence, the ability of EHEC to induce proinflammatory cytokine responses, in particular in intestinal epithelial cells (IECs), which are the first cellular targets encountered by the pathogens during infection, may be a critical step in the pathogenesis of D+ HUS. EHEC O157 bacteria, as well as single bacterial components including purified virulence proteins and O157 LPS, are capable of inducing secretion of proinflammatory cytokines from human intestinal epithelial and other cells (Berin et al., 2002; Guessous et al., 2005; Jandhyala et al., 2010; Miyamoto et al., 2006; Thorpe et al., 1999; Zhang et al., 2012). However, the proinflammatory potentials of EHEC O157 OMVs remain unknown. The aim of this study was to investigate the abilities of EHEC O157 OMVs to induce production of proinflammatory cytokines in human IECs using Caco-2, HCT-8, and HT-29 cell lines as models. Furthermore, we identified OMV components involved in these proinflammatory responses and the underlying signaling pathways.

2. Materials and methods

2.1. Isolation and purification of OMVs and OMV doses used in experiments

OMVs were isolated from EHEC O157 strains 5791/99 (NSF O157:H7 from D+ HUS patient), 493/89 (SF O157:H$^-$ from D+ HUS patient), and 493/89Δ*stx*$_{2a}$ (a spontaneous *stx*$_{2a}$-negative derivative of 493/89), and purified with OptiPrep (Sigma-Aldrich, Taufkirchen, Germany) density gradient ultracentrifugation as described previously (Bielaszewska et al., 2017). The total protein concentrations in purified OMVs were determined with Roti-Nanoquant reagent (Carl Roth, Karlsruhe, Germany), concentrations of LPS with LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific, Bonn, Germany), and concentrations of virulence factors (Stx2a, EHEC-Hly, CdtV, and H7 flagellin) using calibration curves generated from purified proteins (Bielaszewska et al., 2017). The spectra of OMV-associated virulence factors and the OMV concentrations of total proteins, virulence factors, and LPS used in experiments are shown in Table 1. OMVs from strain TA153 (*E. coli* MC1061 harboring *cdt*V operon from strain 493/89 in SuperCos I) and the respective vector control TA154 (*E. coli* MC1061 harboring SuperCos I) were described earlier (Bielaszewska et al., 2017).

2.2. Purified O157 LPS, H7 flagellin, Stx2a and EHEC-Hly

Purified O157 LPS was purchased from BioTrend Chemikalien (Cologne, Germany). H7 flagellin was isolated from strain 5791/99 by the procedure described for H4 flagellin (Kunsmann et al., 2015). Purified Stx2a and EHEC-Hly were prepared as described earlier (Aldick et al., 2007; Bauwens et al., 2011) and biological activities of the preparations were confirmed by Vero cell assay (Bauwens et al., 2011) and a microtiter hemolytic assay (Aldick et al., 2007). Protein concentrations in purified H7 flagellin, Stx2a, and EHEC-Hly were 550 µg/ml, 2.8 mg/ml, and 30.5 µg/ml, respectively.

2.3. Antibodies and inhibitors

Antibody against *E. coli* H7 flagellin (prepared in rabbits) was described previously (Bielaszewska et al., 2017). Anti-TLR4 neutralizing antibody, anti-TLR5 neutralizing antibody, and the respective rat IgG control were from InvivoGen (San Diego, CA, USA). Phospho-NF-κB p65 (Ser536) antibody (rabbit) was from Cell Signaling Technology (Leiden, The Netherlands), and anti-nuclear matrix protein p84 antibody (rabbit) was from Abcam (Cambridge, UK). Alkaline-phosphatase-conjugated goat anti-rabbit IgG was from Dianova (Hamburg, Germany). Polymyxin B was from Sigma-Aldrich, and NF-κB inhibitors SN50 [6-amino-4-(4-phenoxyphenylethylamino) quinazoline] and TPCK (N-α-p-tosyl-l-phenylalanine chloromethyl ketone) were from Calbiochem (San Diego, CA, USA) and Sigma-Aldrich, respectively.

2.4. Cell cultures

Human colonic adenocarcinoma cell lines Caco-2 and HT-29 (ACC 169 and ACC 299, respectively), and human colorectal adenocarcinoma cell line HCT-8 (ATCC CCL-244) were used in passages 24 to 30, 17 to 22, and 10 to 19, respectively. Caco-2 cell line was cultured in Eagle minimal essential medium with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 1% nonessential amino acids. HCT-8 cell line was cultured in RPMI 1640 with 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate, and HT-29 cell line in McCoy's 5 A with 2 mM L-glutamine and 10% FCS. Cell culture media and reagents were from Lonza (Cologne, Germany).

2.5. Cytokine production induced by OMVs

Caco-2, HCT-8, and HT-29 cells were grown until confluence in 96-well plates and incubated for 24 h with purified OMVs from strains 5791/99, 493/89, or 493/89Δ*stx*$_{2a}$ (for OMV concentrations of the total proteins, virulence factors, and LPS see Table 1). Cell culture supernatants were analyzed for the presence of 12 cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 A, interferon-γ, tumor necrosis factor-α, and granulocyte macrophage colony-stimulating factor) using

Table 1
Origins of OMVs and concentrations of OMV-associated total proteins, virulence factors, and LPS used in experiments.

OMVs from strain	Strain characteristics[a]	Relevant components (ng/ml) in OMVs used for experiments[b]					
		Total protein	Stx2a	CdtV	EHEC-Hly	H7 flagellin	O157 LPS
5791/99	O157:H7 (NSF)	450	51	38	2	73	962
493/89	O157:H$^-$ (SF)	450	51	38	0	0	965
493/89Δ*stx*$_{2a}$	O157:H$^-$ (SF)	450	0	38	0	0	965
TA153	*E. coli* MC1061 harboring *cdt*V-*ABC*$_{493/89}$ in SuperCos I	398	0	38	0	0	0
TA154	*E. coli* MC1061 harboring SuperCos I	365	0	0	0	0	0

[a] NSF, non-sorbitol-fermenting; SF, sorbitol-fermenting; H$^-$, nonmotile.
[b] Stx2a, Shiga toxin 2a; CdtV, cytolethal distending toxin V; EHEC-Hly, EHEC hemolysin; LPS, lipopolysaccharide.

the Multi-Analyte ELISArray Kit (Qiagen, Hilden, Germany).

2.6. Kinetics of IL-8 production induced by OMVs and contributions of various OMV components to OMV-mediated IL-8 production

Caco-2, HCT-8, and HT-29 cells grown in 96-well plates were incubated with OMVs 5791/99, 493/89 or 493/89Δ*stx*$_{2a}$ (for concentrations of the relevant components see Table 1) or with purified H7 flagellin (73 ng/ml), Stx2a (51 ng/ml), EHEC-Hly (2 ng/ml) or O157 LPS (965 ng/ml) for 15 min, 1 h, 3 h, and 24 h. IL-8 production was quantified at each time point using the Single-Analyte IL-8 ELISArray Kit (Qiagen) according to the manufacturer´s instructions. Since CdtV occurs solely as an OMV-associated protein (Bielaszewska et al., 2017), we used for these experiments recombinant CdtV from strain 493/89 carried by OMVs TA153 (Table 1). The net effect of CdtV on IL-8 production was determined by subtracting the background IL-8 amounts induced by vector control (TA154) OMVs from those induced by TA153 OMVs.

2.7. Dose-dependence of IL-8 production elicited by EHEC O157 OMVs

To determine the minimum OMV doses capable of eliciting IL-8 production, Caco-2, HCT-8 and HT-29 cells grown as above were exposed for 24 h to 10-fold OMV dilutions containing H7 flagellin (5791/99 OMVs), O157 LPS, and CdtV (all OMVs) in the doses ranging from 73 ng/ml, 962 ng/ml, and 38 ng/ml, respectively, to 0.73 ng/ml, 9.62 ng/ml, and 0.38 ng/ml, respectively. IL-8 was quantified with Single-Analyte IL-8 ELISArray Kit (detection limit 16.5 pg/ml).

2.8. OMV-mediated signaling

To analyze the role of H7 flagellin-TLR5 signaling in EHEC O157 OMV-mediated IL-8 response, IL-8 secretion was quantified (Single-Analyte IL-8 ELISArray Kit) in Caco-2, HCT-8, and HT-29 cells exposed for 24 h to: (i) OMVs or purified H7 flagellin (73 ng/ml) in the absence of antibodies; (ii) OMVs or H7 flagellin which had been preincubated with rabbit anti-H7 antibody (50 μg/ml, 1 h, 37 °C); (iii) OMVs or H7 flagellin after the cell preincubation with rat anti-TLR5 neutralizing antibody (60 μg/ml, 1 h, 37 °C); (iv) OMVs or H7 flagellin which had been preincubated with anti-H7 antibody after the cell pretreatment with anti-TLR5 antibody. OMVs or H7 flagellin preincubated with preimmune rabbit serum (50 μg/ml, 1 h, 37 °C) and cells preincubated with preimmune rat IgG (60 μg/ml, 1 h, 37 °C) were used to control the specificities of the antibody-mediated inhibitory effects. To analyze the role of O157 LPS-TLR4/MD-2 signalling in EHEC O157 OMV-mediated IL-8 response, IL-8 secretion was quantified in Caco-2, HCT-8, and HT-29 cells exposed for 24 h to: (i) OMVs or isolated O157 LPS (965 ng/ml) in the absence of any pretreatment; (ii) OMVs or O157 LPS which had been preincubated (1 h, room temperature) with polymyxin B (100 μg/ml); (iii) OMVs or O157 LPS after the cell preincubation with rat anti-TLR4 neutralizing antibody (60 μg/ml, 1 h, 37 °C). Polymyxin B (100 μg/ml) added to cells without OMVs and cells preincubated with preimmune rat IgG (60 μg/ml, 1 h, 37 °C) were used as inhibition specificity controls.

To determine activation (i.e., nuclear translocation and phosphorylation) of NF-κB, HT-29 cells grown until confluence were incubated for 30 min to 3 h with OMVs (for concentrations of relevant components see Table 1) or with purified H7 flagellin (73 ng/ml) or O157 LPS (965 ng/ml) used as positive controls (Cario et al., 2000; Miyamoto et al., 2006), or with OMV buffer (20 mM TRIS − HCl, pH 8.0) or cell culture medium (negative controls). Nuclear extracts were prepared with the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA), and 50 μg of protein/lane were analyzed by immunoblot (Bielaszewska et al., 2017) with antibody against phosphorylated p65 NF-κB protein or nuclear matrix protein p84 (loading control) and alkaline-phosphatase-conjugated goat anti-rabbit IgG secondary antibody. Signals were developed with NBT/BCIP substrate (Roche, Mannheim, Germany), visualized with Chemi Doc XRS imager (BioRad, Munich, Germany), and quantified by densitometry (Quantity One, BioRad). To inhibit NF-κB activation, Caco-2, HCT-8, and HT-29 cells were preincubated with NF-κB inhibitors SN50 (20 μM) and/or TPCK (50 μM) for 30 min before adding samples, and incubated with the samples in the presence of inhibitors for 24 h. IL-8 production was measured with Single-Analyte IL-8 ELISArray Kit.

2.9. Statistical analysis

Data were analyzed with one-way ANOVA (analysis of variance). *P* values < 0.05 were considered significant.

3. Results

3.1. EHEC O157 OMVs induce IL-8 production in human intestinal epithelial cells

Screening for 12 cytokines (see Materials and Methods) in supernatants of Caco-2, HCT-8, and HT-29 cells, which were exposed for 24 h to OMVs from EHEC O157 strains 5791/99, 493/89, or 493/89Δ*stx*$_{2a}$ demonstrated that all cell lines produced IL-8 in response to each OMV preparation. Analyses of the kinetics of the IL-8 responses demonstrated that IL-8 production rapidly increased between 15 min and 1 h in HCT-8 cells, and between 15 min and 3 h in Caco-2 and HT-29 cells, and further steadily increased until 24 h (Fig. 1A). In each cell line, the IL-8 amount elicited by 5791/99 OMVs (containing 73 ng/ml of flagellin) was significantly higher after 24 h (and in HCT-8 and HT-29 cells also after 1 h and/or 3 h) than those elicited by flagellin-negative OMVs 493/89 and 493/89Δ*stx*$_{2a}$, respectively (Fig. 1A). The latter two OMV preparations caused similar IL-8 responses in each cell line (Fig. 1A), though OMVs from strain 493/89 contain Stx2a (51 ng/ml), whereas OMVs from strain 493/89Δ*stx*$_{2a}$ lack Stx2a (Table 1). These data suggested that H7 flagellin substantially contributes to EHEC O157 OMV-mediated IL-8 production, whereas Stx2a is dispensable for this process.

3.2. Contributions of O157 OMV components to IL-8 responses induced by OMVs

To determine the contributions of OMV-associated virulence proteins and LPS to IL-8 production induced by EHEC O157 OMVs, we compared the kinetics and magnitudes of IL-8 responses elicited by OMVs with those elicited by purified H7 flagellin, O157 LPS, Stx2a, EHEC-Hly, and recombinant OMV-carried CdtV, which were applied to the cells in the same amounts as are those present in O157 OMVs (Table 1). H7 flagellin (73 ng/ml) induced IL-8 production in each cell line with a similar kinetics as did the OMV preparations (Fig. 1B-D). The IL-8 amounts elicited by purified H7 flagellin were slightly, but not significantly, higher than those elicited by flagellin-containing OMVs 5791/99, but were significantly higher than those elicited by flagellin-lacking OMVs 493/89 and 493/89Δ*stx*$_{2a}$, respectively (Fig. 1B-D). Purified O157 LPS (965 ng/ml) caused a steadily increasing IL-8 production in each cell line (Fig. 1B-D). The IL-8 amounts induced by O157 LPS were, except for Caco-2 cells, similar to those induced by OMVs 493/89 and 493/89Δ*stx*$_{2a}$, in particular after 24 h (Fig. 1B-D), but were significantly lower than those induced by OMVs 5791/99 (Fig. 1B-D). Likewise, recombinant CdtV (38 ng/ml) elicited similar IL-8 amounts as did OMVs 493/89 and 493/89Δ*stx*$_{2a}$, but significantly less IL-8 than did OMVs 5791/99 (Fig. 1B-D). In contrast to H7 flagellin, O157 LPS, and CdtV, purified Stx2a induced a minimum IL-8 production (< 60 pg/ml) and EHEC-Hly no measurable IL-8 production in each cell line (Fig. 1B-D). Altogether, these data demonstrated that H7 flagellin is a major IL-8-inducing component of EHEC O157:H7 OMVs, whereas CdtV and O157 LPS substantially contribute to IL-8 production induced by flagellin-negative SF EHEC O157:H$^-$ OMVs. Stx2a and EHEC-Hly play

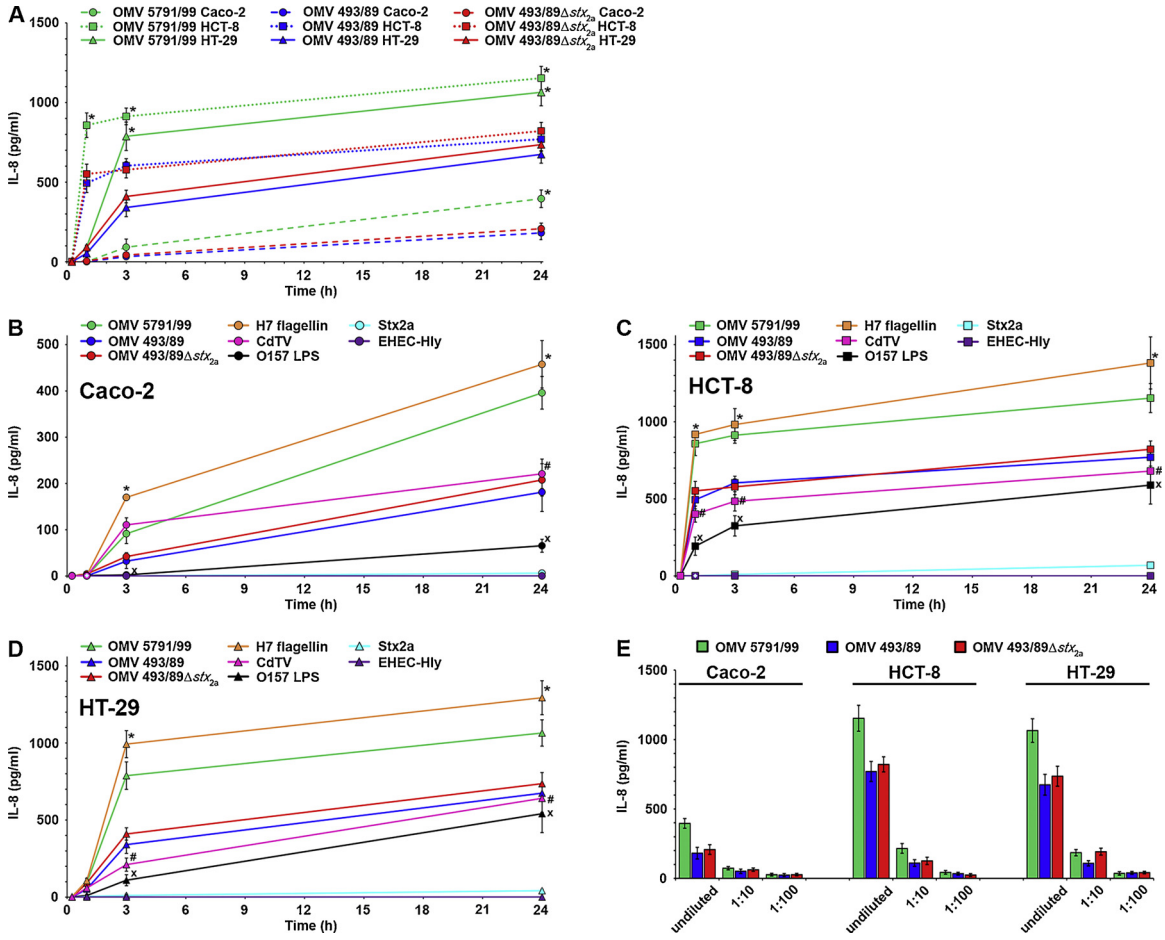

Fig. 1. IL-8 production induced by EHEC O157 OMVs and OMV components in human IECs. (A) Kinetics of IL-8 production (measured by Single-Analyte IL-8 ELISArray Kit) in Caco-2, HCT-8, and HT-29 cells which were incubated for the times indicated with OMVs from EHEC O157:H7 strain 5791/99 or from SF EHEC O157:H$^-$ strains 493/89 or 493/89Δ*stx*$_{2a}$ (for concentrations of relevant components see Table 1). *P < 0.05 for comparison between IL-8 amounts elicited by flagellin-containing OMVs 5791/99 and flagellin-lacking OMVs 493/89 and 493/89Δ*stx*$_{2a}$, respectively. (B–D) Comparison of IL-8 production induced by EHEC O157 OMVs and the indicated OMV components in Caco-2 (B), HCT-8 (C), and HT-29 cells (D). Cells were incubated with OMVs or with purified H7 flagellin (73 ng/ml), O157 LPS (965 ng/ml), Stx2a (51 ng/ml), EHEC-Hly (2 ng/ml), or recombinant CdtV (38 ng/ml) carried by TA153 OMVs for 15 min, 1 h, 3 h, and 24 h. IL-8 was measured as described in A. The net effect of CdtV on IL-8 production was determined by subtracting the background IL-8 amounts induced by vector control OMVs from those induced by TA153 OMVs. *P < 0.05 for comparison between IL-8 amounts elicited by purified H7 flagellin and flagellin-negative OMVs 493/89 and 493/89Δ*stx*$_{2a}$, respectively. #P < 0.05 for comparison between IL-8 amounts elicited by recombinant CdtV and OMVs 5791/99. xP < 0.05 for comparison between IL-8 amounts elicited by purified O157 LPS and OMVs 5791/99. (E) Dose-dependence of IL-8 responses elicited by EHEC O157 OMVs. Caco-2, HCT-8, and HT-29 cells were incubated for 24 h with 10-fold OMV dilutions containing H7 flagellin (OMV 5791/99), O157 LPS, and CdtV (all OMVs) in the doses ranging from 73 ng/ml, 962 ng/ml, and 38 ng/ml, respectively, to 0.73 ng/ml, 9.62 ng/ml, and 0.38 ng/ml, respectively. IL-8 was measured as described in A. Data in all panels are presented as means ± standard deviations from three independent experiments. Statistical calculations were performed using one-way ANOVA.

minor, if any, roles in EHEC O157 OMV-mediated IL-8 responses in human IECs *in vitro*.

3.3. Dose-dependence of IL-8 production elicited by EHEC O157 OMVs

The IL-8 responses induced by EHEC O157 OMVs were dose-dependent in each cell line (Fig. 1E). The lowest OMV doses which elicited a measurable IL-8 production after 24 h contained 0.73 ng/ml of H7 flagellin, 9.62 ng/ml of O157 LPS, and 0.38 ng/ml of CdtV (5791/99 OMVs), and 9.65 ng/ml of O157 LPS, and 0.38 ng/ml of CdtV (493/89 and 493/89Δ*stx*$_{2a}$ OMVs).

3.4. EHEC O157 OMVs induce IL-8 production in human IECs by flagellin-mediated signaling via TLR5 and O157 LPS-mediated signaling via TLR4/MD-2

OMV-induced production of proinflammatory cytokines is governed by signaling cascades triggered by recognition of OMV-associated PAMPs by the host pattern recognition receptors, in particular TLRs (Kaparakis-Liaskos and Ferrero, 2015). Flagellin and LPS, putative PAMPs of EHEC O157 OMVs (Fig. 1B-D), signal via TLR5 and TLR4/MD-2 complex, respectively (Akashi et al., 2000; Hayashi et al., 2001), which are expressed in Caco-2, HCT-8, and HT-29 cell lines (Cario et al., 2000; Kunsmann et al., 2015) that we used as models for human IECs. To gain insight into EHEC O157 OMV-induced signaling pathways leading to IL-8 production in Caco-2, HCT-8, and HT-29 cells and into the roles of H7 flagellin and O157 LPS in this signaling, we analyzed the impact of inhibiting the flagellin-mediated and the LPS-mediated signaling, respectively, on OMV abilities to induce IL-8 responses. Preincubation of O157 OMVs (or purified H7 flagellin as a control) with anti-H7 antibody and/or cells with anti-TLR5 neutralizing antibody significantly decreased IL-8 production induced by flagellin-containing 5791/99 OMVs and by H7 flagellin, respectively, but had no inhibitory effects on IL-8 production induced by flagellin-lacking OMVs 493/89 and 493/89Δ*stx*$_{2a}$ (Fig. 2A). Preincubation of OMVs (or purified O157 LPS as a control) with polymyxin B, which prevents LPS recognition by TLR4/MD-2 complex (Ellis et al., 2010), or preincubation of cells with anti-TLR4 neutralizing antibody significantly decreased IL-8 amounts

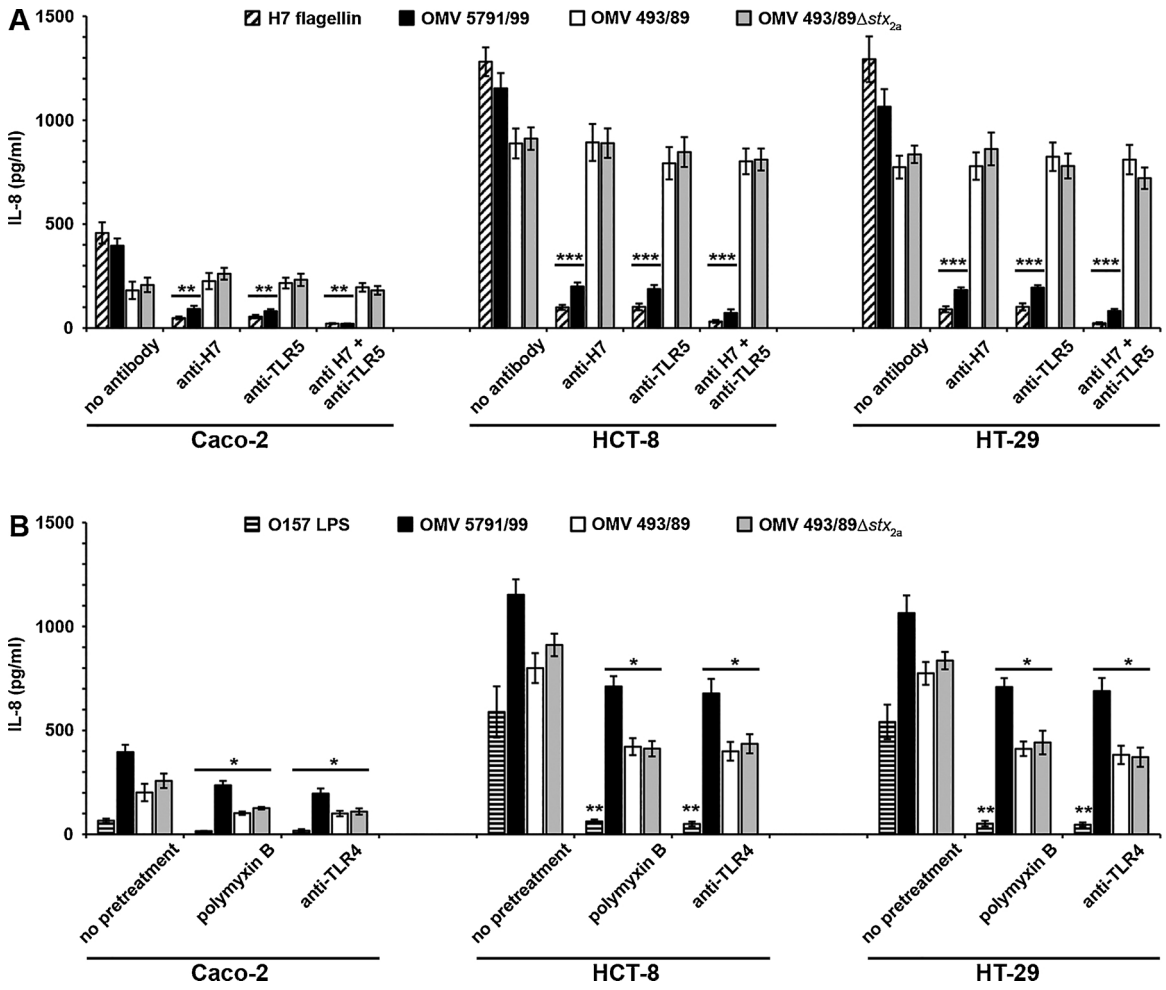

Fig. 2. EHEC O157 OMVs induce IL-8 production in human IECs by H7 flagellin-mediated signaling via TLR5 and O157 LPS-mediated signaling via TLR4/MD-2. (A) Caco-2, HCT-8, and HT-29 cells were exposed for 24 h to: OMVs or purified H7 flagellin (73 ng/ml) in the absence of antibodies; OMVs or H7 flagellin which had been preincubated with anti-H7 antibody; OMVs or H7 flagellin after the cell preincubation with anti-TLR5 antibody; and OMVs or H7 flagellin which had been preincubated with anti-H7 antibody after the cell preincubation with anti-TLR5 antibody. IL-8 production was quantified with Single-Analyte IL-8 ELISArray Kit. $^{**}P < 0.01$ or $^{***}P < 0.001$ for comparison between IL-8 production elicited by H7 flagellin or 5791/99 OMVs in the absence of antibodies and after each respective antibody pretreatment. (B) Caco-2, HCT-8, and HT-29 cells were exposed for 24 h to: OMVs or purified O157 LPS (965 ng/ml) in the absence of any pretreatment; OMVs or purified O157 LPS which had been preincubated with polymyxin B (100 μg/ml); and OMVs or purified O157 LPS after the cell preincubation with anti-TLR4 antibody. IL-8 production was quantified as described in A. $^{*}P < 0.05$ or $^{**}P < 0.01$ for comparison between IL-8 production elicited by OMVs or by O157 LPS in the absence of any pretreatment and after each respective pretreatment. All data are presented as means ± standard deviations from three independent experiments. Statistical calculations were performed using one-way ANOVA.

induced by both flagellin-containing (5791/99) and flagellin-lacking (493/89 and 493/89Δstx_{2a}) OMVs, as well as by O157 LPS (Fig. 2B). No inhibition of IL-8 production was caused by control preimmune sera or polymyxin B alone (data not shown) confirming the specificities of the inhibitory effects. Altogether, these data demonstrated that H7 flagellin and O157 LPS are important EHEC O157 OMV-associated PAMPs, which trigger the cascades leading to OMV-mediated IL-8 production in human IECs by signaling via TLR5 and TLR4/MD-2, respectively. Whereas the H7 flagellin-TLR5 signaling predominates in IL-8 induction by EHEC O157:H7 OMVs, the O157 LPS-TLR4/MD-2 signaling is involved in IL-8 induction by both EHEC O157:H7 OMVs and flagellin-lacking SF EHEC O157:H^- OMVs.

3.5. EHEC O157 OMV-induced TLR signaling leads to activation of the nuclear factor NF-κB

TLR-mediated signaling ultimately leads to activation of the nuclear factor κB (NF-κB), a major transcription regulator of proinflammatory cytokines (Carmody and Chen, 2007). Activation of NF-κB involves its release from the cytoplasmic IκB inhibitory complex and subsequent translocation to the nucleus where it binds DNA (Carmody and Chen, 2007; Henkel et al., 1993). To determine if the EHEC O157 OMV-induced signaling via TLR4 and TLR5 leads to activation of NF-κB in human IECs, we incubated HT-29 cells with OMVs (or with purified H7 flagellin or O157 LPS as controls) for different time intervals and monitored activation of NF-κB by immunoblot of nuclear extracts with an antibody against phosphorylated NF-κB p65 subunit. Immunoreactive bands demonstrating intranuclear translocation of NF-κB were elicited by all OMV preparations already after 30 min of incubation, and the band intensities increased until 3 h (Fig. 3A and B). Strong NF-κB activation was also observed at each time point in HT-29 cells exposed to purified H7 flagellin and O157 LPS, respectively (Fig. 3A).

To determine if NF-κB activation is necessary for IL-8 production induced by EHEC O157 OMVs, IL-8 was quantified in Caco-2, HCT-8, and HT-29 cells which had been incubated for 24 h with OMVs (or purified H7 flagellin or O157 LPS as controls) in the absence or in the presence of NF-κB inhibitors SN50 and/or TPCK, which block different steps in NF-κB activation (Henkel et al., 1993; Lin et al., 1995). Each inhibitor alone significantly reduced IL-8 amounts elicited by O157 OMVs; combination of both inhibitors further decreased (in HCT-8 and

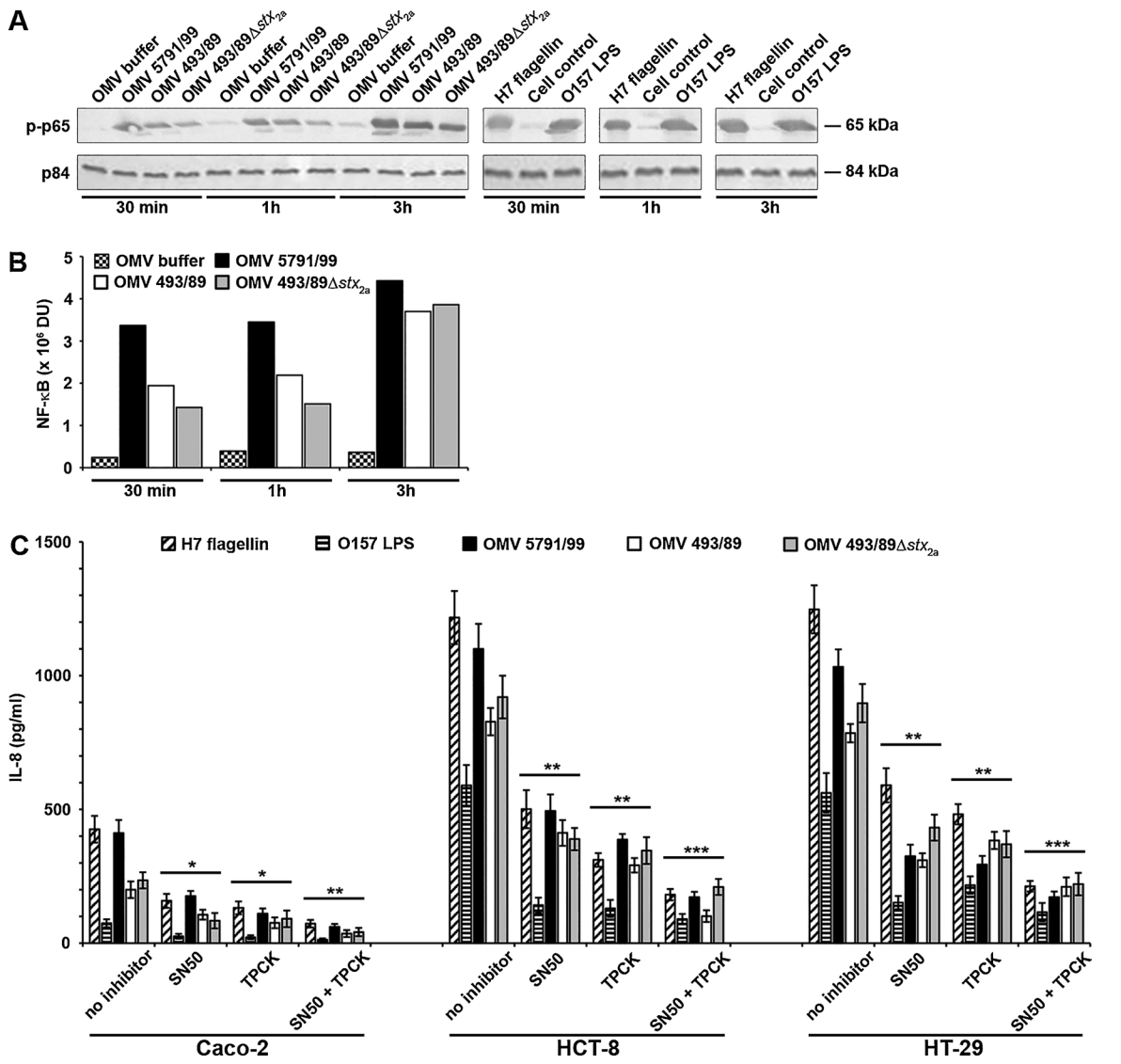

Fig. 3. Activation of the nuclear factor NF-κB by EHEC O157 OMVs is essential for OMV-mediated IL-8 production in human IECs. (A, B) Kinetics of OMV-induced NF-κB activation in HT-29 cells. (A) HT-29 cells were incubated for the times indicated with OMVs or with purified H7 flagellin or O157 LPS (positive controls) or with OMV buffer or cell culture medium (negative controls). Nuclear extracts were analyzed for activated NF-κB by immunoblot with antibody against phosphorylated NF-κB p65 subunit (p-p65). Nuclear matrix protein p84 served as a loading control. (B) NF-κB signals produced by OMVs were quantified by densitometry and expressed in arbitrary densitometric units (DU). (C) Inhibition of NF-κB activation inhibits IL-8 production induced by EHEC O157 OMVs, H7 flagellin, and O157 LPS in Caco-2, HCT-8, and HT-29 cells. Cells were incubated for 24 h with each OMV preparation or with purified H7 flagellin or O157 LPS either in the absence or in the presence of NF-κB inhibitors SN50 (20 μM) and/or TPCK (50 μM). IL-8 was measured with Single-Analyte IL-8 ELISArray Kit. Data are means ± standard deviations from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for comparison between IL-8 production elicited by each sample in the absence and in the presence of the respective inhibitor(s). Statistical calculations were performed using one-way ANOVA.

HT-29 cells) or almost abolished (in Caco-2 cells) OMV-mediated IL-8 production (Fig. 3C). Similar effects had the NF-κB inhibitors on IL-8 production elicited by purified H7 flagellin and O157 LPS, respectively (Fig. 3C). These data demonstrated that activation of NF-κB is a crucial step in EHEC O157 OMV-mediated signaling leading to IL-8 production in human IECs, and that OMV-associated H7 flagellin and O157 LPS essentially contribute to the OMV-induced NF-κB activation.

4. Discussion

We demonstrate that OMVs released by EHEC O157 strains induce production of IL-8 in human IECs. This proinflammatory potential of EHEC O157 OMVs may have implications in the pathogenesis of EHEC O157-mediated diseases as well as in the host immune responses against the infection. The pathogenetic implications of the OMV-induced IL-8 production arise from the role of IL-8 as a potent neutrophil chemoattractant and activator (Baggiolini and Clark-Lewis, 1992; Fitzpatrick et al., 1992). The IL-8-inducing capacity of OMVs released by EHEC O157 colonizing the human colon during infection might contribute to the acute inflammatory infiltration of the colonic mucosa and presence of fecal leukocytes observed in a subset of EHEC O157-infected patients (Griffin et al., 1990). In addition, the proinflammatory potential of EHEC OMVs might have been involved in the reported ability of EHEC strains to induce basolateral-to-apical transmigration of neutrophils across polarized IECs, which partially correlated with EHEC-induced IL-8 production and substantially increased Stx translocation in the opposite direction (Hurley et al., 2001). Such a process, which simulates the toxin translocation from the intestinal lumen to the circulation during EHEC infection is critical for the systemic spread of Stx and thus for development of D+ HUS (Proulx et al., 2001; Tarr et al., 2005; Zoja et al., 2010). Moreover, IL-8-induced activation of neutrophils (Fitzpatrick et al., 1992) essentially contributes to the

neutrophil-mediated endothelial injury during D+ HUS (Forsyth et al., 1989), which is a histopathological hallmark of this disorder (Proulx et al., 2000; Zoja et al., 2010). Hence, the proinflammatory capacities of EHEC O157 OMVs, in particular their ability to induce IL-8 production, further expand the roles of these nanostructures in the pathogenesis of EHEC-mediated diseases beyond their serving as carriers for EHEC O157 virulence factors and delivery tools for the virulence cargoes into the host cells (Bielaszewska et al., 2017). Notably, the nanosize of OMVs and their internalization by the host cells (Bielaszewska et al., 2017) allow these structures to reach the sites within the host tissues that are not accessible to EHEC, which are non-invasive pathogens. The importance of OMVs in the pathogenesis of EHEC O157 infections is supported by a significant upregulation of OMV production by the conditions encountered by EHEC O157 in the human gastrointestinal tract (Bauwens et al., 2017).

Although OMVs from both EHEC O157:H7 and SF EHEC O157:H$^-$ induce IL-8 production in IECs, the major components accounting for the IL-8 responses differ between these OMVs. The key role of OMV-associated H7 flagellin in IL-8 production induced by EHEC O157:H7 OMVs is in accordance with H7 flagellin being the major component of EHEC O157:H7 bacteria involved in IL-8 secretion from human IECs *in vitro* and *in vivo* (Berin et al., 2002; Miyamoto et al., 2006). The predominant role of H7 flagellin as an OMV-associated PAMP involved in IL-8 production is supported by the significantly higher proinflammatory potential of EHEC O157:H7 OMVs compared to that of flagellin-lacking OMVs from SF EHEC O157:H$^-$ (Fig. 1A). In the latter OMVs, O157 LPS and CdtV are the major contributors to IL-8 production. Unlike LPS, which has also been shown to play a role in IL-8 production induced by OMVs from EHEC O104:H4 (Kunsmann et al., 2015) and from other pathogens such as *Pseudomonas aeruginosa* (Ellis et al., 2010), the proinflammatory effect of OMV-associated CdtV was, to the best of our knowledge, first demonstrated in our study. This proinflammatory potential of EHEC O157 OMV-associated CdtV is in contrast to the reported lack of Cdt involvement in the inflammatory response elicited by Cdt-containing OMVs from *Campylobacter jejuni* (Elmi et al., 2012). Notably, in contrast to H7 flagellin, O157 LPS and CdtV, Stx2a was dispensable for EHEC O157 OMV-induced IL-8 production as has also been reported for EHEC O157 bacterial cells (Berin et al., 2002; Miyamoto et al., 2006) and for OMVs produced by EHEC O104:H4 (Kunsmann et al., 2015). Sharing the IL-8-inducing components by EHEC O157 OMVs and bacterial cells supports the hypothesis that EHEC O157 OMVs are proinflammatory facsimiles of their parental bacteria, as has been demonstrated for OMVs from *Salmonella typhiumurium* (Alaniz et al., 2007). This is further supported by similar signaling pathways used to induce IL-8 secretion by EHEC O157 bacteria (Berin et al., 2002; Cario et al., 2000; Jandhyala et al., 2010; Miyamoto et al., 2006) and by O157 OMVs, specifically the H7 flagellin-mediated TLR5 signaling and O157 LPS-mediated TLR4/MD-2 signaling (Fig. 2) followed by NF-κB activation (Fig. 3). While the flagellin-TLR5 signaling and LPS-TLR4/MD-2 signaling, respectively, were also identified as pathways involved in IL-8 production induced by EHEC O104:H4 OMVs (Kunsmann et al., 2015), the signaling pathways underlying IL-8 production induced by OMV-associated CdtV remain to be determined.

In conclusion, EHEC O157 OMVs induce production of IL-8, a key player in neutrophil recruitment and activation. The proinflammatory capacities of EHEC O157 OMVs have implications in the pathogenesis of D+ HUS. Moreover, the immunomodulatory potential of EHEC O157 OMVs combined with their functions as carriers of a cocktail of major EHEC O157 virulence factors and their intracellular delivery tools (Bielaszewska et al., 2017) provide a rationale for considering EHEC O157 OMVs as vaccine candidates. Studies on immunogenic properties of EHEC O157 OMVs and their abilities to induce protective immunity against EHEC O157 infections are underway.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

We thank Angelika Fruth (Robert Koch Institute, Wernigerode, Germany) for providing us with anti-H7 antibody, and Margarete Junge (Münster) for skillful technical assistance.

Funding

This study was supported by the German Research Foundation (DFG) [grant SFB 1009, project B04; and grant KA 717/5-3], by the Interdisciplinary Center of Clinical Research (IZKF) Münster [grant Me2/010/16], and by the Ministry of Health of the Czech Republic [funding project Conceptual development of research organization ("The National Institute of Public Health - NIPH 75010330")].

References

Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M., Miyake, K., 2000. Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J. Immunol. 164, 3471–3475.

Alaniz, R.C., Deatherage, B.L., Lara, J.C., Cookson, B.T., 2007. Membrane vesicles are immunogenic facsimiles of *Salmonella typhimurium* that potently activate dendritic cells, prime B and T cell responses, and stimulate protective immunity in vivo. J. Immunol. 179, 7692–7701.

Aldick, T., Bielaszewska, M., Zhang, W., Brockmeyer, J., Schmidt, H., Friedrich, A.W., Kim, K.S., Schmidt, M.A., Karch, H., 2007. Hemolysin from Shiga toxin-negative *Escherichia coli* O26 strains injures microvascular endothelium. Microbes Infect. 9, 282–290.

Baggiolini, M., Clark-Lewis, I., 1992. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 307, 97–101.

Bauwens, A., Bielaszewska, M., Kemper, B., Langehanenberg, P., von Bally, G., Reichelt, R., Mulac, D., Humpf, H.U., Friedrich, A.W., Kim, K.S., Karch, H., Müthing, J., 2011. Differential cytotoxic actions of Shiga toxin 1 and Shiga toxin 2 on microvascular and macrovascular endothelial cells. Thromb. Haemost. 105, 515–528.

Bauwens, A., Kunsmann, L., Marejková, M., Zhang, W., Karch, H., Bielaszewska, M., Mellmann, A., 2017. Intrahost milieu modulates production of outer membrane vesicles, vesicle-associated Shiga toxin 2a and cytotoxicity in *Escherichia coli* O157:H7 and O104:H4. Environ. Microbiol. Rep. 9, 626–634.

Berin, M.C., Darfeuille-Michaud, A., Egan, L.J., Miyamoto, Y., Kagnoff, M.F., 2002. Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. Cell Microbiol. 4, 635–648.

Bielaszewska, M., Rüter, C., Bauwens, A., Greune, L., Jarosch, K.A., Steil, D., Zhang, W., He, X., Lloubes, R., Fruth, A., Kim, K.S., Schmidt, M.A., Dobrindt, U., Mellmann, A., Karch, H., 2017. Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic *Escherichia coli* O157: intracellular delivery, trafficking and mechanisms of cell injury. PLoS Pathog. 13 (2), e1006159.

Buvens, G., Piérard, D., Hachimi-Idrissi, S., Lauwers, S., 2009. First sorbitol-fermenting Verocytotoxin-producing *Escherichia coli* O157: H- isolated in Belgium. Acta Clin. Belg. 64, 59–64.

Cario, E., Rosenberg, I.M., Brandwein, S.L., Beck, P.L., Reinecker, H.C., Podolsky, D.K., 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. J. Immunol. 164, 966–972.

Carmody, R.J., Chen, Y.H., 2007. Nuclear factor-kappaB: activation and regulation during toll-like receptor signaling. Cell. Mol. Immunol. 4, 31–41.

Ellis, T.N., Kuehn, M.J., 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. Microbiol. Mol. Biol. Rev. 74, 81–94.

Ellis, T.N., Leiman, S.A., Kuehn, M.J., 2010. Naturally produced outer membrane vesicles from *Pseudomonas aeruginosa* elicit a potent innate immune response via combined sensing of both lipopolysaccharide and protein components. Infect. Immun. 78, 3822–3831.

Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D.C., Inglis, N.F., Manson, E., Imrie, L., Bajaj-Elliott, M., Wren, B.W., Smith, D.G., Dorrell, N., 2012. *Campylobacter jejuni* outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. Infect. Immun. 80, 4089–4098.

Fitzpatrick, M.M., Shah, V., Trompeter, R.S., Dillon, M.J., Barratt, T.M., 1992. Interleukin-8 and polymorphoneutrophil leucocyte activation in hemolytic uremic syndrome of childhood. Kidney Int. 42, 951–956.

Forsyth, K.D., Simpson, A.C., Fitzpatrick, M.M., Barratt, T.M., Levinsky, R.J., 1989. Neutrophil mediated endothelial injury in haemolytic uraemic syndrome. Lancet 2, 411–414.

Griffin, P.M., Olmstead, L.C., Petras, R.E., 1990. *Escherichia coli* O157:H7-associated colitis. A clinical and histological study of 11 cases. Gastroenterology 99, 142–149.

Guessous, F., Marcinkiewicz, M., Polanowska-Grabowska, R., Kongkhum, S., Heatherly,

D., Obrig, T., Gear, A.R., 2005. Shiga toxin 2 and lipopolysaccharide induce human microvascular endothelial cells to release chemokines and factors that stimulate platelet function. Infect. Immun. 73, 8306–8316.
Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., Eng, J.K., Akira, S., Underhill, D.M., Aderem, A., 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 410, 1099–1103.
Henkel, T., Machleidt, T., Alkalay, I., Krönke, M., Ben-Neriah, Y., Baeuerle, P.A., 1993. Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. Nature 365, 182–185.
Hurley, B.P., Thorpe, C.M., Acheson, D.W., 2001. Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. Infect. Immun. 69, 6148–6155.
Jandhyala, D.M., Rogers, T.J., Kane, A., Paton, A.W., Paton, J.C., Thorpe, C.M., 2010. Shiga toxin 2 and flagellin from shiga-toxigenic *Escherichia coli* superinduce interleukin-8 through synergistic effects on host stress-activated protein kinase activation. Infect. Immun. 78, 2984–2994.
Kaparakis-Liaskos, M., Ferrero, R.L., 2015. Immune modulation by bacterial outer membrane vesicles. Nat. Rev. Immunol. 15, 375–387.
Karch, H., Tarr, P.I., Bielaszewska, M., 2005. Enterohaemorrhagic *Escherichia coli* in human medicine. Int. J. Med. Microbiol. 295, 405–418.
King, L.A., Loukiadis, E., Mariani-Kurkdjian, P., Haeghebaert, S., Weill, F.X., Baliere, C., Ganet, S., Gouali, M., Vaillant, V., Pihier, N., Callon, H., Novo, R., Gaillot, O., Thevenot-Sergentet, D., Bingen, E., Chaud, P., de Valk, H., 2014. Foodborne transmission of sorbitol-fermenting *Escherichia coli* O157:[H7] via ground beef: an outbreak in northern France, 2011. Clin. Microbiol. Infect. 20, O1136–O1144.
Kolling, G.L., Matthews, K.R., 1999. Export of virulence genes and Shiga toxin by membrane vesicles of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 65, 1843–1848.
Kunsmann, L., Rüter, C., Bauwens, A., Greune, L., Glüder, M., Kemper, B., Fruth, A., Wai, S.N., He, X., Lloubes, R., Schmidt, M.A., Dobrindt, U., Mellmann, A., Karch, H., Bielaszewska, M., 2015. Virulence from vesicles: Novel mechanisms of host cell injury by *Escherichia coli* O104:H4 outbreak strain. Sci. Rep. 5, 13252.
Lin, Y.Z., Yao, S.Y., Veach, R.A., Torgerson, T.R., Hawiger, J., 1995. Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J. Biol. Chem. 270, 14255–14258.
Marejková, M., Bláhová, K., Janda, J., Fruth, A., Petráš, P., 2013. Enterohemorrhagic *Escherichia coli* as causes of hemolytic uremic syndrome in the Czech Republic. PLoS One 8 (9), e73927.
Mellmann, A., Bielaszewska, M., Köck, R., Friedrich, A.W., Fruth, A., Middendorf, B., Harmsen, D., Schmidt, M.A., Karch, H., 2008. Analysis of collection of hemolytic uremic syndrome-associated enterohemorrhagic *Escherichia coli*. Emerg. Infect. Dis. 14, 1287–1290.
Miyamoto, Y., Iimura, M., Kaper, J.B., Torres, A.G., Kagnoff, M.F., 2006. Role of Shiga toxin versus H7 flagellin in enterohaemorrhagic *Escherichia coli* signalling of human colon epithelium in vivo. Cell. Microbiol. 8, 869–879.
Orth, D., Grif, K., Zimmerhackl, L.B., Würzner, R., 2009. Sorbitol-fermenting Shiga toxin-producing *Escherichia coli* O157 in Austria. Wien. Klein. Wochenschr. 121, 108–112.
Pollock, K.G., Locking, M.E., Beattie, T.J., Maxwell, H., Ramage, I., Hughes, D., Cowieson, J., Allison, L., Hanson, M., Cowden, J.M., 2010. Sorbitol-fermenting *Escherichia coli* O157, Scotland. Emerg. Infect. Dis. 16, 881–882.
Proulx, F., Seidman, E.G., Karpman, D., 2001. Pathogenesis of Shiga toxin-associated hemolytic uremic syndrome. Pediatr. Res. 50, 163–171.
Siegler, R.L., 2003. Postdiarrheal Shiga toxin-mediated hemolytic uremic syndrome. JAMA 290, 1379–1381.
Tarr, P.I., Gordon, C.A., Chandler, W.L., 2005. Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. Lancet 365, 1073–1086.
Thorpe, C.M., Hurley, B.P., Lincicome, L.L., Jacewicz, M.S., Keusch, G.T., Acheson, D.W., 1999. Shiga toxins stimulate secretion of interleukin-8 from intestinal epithelial cells. Infect. Immun. 67, 5985–5993.
Vygen-Bonnet, S., Rosner, B., Wilking, H., Fruth, A., Prager, R., Kossow, A., Lang, C., Simon, S., Seidel, J., Faber, M., Schielke, A., Michaelis, K., Holzer, A., Kamphausen, R., Kalhöfer, D., Thole, S., Mellmann, A., Flieger, A., Stark, K., 2017. Ongoing haemolytic uraemic syndrome (HUS) outbreak caused by sorbitol-fermenting (SF) Shiga toxin-producing *Escherichia coli* (STEC) O157, Germany, December 2016 to May 2017. Euro Surveill. 22 (21) Pii, 30541.
Walters, M.D., Matthei, I.U., Kay, R., Dillon, M.J., Barratt, T.M., 1989. The polymorphonuclear leucocyte count in childhood haemolytic uraemic syndrome. Pediatr. Nephrol. 3, 130–134.
Westerholt, S., Hartung, T., Tollens, M., Güstrau, A., Oberhoffer, M., Karch, H., Klare, B., Pfeffer, K., Emmrich, P., Oberhoffer, R., 2000. Inflammatory and immunological parameters in children with haemolytic uremic syndrome (HUS) and gastroenteritis-pathophysiological and diagnostic clues. Cytokine 12, 822–827.
Zhang, X., Cheng, Y., Xiong, Y., Ye, C., Zheng, H., Sun, H., Zhao, H., Ren, Z., Xu, J., 2012. Enterohemorrhagic *Escherichia coli* specific enterohemolysin induced IL-1β in human macrophages and EHEC-induced IL-1β required activation of NLRP3 inflammasome. PLoS One 7 (11), e50288.
Zoja, C., Buelli, S., Morigi, M., 2010. Shiga toxin-associated hemolytic uremic syndrome: pathophysiology of endothelial dysfunction. Pediatr. Nephrol. 25, 2231–2240.